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Small-Molecule Tools for Advanced Microscopy Techniques

By

Neville Dadina

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Chemistry

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Alanna Schepartz, Chair

Professor Evan Miller

Professor Roberto Zoncu

Spring 2025

Small-Molecule Tools for Advanced Microscopy Techniques

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By

Neville Dadina

Abstract

Small-Molecule Tools for Advanced Microscopy Techniques

By
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Doctor of Philosophy in Chemistry
University of California, Berkeley

Professor Alanna Schepartz, Chair

Recent advances in fluorescence microscopes, such as super-resolution microscopy and fluorescence lifetime imaging microscopy (FLIM), have expanded our capabilities to decipher biological systems. However, the full potential of these technologies remains limited by the molecular tools available to label and track biological components. While genetically encoded fluorescent proteins and self-labeling enzymes have become standard, they often impose significant cellular perturbations.

Small-molecule fluorophores and labeling strategies offer an alternative—one that bypass the need for genetic manipulation. Recent innovations in bioorthogonal chemistry have expanded the capabilities of small-molecule probes, enabling site-specific labeling and incorporation of extremely photostable dyes. This dissertation explores the development and application of chemically driven imaging tools that leverage the technologies that modern fluorescence microscopy has to offer.

Chapter One provides a comprehensive overview of small-molecule probes designed to visualize organelle dynamics, highlighting how these tools can be applied across microscopy modalities and biological systems. Chapter Two presents the design and implementation of MAO-SiR, a novel high-density environmentally sensitive (HIDE) probe specifically developed for the inner mitochondrial membrane. MAO-SiR enables prolonged super-resolution imaging of mitochondrial dynamics, addressing a critical need for stability and spatial fidelity in live-cell imaging. Chapter Three describes a new FLIM-based multiplexing platform that employs bioorthogonal chemistry to control fluorescence lifetime through ligation strategies. This approach enables simultaneous imaging of three organelles using a single dye, without genetic manipulation, expanding the capabilities of FLIM multiplexing.

Together, these chapters demonstrate how small-molecule tools can overcome the limitations of genetically encoded systems, providing new avenues for precise, non-invasive, and scalable live-cell imaging.

Dedicated to my parents:

*While life, oceans, time zones, and circumstances may part us,
you have showed up to support me every day.*

*I am blessed to have you as my parents. I could not have done this without your
unwavering love, support, and encouragement.*

Love you.

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Acknowledgements

On a personal note...

About five years ago, I took an Uber to SFO airport and had one of the most enlightening conversations that would change the way I thought about life. I was flying to Boston to see a friend, and I was struggling with my decision to do a Ph.D. I started chatting with the driver, and he picked up on my anxiety. He then asked me a simple question: "When planning this trip, did you make a choice or a decision?" It was a strange question. "Those two words mean the same thing," I said. "Yes, " he replied, "But what they stand for is very different."

A decision is logical. Objectively, the pros outweigh the cons, and you choose the path with more pros. Buying an affordable car with good mileage and plenty of room is a good decision. A choice is personal. The pros might not outweigh the cons—sometimes they do—but you choose the path that speaks to you more. Ditch that car and get a motorcycle.

Ph.D. is a choice, not a decision. It's a grueling time in your life, and it is a thankless job marred by red tape and events out of your control. You take an immense financial hit to build knowledge that will impact an insignificant percentage of humanity, or, that knowledge gets buried in the depths of "the literature"—don't expect to see the benefits. You trade your time with your family for late nights in lab to earn a piece of paper that adds five letters to your legal name. It is, objectively, a bad decision.

But it is a good choice. The Ph.D. taught me to fail more than succeed. I used to fear failure—it made me feel incompetent. But I have failed so often for six years that I am wildly comfortable with it. I embrace it, and I try to enjoy it. Failure has taught me more than success could ever dream of.

The Ph.D. spotlights sacrifice—you realize yours every time an experiment goes wrong. But the sacrifice drew into focus the little things: morning coffee walks, Californian road trips, a burrito after climbing, late-night Tango classes. The little things that brought me joy every day. I realized that I had a lot to be grateful for. Then, I started to **really** value the bigger things: a loving family, supportive friends, excellent colleagues, and boundless opportunity. Over time, the Ph.D. taught me to accept what I cannot control, enjoy living in the present, and be grateful for my life. As a plus, I got a piece of paper which adds five letters to my name in perpetuity.

A Ph.D. is a bad decision, but it's a great choice. It made me grow in ways I couldn't imagine, it showed me strength I did not know I had, it taught me to appreciate what I have. So, if you're reading this, and you're doing a Ph.D. (or thinking of doing one) here's my take: I made a choice. I ditched the car and got the metaphorical motorcycle. I took some falls, had some accidents, but at the end of the day, I had a blast. I had fun. I learned. Would I do it again? Hell no. It took six years. I'm passionate, not delusional. Like skydiving out of a plane without a parachute, it's an activity that you only do once. But if I went back in time and had to make the same choice, I would. I loved it. Ups and downs, every single moment.

To the people:

While I worked tirelessly on this chapter of my life, the people around me supplied the ink and the paper that brought these words to life. Without them, I wouldn't be the person I am today. Thank you for your love and support; I am the best version of myself because of you.

First, a giant whose shoulders I stand on. In my case, she's lifted me up above valley walls to peer at the horizon that is scientific discovery. For the last five (some say six) years, I have worked with Dr. Alanna Schepartz, and this period in my life has been memorable and formative. To give you a sense of what it's like to work with her, after a meeting in her office, I told her, "I thought a Ph.D. was like a marathon; you gotta pace yourself." She said, "No, it's a sprint, the whole way through, all your years. But don't tell anyone I said that." Sorry for blowing your cover, Alanna. But yes, it has been a sprinting marathon. It's been entertaining, engaging, nerve-racking, trying, inspiring, empowering, poetic, pedantic, surprising, and, most importantly, fulfilling. Would I do it again? In a heartbeat. I have had the privilege, the joy, and the opportunity of a lifetime to work side-by-side with an incredible scientist. So, Alanna, thank you for your mentorship, guidance, and trust. Working for you has been a pivotal moment in my life.

I would also like to thank Dr. Evan Miller. Your door was always open, and you always welcomed me with warmth. I have always enjoyed working with the Miller lab. I am truly grateful for all the help and advice throughout my time at Berkeley. Your mentorship and guidance have been an integral part of my Ph.D. experience.

My journey as a mentee at Berkeley would not be complete without Dr. Jonathan Tyson, M.Sc., Dr. Deepto Mozumdar, and Dr. Shuai Zheng. These people played an essential role in building me as a scientist. The pandemic really gave us some quality time together. Shuai and I wrestled with vacuum pumps and hoses and set up schlenk lines (oh god, the number of Schlenk lines), Deepto taught me how to "wash once, wash twice, wash three times" every time I worked with mammalian cells, and Tyson and I spent days debating science and shirking bench work (these conversations were memorable and foundational to my education). These individuals taught me valuable lessons that I will pass on. However, the one lesson they all taught in unison: don't doubt yourself. Thank you for your confidence and patience.

The Schepartz Lab. The HIDE subgroup has been home for the last few years and is the most collaborative group I have ever known. Shuai, Lauren, Charmaine, and Justin have been incredible people to work with. Lauren and I have been desk and bench mates for the last five years. She was a force when she joined the lab and remains one. She is one of the greatest scientific minds I've encountered and a role model for meal prepping. Justin was the latest addition to the group, but he seemed like a long-lost member. We worked as a team for the last year on Chapter Three of this thesis. I couldn't have done it without his support—he is phenomenal. Finally, Charmaine. Charmaine was my first and

only undergraduate mentee. There is so much that I could say about her—an exquisite chemist, a brilliant scientist, a hard worker, a great mentee. Above all else, a good friend.

Truly, one of the most beautiful things about the Schepartz Lab is how easy it is to make lifelong friends. Everyone in the lab is warm and welcoming; we have a healthy level of banter, and people are humble but brilliant. It's rare to work in an environment that fosters kindness and brilliance; I am truly fortunate to have experienced it. I will truly miss Maddie (and her love for ranch), Izzi (for always being gullible), Leah (for emailing the chem department for her wallet), and Dan (for being the most dedicated Francis lab alum). Lunches, coffee, moments to sit around someone's desk and talk about the world—your support got me through tough times, and your laughter kept me afloat in good times. Of course, I cannot forget the clan of the climbing shirts, Brother Noah and Brother Isaac. From climbing till 11 p.m. to working late nights to meeting at Cheeseboard for a leisurely start, some of my fondest memories in grad school are with you. It would take an entire chapter to capture all the stories, shenanigans, banter, and memories we've shared. All of you have been an integral part of my journey as a scientist and as a human being.

One of the aspects of my Ph.D. that has made it so enjoyable is the love and support of my friends. To my housemates—Aidan, Forrest, Jon, Ryan, Kris, Facu, Nels, Michal, and Jeff—thank you for creating a home away from home. I always looked forward to laughing after a long day. Katherine, thank you for helping me write this behemoth. Aryan, thank you for distracting me from writing this behemoth. Orion, thank you for slowing down the Ph.D. with our morning walks and coffee. It might have cost us a few months, but I would not trade those moments for anything. To the Lalas, Java, Un, Mustu, Tony, Shanta, Khumail, and Prathamesh: your support from India has kept me alive when I missed home. Waking up to 400+ WhatsApp messages seemed like a pain, but now I look forward to them every morning. Finally, Sarica, your camaraderie and companionship through this degree have been unwavering. Our friendship has been quick, strong, and deep. You are one of my biggest champions. Thank you for being in my life.

Some say you don't choose your family. Sometimes, Brown University chooses them for you. I always thought it was a bit of a coincidence that they threw an Indian and a Pakistani in the same freshman dorm room. But truly, that was the best thing that ever happened to me. I met Aqil Rashid ten years ago. Since then, he has become my best friend and the brother I never had. Aqil, you have supported me unconditionally throughout this degree. My life has been more enjoyable and more fulfilled because of your presence. You showed up for me every day, no strings attached. We celebrated birthdays, mourned deaths, and created memories. Thank you for being my family.

Lastly, I want to thank the people I am most grateful for: my family. My grandmother Zarin Dadina, whose spirit has been with me and watched over me. She taught me compassion and how to treat people with dignity and respect. In moments that I miss her, I know she has kept me safe. My grandfather, Pessi Dadina. Secretly, he brainwashed me into becoming a chemist. Grampoo, I owe my curiosity and love for science to you. Thank you for being the kindling to my scientific journey. My aunt, Varez Dadina. She really is the epitome of "cool aunt." I love chatting with her, talking about life, and laughing about the

absurdity of her actions. Besides that, she is a rock in my life and a trusted confidant. You're why I made it to Brown, and finally to Berkeley. Finally, my parents, Avan and Navzer Dadina. Mum and Dad, I am blessed to be your child. You've supported me unconditionally throughout my career. You've sent me off into the world and truly let me chart my own course, even when that course took me further away from you. On days that I'm low, you talk me up. You might not know this, but I save every voicemail you leave me. Moments when I can't call, I listen to them, and I feel your love and presence. Circumstances, choices, and geography separate us. But I carry you in my heart every day and every step of the way. I love you very much, and this dissertation is for you.

P.S. To the reader: Thank you for picking up this dissertation. You may have picked it up just to read this section; I certainly have for most dissertations. It's the most interesting section of a dissertation. It is the only section of the thesis where you get to know the author on a human level. In today's world, that human element is taken for granted. This work is not perfect, but it is what I dedicated six years of my life to. And I am proud of it. And I am humbled that you chose to read it. So, from the bottom of my heart, thank you for reading. Thank you for your time. Thank you for your patience.

Chapter 1: Imaging organelle membranes in live cells at the nanoscale with lipid-based fluorescent probes

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Abstract

Understanding how organelles interact, exchange materials, assemble, disassemble, and evolve as a function of space, time, and environment is an exciting area at the very forefront of chemical and cell biology. Here we bring attention to recent progress in the design and application of lipid-based tools to visualize and interrogate organelles in live cells, especially at super-resolution. We highlight strategies that rely on modification of natural lipids or lipid-like small molecules *ex cellula*, where organelle specificity is provided by the structure of the chemically modified lipid or *in cellula* using cellular machinery, where an enzyme labels the lipid *in situ*. We also describe recent improvements to the chemistry upon which lipid probes rely, many of which have already begun to broaden the scope of biological questions that can be addressed by imaging organelle membranes at the nanoscale.

Introduction

Organelles are the way the cell segregates biochemistry. Alone and together, organelles drive eukaryotic cell biology. Many cellular functions are marked by dynamic changes in organelle structure and interaction, and organelle dysfunction underlies a plethora of metabolic and neurologic disease. Understanding how organelles interact, exchange materials, assemble, disassemble, and evolve as a function of space, time, and environment is an exciting area at the very forefront of chemical and cell biology.

Historically, changes in organelle structure, function, and interaction have been visualized using fluorescence microscopy and organelle-specific proteins tagged with a fluorescent protein or small molecule dye. Recently, lipids and lipid-like small molecules have been shown to offer distinct and unique advantages for imaging organelle structure, function, and interaction, especially at the nanoscale.

Besides demarking a more continuous organelle boundary, one advantage of labeling an organelle membrane with a lipid probe as opposed to a membrane-embedded protein is labeling density [1]. Excepting polymeric proteins such as actin and tubulin, the density of lipid in a membrane exceeds that of protein by > 100-fold [2]. This increase in density facilitates super-resolution microscopy (SRM), as resolution depends integrally on the number of detectable molecules; the Nyquist-Shannon sampling criterion demands that the labeling density be greater than the desired resolution by at least a factor of two [3]. Additionally, the increase in labelling density also results in an almost equivalent increase in imaging time, even at super-resolution. Moreover, as lipids and lipid-like small molecules (or their precursors) are often cell-permeant, they permit experimentation with primary or patient-derived cells as well as cultured cell lines that are notoriously difficult to genetically manipulate.

In this current opinion, we bring attention to recent progress in the design and application of tools that visualize organelles—predominantly in live cells—at super-resolution using stimulated emission depletion (STED) [4,5] and single molecule localization microscopy (SMLM) [6–9]. For those interested in tools suitable for predominantly confocal methods, the reader is referred to these excellent recent reviews: [10–12]. We focus broadly on three approaches: (1) modification of natural lipids *ex cellula*, where organelle specificity is provided by the structure of the chemically modified lipid (**Figure 1A**), (2) modification of lipids *in cellula* using cellular machinery, where an enzyme labels the lipid *in situ* (**Figure 1B**), and (3) the design of lipid-like small molecules that localize selectively within specific organelle membranes (**Figure 1C**). We also highlight improvements to the chemistry upon which lipid probes rely, including strategies to encode high fluorogenicity, and new, bright, versatile fluorophores and bioorthogonal reactions, many of which have already begun to broaden the scope of biological questions that can be addressed by imaging organelle membranes at the nanoscale.

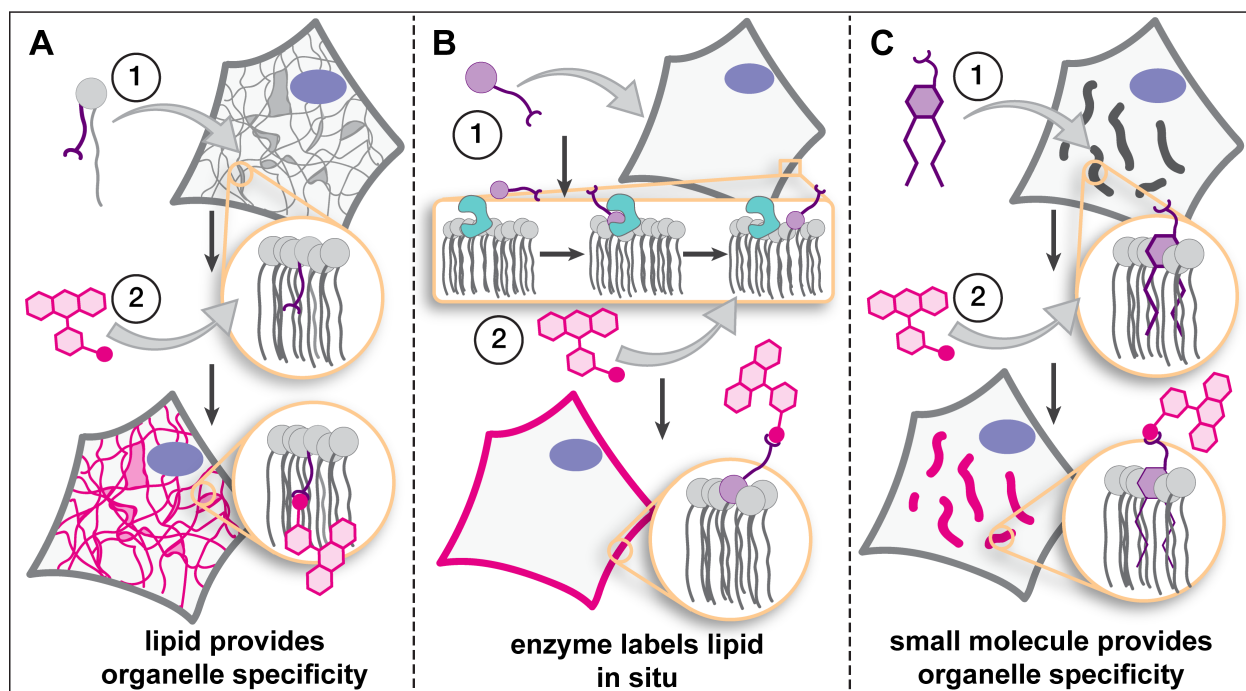


Figure 1. General strategies to image organelles and/or track lipids selectively, often at the nanoscale, that exploit (a) lipid structure; (b) organelle-restricted enzymes; and (c) organelle-targeting small molecules to limit fluorescence to the organelle of interest.

Imaging organelles at the nanoscale with lipid probes

One way to image organelles selectively for nanoscopy is to fluorescently tag the molecules that comprise the membranes themselves—the lipids. This strategy exploits the fact that different organelles are often enriched in different lipids [13]. Pioneering work by Pagano [14,15] demonstrated over thirty years ago that ceramide-like lipids tagged with a boron dipyrromethene difluoride (BODIPY) or nitrobenzoxadiazole (NBD) fluorophore could visualize the Golgi apparatus and interrogate Golgi-plasma membrane trafficking. The utility of BODIPY-Cer and NBD-Cer catalyzed the development of commercially available ceramide-derived fluorescent probes to interrogate Golgi trafficking and sphingolipid transport/metabolism. Although the commercially available probes are used widely, their reliance on relatively non-photostable NBD and BODIPY fluorophores limits their utility for super-resolution methods such as STED and SMLM.

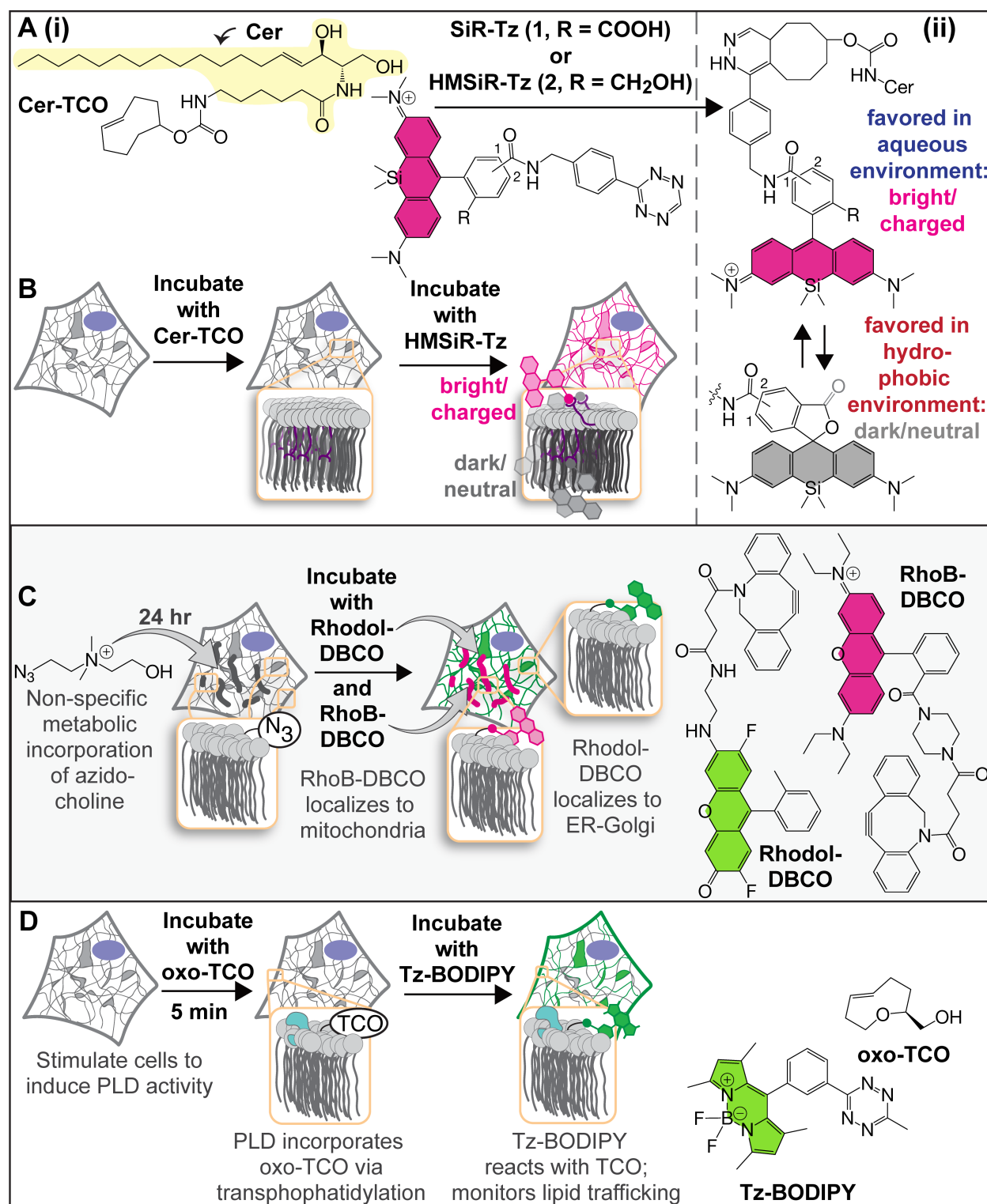


Figure 2. Lipid-focused strategies to image organelles and/or study their trafficking. (a) (i) Cer-TCO, which localizes in a temperature-dependent way to the Golgi or ER and reacts and (ii) the environment-dependent spirocyclization equilibria of SiR and HMSiR that effectively extends imaging times by “hiding” a reservoir of dark-state dyes in the membrane. (b) *in cellula* with tetrazine-containing fluorophores such as SiR and HMSiR to enable STED and SMLM, respectively. (c) Biosynthetic incorporation of azido-choline into phospholipids followed by coincubation with a click-compatible dye fused to an organelle-targeting

small molecule results in selective localization of the labeled lipid to the organelle of interest. (d) Incorporation of oxo-TCO handle via PLD-mediated transphosphatodilation, followed by fluorogenic Tz-BODIPY results in real-time visualization of PLD activity.

One strategy [16,17] to expand the utility of ceramide-like probes for super-resolution imaging employs bespoke analogs that carry photostable fluorophores such as SiR [18] (useful for STED) and HMSiR [19] (useful for SMLM). These super-resolution lipid probes are assembled *in cellula*: a ceramide-derivative such as Cer-TCO (**Figure 2A(i)**) is added to cells, whereupon it localizes to the Golgi or the ER depending on temperature [20–22]. Next, a tetrazine-tagged fluorophore such as SiR-Tz or HMSiR-Tz is added, tetrazine ligation [23,24] ensues, and the organelle membrane is labeled selectively (**Figure 2B**). In this case, organelle selectivity is provided by the inherent trafficking/localization properties of the lipid (**Figure 1A**); subsequent reaction with the fluorophore enables 3D live-cell nanoscopy [16,17]. In the case of the probe assembled from Cer-TCO and HMSiR-Tz, SMLM is achieved without toxic additives or laser-induced photoswitching.

A less obvious advantage of labeling an organelle membrane directly is a truly significant increase in imaging time [17]. In a particularly dramatic example, labeling the ER with the lipid probe generated by reaction of Cer-TCO and HMSiR-Tz generates SMLM images that last almost 30 min; when the ER is labeled with HMSiR attached to a HaloTag-Sec61 β fusion protein, the images last for only a minute [17]. The extended imaging times also influence image quality for SMLM images, allowing a user to coalesce more blinking events over multiple frames for improved reconstruction. The increase in imaging time is due to the fact that dyes such as HMSiR and SiR exist in two states, only one of which is fluorescent (**Figure 2A(ii)**), and the non-fluorescent form is favored within the membrane environment. Thus the membrane holds an excess reservoir of dye molecules that are “hiding” in the lipid and can be used to replenish dyes that are bleached over time. This modular two-component approach using HIDE probes demonstrates outstanding SRM imaging times when compared to previous ceramide derivatives and protein-based labeling techniques.

Enzymatically modifying lipids for super-resolution imaging

A conceptually related approach to image organelles was reported recently by Hamachi [25**] (**Figure 2C**). In this case, azido-choline is enzymatically incorporated *in cellula*, to modify a choline-containing phospholipid. This modified phospholipid is then capable of a bioorthogonal click (SPAAC) [26–28] reaction for subsequent fluorophore labelling. Coincubation with a click-compatible dye fused to an organelle-targeting small molecule results in selective localization of the labeled lipid to the organelle of interest. This strategy is effectively the inverse of the HIDE strategy - rather than using the lipid to localize the dye, in this case the dye is used to localize the lipid (**Figure 1B**). This strategy enabled structured illumination microscopy (SIM) of the mitochondria and confocal studies of inter-organelle translocation of phosphatidylcholine (PC).

Baskin and coworkers also recently reported a third creative way to selectively image organelle lipids, in this case by exploiting the promiscuity of phospholipase D [29,30**](**Figure 2D**). Phospholipase D (PLD) hydrolyzes phospholipids, but can make use of alcohols as substrates in place of water - the result is a trans-phosphatidylation reaction, not hydrolysis. If the alcohol carries a trans-cyclooctene, the newly synthesized phospholipid can react with a fluorogenic tetrazine dye. Using this enzymatic tool, Baskin and coworkers achieved adequate temporal resolution to directly observe PLD activity and subsequent trafficking of its lipid substrates. While this work did not report super-resolution experiments, the chemistry described could easily be adapted for this purpose. Lipid biosynthesis offers many potential avenues to selectively label lipids *in cellula* to observe their trafficking and metabolism [31–35].

Imaging organelle membranes at the nanoscale with lipid-like small molecules

Another versatile method for super-resolution imaging of organelle membranes exploits lipid-like small molecules that interact selectively with organelle membranes. Indeed, some of the earliest examples of live-cell super-resolution microscopy described by Zhuang exploited small molecule fluorophores such as DiI, MitoTracker Red, ER-Tracker Red, and LysoTracker Red. These molecules could be induced to blink by strong laser excitation and subsequent reactivation by ultraviolet light to visualize the plasma membrane, mitochondrial inner membrane, endoplasmic reticulum, and lysosome, respectively using STORM [1].

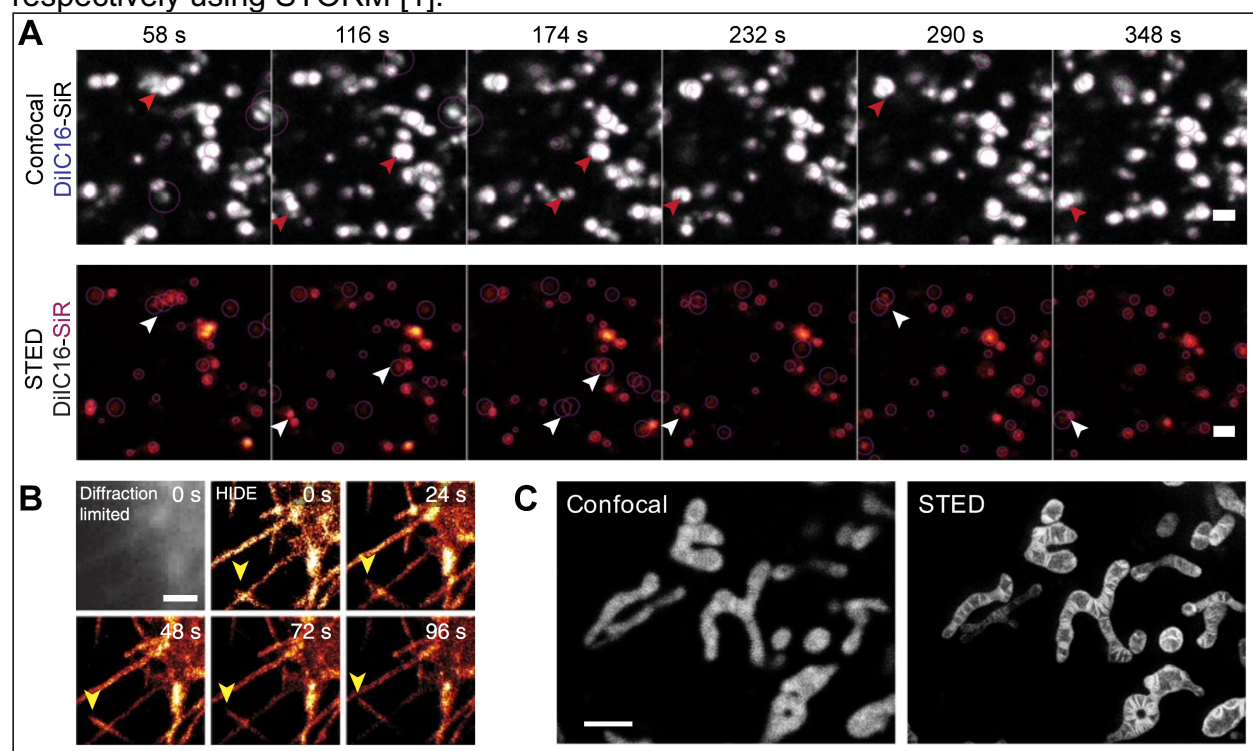


Figure 3. Membrane-targeting small-molecule probes for super-resolution microscopy of organelles. (a) Visualizing endosomal motility defects with DiI-C16-SiR in wild-type fibroblasts from patients with Niemann-Pick C disease; scale bar: 1 μ m. Reprinted by permission from Springer Nature

Customer Service Centre GmbH: Nature Chemical Biology, Endosome motility defects revealed at super-resolution in live cells using HIDE probes, Gupta et al., 2020 (b) SMLM time-lapse images of plasma membrane filopodia acquired using Dil-HMSiR; scale bar: 1 μ m. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Nature Biotechnology, Long time-lapse nanoscopy with spontaneously blinking membrane probes, Takakura et al., 2017 (c) Confocal vs. STED images of the mitochondria in HeLa cells using MitoPB Yellow; scale bar: 2 μ m. Reprinted from Wang C et al.,: A photostable fluorescent marker for the superresolution live imaging of the dynamic structure of the mitochondrial cristae. Proc Natl Acad Sci U S A 2019, 116:15817–15822.

One strategy to avoid the need for multiple lasers or additives to induce photoswitching makes use of the spontaneously blinking fluorophore HMSiR [19] paired with some of the small molecule fluorophores described above, but tagged with trans-cyclooctene to enable *in cellula* tetrazine ligation [17,36**,37]. This strategy has been used to image multiple different organelles using SMLM methods, including the plasma membrane (HMSiR-Dil) (**Figure 3B**). and mitochondria (HMSiR-RhoB) both of which are assembled *in cellula* via a tetrazine ligation. Analogous experiments using Dil-C16-SiR, a lipid-like small molecule that localizes to endolysosomes, could detect both subtle and dramatic endosome motility defects in Niemann Pick patient samples using STED (**Figure 3A**). In all of these cases, because HMSiR and SiR exist in two states, imaging times are exceptionally long [36**,38**]. It should be noted that the membrane-targeting molecules in the above examples are intrinsically fluorescent and thereby occupy an imaging channel. For multi-color experiments targeting multiple organelles, this limits the number of channels that can be acquired simultaneously.

More recent examples of small molecules that enable plasma membrane-specific super-resolution include multi-colored, fluorogenic MemBright dyes [39*] (**Figure 3C**). These cyanine-derived molecules are amphiphilic and form non-fluorescent soluble aggregates under cell culture conditions; the dyes light up when they distribute throughout the membrane. MemBright Cy3.5 supported the use of 3D STORM to dynamically image a synapse coiling around a dendrite, and in combination with immunostaining, multicolor STORM revealed the accumulation of glutamate receptors at axon-dendritic junctions in fixed cells. Styryl dyes were also recently improved for enhanced brightness and photostability, and shown to support imaging of hippocampal neurons *via* STED [40*].

A unique and enabling attribute of certain fluorophores, such as Nile Red (**Figure 3D**), is solvatochromism, in which the emission spectrum varies as a function of solvent polarity. In early work, Xu and coworkers used Nile Red to image spatially resolved polarity changes across the plasma membrane and the membranes of nanoscale intracellular organelles in an unbiased manner [41]. More recently, Nile Red derivatives carrying lipid chains were shown to localize the dye to only the plasma membrane. Short-chain and long-chain derivatives with improved photophysical properties have been designed that display reversible and irreversible membrane binding, respectively [42*]. Irreversible binding is desirable for traditional confocal microscopy, and reversible labeling was utilized in SR-PAINT microscopy to image nanoscale heterogeneity in lipid order within

the plasma membrane. This kind of reversible binding shows promise for time-lapse STED microscopy [43], as new molecules are available to replace photobleached molecules over time.

A naphthophosphone fluorophore demonstrating remarkable photostability (MitoPB Yellow) was developed with a mitochondria-targeting triphenylphosphine moiety and a protein-crosslinking epoxide, facilitating STED imaging (**Figure 3C**) even after membrane potential loss due to fixation [44*]. Cyanine- and coumarin-based derivatives have also been reported for imaging lipid droplets with two-photon excitation microscopy and STED [45,46], and 3D STED has enabled the quantification of lipid droplet count and size in cells. All the probes that have been discussed above have been categorized and listed in a supplementary table (**Figure S1**).

Looking to the future: Enabling chemistry for more sophisticated experimentation

There has also been excellent recent progress in technologies that increase the sophistication of experiments possible with lipid and lipid-like small molecule probes. One big issue is labeling specificity, which can be improved with dyes whose emission intensity increases only when properly localized or assembled. Several groups have reported fluorogenic tetrazine and azide probes whose fluorescence is quenched until bound by its target [47–52]. A particularly creative approach to improved fluorogenicity was reported by Wombacher and coworkers, yielding a spontaneously blinking HMSiR analog (**f-HM-SiR**) useful for live-cell SMLM whose fluorescence increases 10-fold upon reaction with bicyclo[6.1.0]non-4-yne (**BCN**). **f-HM-SiR** allows for no-wash imaging and reduces unwanted background signal to improve localization precision [53**] (**Figure 4A**). Another potentially useful approach utilizes polarity-dependent photoactivation, which leads to a fluorescent product only within apolar media. A probe based on this molecular logic gate was demonstrated to label lipid droplets with very high specificity [54].

All of the experiments described above would be impossible without photostable fluorophores whose physical properties—absorption and emission maxima, quantum yield, spirocyclization equilibria (pK_{cycle}), dielectric constant, fluorogenicity—are appropriate for the super-resolution modality (STED, SMLM, SIM, etc.), cell environment (pH) and optical set-up of the commercial or custom instrument employed. Lavis and coworkers developed a number of azetidine-containing Janelia dyes with improved brightness, photostability, and fluorogenicity relative to classical tetramethyl-rhodamines such as the commonly employed SiR and tetramethyl rhodamine (TMR) [55,56]. In particular, JF646 and JF635 (**Figure 4B**) exhibit higher quantum yields (0.54 and 0.56, respectively) [55] than SiR (0.41) but emit at approximately the same wavelength [56].

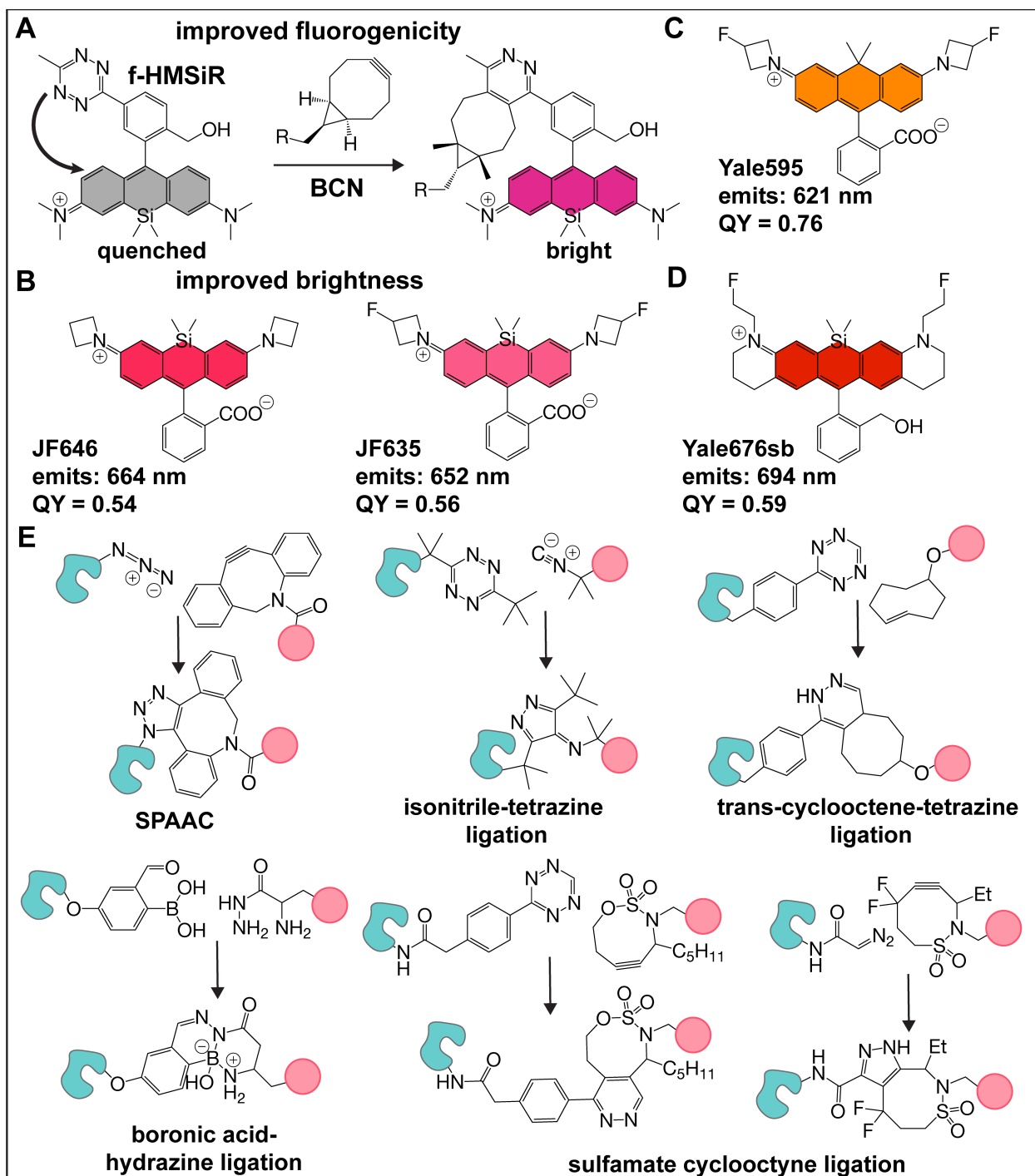


Figure 4. (a) Fluorogenic spontaneously-blinking fluorophores reported by Wombacher and coworkers. Upon reacting with bicyclo[6.1.0]non-4-yne (BCN), the fluorescence intensity of the HM-SiR analog increases, thereby enabling no-wash, live-cell SRM. (b) Brighter fluorophores. Improvements to SiR by Lavis and coworkers resulted in near-infrared fluorophores that absorb at similar wavelengths to SiR, but with increased brightness and better photophysical properties. (c) Tuning dyes for hydrophobic environments. Yale595 was developed by Chu et al. to high brightness in hydrophobic environments. (d) Development of a near-infrared, spontaneously blinking fluorophore. Yale676sb was developed as a bright,

spectrally separable fluorophore from HMSiR. The development of this fluorophore has enabled two-color ratiometric SMLM, using NIR dyes and a single laser. (e) Mutually orthogonal bioorthogonal ligations. Improvements to the isonitrile-tetrazine ligation by Franzini and coworkers, and development of the sulfamate-cyclooctyne ligation by Schomaker and coworkers have led to the development of triply-orthogonal bioorthogonal reaction pairs.

Despite these advances, real challenges remain, most notably the lack of fluorophores that satisfy all the criteria cited above but also emit at a wavelength that does not overlap with widely used silicon-rhodamine dyes such as SiR-COOH (for STED/SIM), JF646 (for STED) or HMSiR (for SMLM). Such dyes are essential for multi-color experiments needed to reveal organelle interactions in both space and time. Notable progress includes the recent report of Yale595, an azetidine dye whose electron-withdrawing substituents have been tuned to promote high brightness even within a non-polar lipid environment [38**]. Yale595 (**Figure 4C**) was used alongside SiR to enable two-color super-resolution (STED) and 3D confocal imaging of the plasma membrane and either the ER or mitochondria. In more recent progress, Tyson *et al.* judiciously balanced the pK_{cycle} and emission wavelength of a spontaneously blinking silicon-rhodamine fluorophore to develop Yale676sb, which was paired with HMSiR to enable two-color SMLM of the ER and mitochondria using a single far-red laser [57**]. Yale676sb (**Figure 4D**) is distinguished not only by its emission wavelength (694 nm) and pK_{cycle} but also by an exceptional quantum yield (0.59).

Several improvements have also been made to expand the bioorthogonal ligation toolkit to include new, mutually orthogonal chemistries. Franzini and coworkers described an isonitrile-tetrazine ligation [58**] (**Figure 4E**) that is promoted by dispersion forces. In addition to favourable reaction kinetics, this new reaction is mutually orthogonal to both SPAAC [26–28] and TCO-tetrazine ligations [23,24]. Similarly, Schomaker and coworkers demonstrated mutual orthogonality between sulfamate-containing cyclooctynes by tuning the electronics of the reaction pairs [59]. Using two newly developed reaction partners, and a boronic acid/hydrazine ligation, they too demonstrated simultaneous triple labelling (**Figure 4E**). These mutually orthogonal chemistries enable the modular delivery of multiple fluorophore-probe pairs for multicolor imaging. With the development of additional mutually orthogonal chemistries, in tandem with other technologies described above [25,29,30], one could easily envision experiments that combine different lipid reporters to interrogate time-dependent lipid trafficking between multiple organelles, in addition to observing individual organelles at sub-diffraction resolutions.

Conclusions

The last few years have witnessed great progress in the design and application of lipids and lipid-like small molecules for imaging organelle structure, function, and interaction, especially at the nanoscale. Multiple approaches have been described for both labeling

and localizing lipids selectively, and in certain cases these events have led to new biological knowledge that would be impossible to obtain without the lipid probe. There has also been much progress in chemistry upon which lipid probes rely, including strategies to encode high fluorogenicity, and new, bright, versatile fluorophores and bioorthogonal reactions. Many of these advances have already begun to broaden the scope of biological questions that can be addressed by imaging organelle membranes at the nanoscale.

References

1. Shim S-H, Xia C, Zhong G, Babcock HP, Vaughan JC, Huang B, Wang X, Xu C, Bi G-Q, Zhuang X: **Super-resolution fluorescence imaging of organelles in live cells with photoswitchable membrane probes**. *Proc Natl Acad Sci U S A* 2012, **109**:13978–13983.
2. Quinn P, Griffiths G, Warren G: **Density of newly synthesized plasma membrane proteins in intracellular membranes II. Biochemical studies**. *J Cell Biol* 1984, **98**:2142–2147.
3. Fernández-Suárez M, Ting AY: **Fluorescent probes for super-resolution imaging in living cells**. *Nat Rev Mol Cell Biol* 2008, **9**:929–943.
4. Klar TA, Jakobs S, Dyba M, Egnér A, Hell SW: **Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission**. *Proc Natl Acad Sci* 2000, **97**:8206–8210.
5. Hell SW, Kroug M: **Ground-state-depletion fluorescence microscopy: A concept for breaking the diffraction resolution limit**. *Appl Phys B* 1995, **60**:495–497.
6. Rust MJ, Bates M, Zhuang X: **Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM)**. *Nat Methods* 2006, **3**:793–796.
7. Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS, Davidson MW, Lippincott-Schwartz J, Hess HF: **Imaging Intracellular Fluorescent Proteins at Nanometer Resolution**. *Science* 2006, **313**:1642–1645.
8. Heilemann M, van de Linde S, Schüttelz M, Kasper R, Seefeldt B, Mukherjee A, Tinnefeld P, Sauer M: **Subdiffraction-Resolution Fluorescence Imaging with Conventional Fluorescent Probes**. *Angew Chem Int Ed* 2008, **47**:6172–6176.
9. Hess ST, Girirajan TPK, Mason MD: **Ultra-High Resolution Imaging by Fluorescence Photoactivation Localization Microscopy**. *Biophys J* 2006, **91**:4258–4272.

10. Flores J, White BM, Brea RJ, Baskin JM, Devaraj NK: **Lipids: chemical tools for their synthesis, modification, and analysis.** *Chem Soc Rev* 2020, doi:10.1039/d0cs00154f.
11. Neef AB, Schultz C: **Selective Fluorescence Labeling of Lipids in Living Cells.** *Angew Chem Int Ed* 2009, **48**:1498–1500.
12. Bumpus TW, Baskin JM: **Greasing the Wheels of Lipid Biology with Chemical Tools.** *Trends Biochem Sci* 2018, **43**:970–983.
13. Meer G van, Voelker DR, Feigenson GW: **Membrane lipids: where they are and how they behave.** *Nat Rev Mol Cell Biol* 2008, **9**:112–124.
14. Pagano RE, Martin OC, Kang HC, Haugland RP: **A novel fluorescent ceramide analogue for studying membrane traffic in animal cells: accumulation at the Golgi apparatus results in altered spectral properties of the sphingolipid precursor.** *J Cell Biol* 1991, **113**:1267–1279.
15. Lipsky N, Pagano R: **A vital stain for the Golgi apparatus.** *Science* 1985, **228**:745–747.
16. Erdmann RS, Toomre D, Schepartz A: **STED Imaging of Golgi Dynamics with Cer-SiR: A Two-Component, Photostable, High-Density Lipid Probe for Live Cells.** In *Super-Resolution Microscopy: Methods and Protocols*. Edited by Erfle H. 2017:65–78.
17. Takakura H, Zhang Y, Erdmann RS, Thompson AD, Lin Y, McNellis B, Rivera-Molina F, Uno S, Kamiya M, Urano Y, et al.: **Long time-lapse nanoscopy with spontaneously blinking membrane probes.** *Nat Biotechnol* 2017, **35**:773–780.
18. Lukinavičius G, Umezawa K, Olivier N, Honigsmann A, Yang G, Plass T, Mueller V, Reymond L, Jr IRC, Luo Z-G, et al.: **A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins.** *Nat Chem* 2013, **5**:132–139.
19. Uno S, Kamiya M, Yoshihara T, Sugawara K, Okabe K, Tarhan MC, Fujita H, Funatsu T, Okada Y, Tobita S, et al.: **A spontaneously blinking fluorophore based on intramolecular spirocyclization for live-cell super-resolution imaging.** *Nat Chem* 2014, **6**:681–689.
20. Kuliawat R, Arvan P: **Protein targeting via the “constitutive-like” secretory pathway in isolated pancreatic islets: passive sorting in the immature granule compartment.** *J Cell Biol* 1992, **118**:521–529.
21. Griffiths G, Pfeiffer S, Simons K, Matlin K: **Exit of newly synthesized membrane proteins from the trans cisterna of the Golgi complex to the plasma membrane.** *J Cell Biol* 1985, **101**:949–964.
22. Simon JP, Ivanov IE, Adesnik M, Sabatini DD: **The production of post-Golgi vesicles requires a protein kinase C-like molecule, but not its phosphorylating activity.** *J Cell Biol* 1996, **135**:355–370.

23. Blackman ML, Royzen M, Fox JM: **Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron-Demand Diels–Alder Reactivity.** *J Am Chem Soc* 2008, **130**:13518–13519.
24. Devaraj NK, Weissleder R, Hilderbrand SA: **Tetrazine-Based Cycloadditions: Application to Pretargeted Live Cell Imaging.** *Bioconjug Chem* 2008, **19**:2297–2299.
25. Tamura T, Fujisawa A, Tsuchiya M, Shen Y, Nagao K, Kawano S, Tamura Y, Endo T, Umeda M, Hamachi I: **Organelle membrane-specific chemical labeling and dynamic imaging in living cells.** *Nat Chem Biol* 2020, **16**:1361–1367.
26. Agard NJ, Prescher JA, Bertozzi CR: **A Strain-Promoted [3 + 2] Azide–Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems.** *J Am Chem Soc* 2004, **126**:15046–15047.
27. Baskin JM, Prescher JA, Laughlin ST, Agard NJ, Chang PV, Miller IA, Lo A, Codelli JA, Bertozzi CR: **Copper-free click chemistry for dynamic in vivo imaging.** *Proc Natl Acad Sci* 2007, **104**:16793–16797.
28. Sletten EM, Bertozzi CR: **From Mechanism to Mouse: A Tale of Two Bioorthogonal Reactions.** *Acc Chem Res* 2011, **44**:666–676.
29. Bumpus TW, Baskin JM: **Clickable Substrate Mimics Enable Imaging of Phospholipase D Activity.** *ACS Cent Sci* 2017, **3**:1070–1077.
30. Liang D, Wu K, Tei R, Bumpus TW, Ye J, Baskin JM: **A real-time, click chemistry imaging approach reveals stimulus-specific subcellular locations of phospholipase D activity.** *Proc Natl Acad Sci* 2019, **116**:15453–15462.
31. Thiele C, Papan C, Hoelper D, Kusserow K, Gaebler A, Schoene M, Piotrowitz K, Lohmann D, Spandl J, Stevanovic A, et al.: **Tracing fatty acid metabolism by click chemistry.** *ACS Chem Biol* 2012, **7**:2004–2011.
32. Pérez AJ, Bode HB: **ω -Azido fatty acids as probes to detect fatty acid biosynthesis, degradation, and modification.** *J Lipid Res* 2014, **55**:1897–1901.
33. Peng T, Hang HC: **Bifunctional fatty acid chemical reporter for analyzing S-palmitoylated membrane protein-protein interactions in mammalian cells.** *J Am Chem Soc* 2015, **137**:556–559.
34. Won SJ, Martin BR: **Temporal profiling establishes a dynamic S-palmitoylation cycle.** *ACS Chem Biol* 2018, **13**:1560–1568.
35. Thinon E, Hang HC: **Chemical Reporters for Exploring Protein Acylation.** *Biochem Soc Trans* 2015, **43**:253–261.
36. Gupta A, Rivera-Molina F, Xi Z, Toomre D, Schepartz A: **Endosome motility defects revealed at super-resolution in live cells using HIDE probes.** *Nat Chem Biol* 2020, **16**:408-+.

37. Thompson AD, Omar MH, Rivera-Molina F, Xi Z, Koleske AJ, Toomre DK, Schepartz A: **Long-Term Live-Cell STED Nanoscopy of Primary and Cultured Cells with the Plasma Membrane HIDE Probe Dil-SiR.** *Angew Chem Int Ed* 2017, **56**:10408–10412.
38. Chu L, Tyson J, Shaw JE, Rivera-Molina F, Koleske AJ, Schepartz A, Toomre DK: **Two-color nanoscopy of organelles for extended times with HIDE probes.** *Nat Commun* 2020, **11**:4271.
39. Collot M, Ashokkumar P, Anton H, Boutant E, Faklaris O, Galli T, Mely Y, Danglot L, Klymchenko AS: **MemBright: A Family of Fluorescent Membrane Probes for Advanced Cellular Imaging and Neuroscience.** *Cell Chem Biol* 2019, **26**:600-+.
40. Collot M, Boutant E, Fam KT, Danglot L, Klymchenko AS: **Molecular Tuning of Styryl Dyes Leads to Versatile and Efficient Plasma Membrane Probes for Cell and Tissue Imaging.** *Bioconjug Chem* 2020, **31**:875–883.
41. Moon S, Yan R, Kenny SJ, Shyu Y, Xiang L, Li W, Xu K: **Spectrally Resolved, Functional Super-Resolution Microscopy Reveals Nanoscale Compositional Heterogeneity in Live-Cell Membranes.** *J Am Chem Soc* 2017, **139**:10944–10947.
42. Danylchuk D, Moon S, Xu K, Klymchenko AS: **Switchable Solvatochromic Probes for Live-Cell Super-resolution Imaging of Plasma Membrane Organization.** *Angew Chem-Int Ed* 2019, **58**:14920–14924.
43. Spahn C, Grimm JB, Lavis LD, Lampe M, Heilemann M: **Whole-Cell, 3D, and Multicolor STED Imaging with Exchangeable Fluorophores.** *Nano Lett* 2018, **19**:500–505.
44. Wang C, Taki M, Sato Y, Tamura Y, Yaginuma H, Okada Y, Yamaguchi S: **A photostable fluorescent marker for the superresolution live imaging of the dynamic structure of the mitochondrial cristae.** *Proc Natl Acad Sci* 2019, **116**:15817–15822.
45. Collot M, Fam TK, Ashokkumar P, Faklaris O, Galli T, Danglot L, Klymchenko AS: **Ultrabright and Fluorogenic Probes for Multicolor Imaging and Tracking of Lipid Droplets in Cells and Tissues.** *J Am Chem Soc* 2018, **140**:5401–5411.
46. Xu H, Zhang H, Liu G, Kong L, Zhu X, Tian X, Zhang Z, Zhang R, Wu Z, Tian Y, et al.: **Coumarin-Based Fluorescent Probes for Super-resolution and Dynamic Tracking of Lipid Droplets.** *Anal Chem* 2019, **91**:977–982.
47. Wieczorek A, Werther P, Euchner J, Wombacher R: **Green- to far-red-emitting fluorogenic tetrazine probes – synthetic access and no-wash protein imaging inside living cells.** *Chem Sci* 2016, **8**:1506–1510.

48. Yang J, Šečkutė J, Cole CM, Devaraj NK: **Live-Cell Imaging of Cyclopropene Tags with Fluorogenic Tetrazine Cycloadditions.** *Angew Chem Int Ed* 2012, **51**:7476–7479.
49. Galeta J, Dzijak R, Obořil J, Dračinský M, Vrabel M: **A Systematic Study of Coumarin–Tetrazine Light-Up Probes for Bioorthogonal Fluorescence Imaging.** *Chem – Eur J* 2020, **26**:9945–9953.
50. Kozma E, Girona GE, Paci G, Lemke EA, Kele P: **Bioorthogonal double-fluorogenic siliconrhodamine probes for intracellular super-resolution microscopy.** *Chem Commun* 2017, **53**:6696–6699.
51. Shieh P, Hangauer MJ, Bertozzi CR: **Fluorogenic Azidofluoresceins for Biological Imaging.** *J Am Chem Soc* 2012, **134**:17428–17431.
52. Wieczorek A, Buckup T, Wombacher R: **Rigid tetrazine fluorophore conjugates with fluorogenic properties in the inverse electron demand Diels–Alder reaction.** *Org Biomol Chem* 2014, **12**:4177–4185.
53. Werther P, Yserentant K, Braun F, Kaltwasser N, Popp C, Baalmann M, Herten D, Wombacher R: **Live-Cell Localization Microscopy with a Fluorogenic and Self-Blinking Tetrazine Probe.** *Angew Chem Int Ed* 2020, **59**:804–810.
54. Eördögh Á, Paganini C, Pinotsi D, Arosio P, Rivera-Fuentes P: **A Molecular Logic Gate Enables Single-Molecule Imaging and Tracking of Lipids in Intracellular Domains.** *ACS Chem Biol* 2020, **15**:2597–2604.
55. Grimm JB, Muthusamy AK, Liang Y, Brown TA, Lemon WC, Patel R, Lu R, Macklin JJ, Keller PJ, Ji N, et al.: **A general method to fine-tune fluorophores for live-cell and in vivo imaging.** *Nat Methods* 2017, **14**:e04236 14.
56. Grimm JB, English BP, Chen J, Slaughter JP, Zhang Z, Revyakin A, Patel R, Macklin JJ, Normanno D, Singer RH, et al.: **A general method to improve fluorophores for live-cell and single-molecule microscopy.** *Nat Methods* 2015, **12**:244–250.
57. Tyson J, Hu K, Zheng S, Kidd P, Dadina N, Chu L, Toomre D, Bewersdorf J, Schepartz A: **Extremely Bright, Near-IR Emitting Spontaneously Blinking Fluorophores Enable Ratiometric Multicolor Nanoscopy in Live Cells.** *ACS Cent Sci* 2021, doi:10.1021/acscentsci.1c00670.
58. Tu J, Svatunek D, Parvez S, Liu AC, Levandowski BJ, Eckvahl HJ, Peterson RT, Houk KN, Franzini RM: **Stable, Reactive, and Orthogonal Tetrazines: Dispersion Forces Promote the Cycloaddition with Isonitriles.** *Angew Chem Int Ed* 2019, **58**:9043–9048.
59. Hu Y, Roberts JM, Kilgore HR, Lani ASM, Raines RT, Schomaker JM: **Triple, Mutually Orthogonal Bioorthogonal Pairs through the Design of Electronically Activated Sulfamate-Containing Cycloalkynes.** *J Am Chem Soc* 2020, **142**:18826–18835.

Supplementary Information

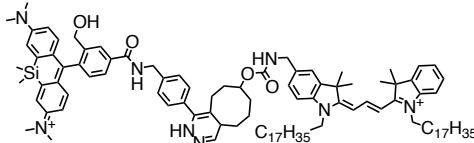
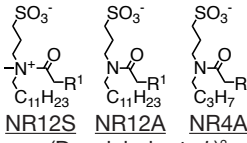
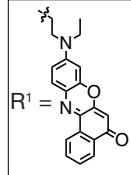
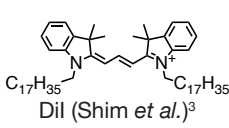
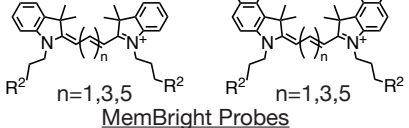
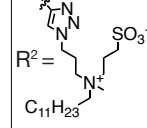
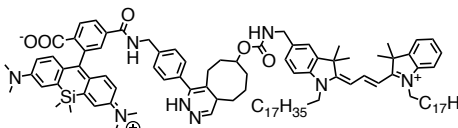
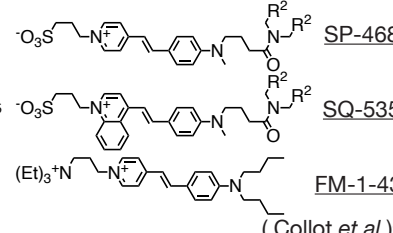
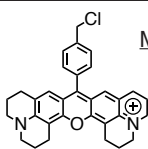
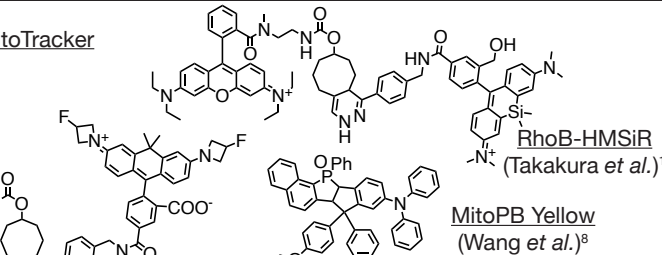
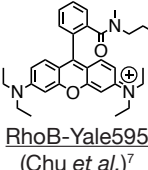
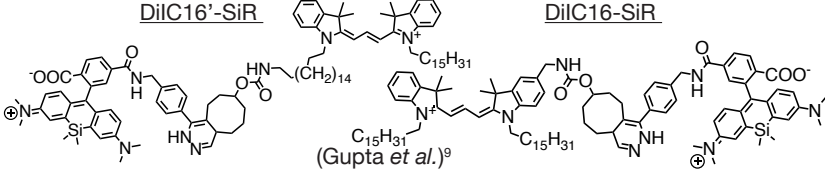
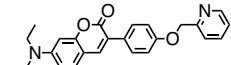
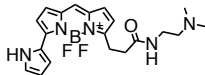
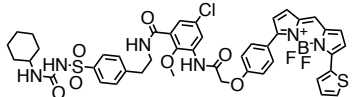
Organelle	Microscopy Technique	Probe
Plasma Membrane	SMLM	 <u>Dil-HMSiR</u> (Takakura <i>et al.</i>) ¹  <u>NR12S</u> <u>NR12A</u> <u>NR4A</u> (Danylichuk <i>et al.</i>) ²  $R^1 =$
		 <u>Dil</u> (Shim <i>et al.</i>) ³  <u>MemBright Probes</u> (Colloet <i>et al.</i>) ⁴  $R^2 =$
	STED	 <u>Dil-SiR</u> (Thompson <i>et al.</i>) ⁶  <u>SP-468</u> <u>SQ-535</u> <u>FM-1-43</u> (Colloet <i>et al.</i>) ⁵
Mitochondria	SMLM	 <u>MitoTracker</u>  <u>RhoB-HMSiR</u> (Takakura <i>et al.</i>) ¹ <u>MitoPB Yellow</u> (Wang <i>et al.</i>) ⁸
	STED	 <u>RhoB-Yale595</u> (Chu <i>et al.</i>) ⁷
Late Endosomes	STED	 <u>DiIC16'-SiR</u> <u>DiIC16-SiR</u> (Gupta <i>et al.</i>) ⁹
Lipid Droplets	STED	 <u>CM2P</u> (Xu <i>et al.</i>) ¹⁰
Lysosome	SMLM	 <u>LysoTracker Red</u> (Shim <i>et al.</i>) ³
ER	SMLM	 <u>ER-Tracker Red</u> (Shim <i>et al.</i>) ³

Table S5. List of organelle membrane-targeting small-molecule probes, their target organelle, and demonstrated super-resolution microscopy technique.

References:

1. Takakura H, Zhang Y, Erdmann RS, Thompson AD, Lin Y, McNellis B, Rivera-Molina F, Uno S, Kamiya M, Urano Y, et al.: **Long time-lapse nanoscopy with spontaneously blinking membrane probes.** *Nat Biotechnol* 2017, **35**:773–780.
2. Danylchuk D, Moon S, Xu K, Klymchenko AS: **Switchable Solvatochromic Probes for Live-Cell Super-resolution Imaging of Plasma Membrane Organization.** *Angew Chem-Int Ed* 2019, **58**:14920–14924.
3. Shim S-H, Xia C, Zhong G, Babcock HP, Vaughan JC, Huang B, Wang X, Xu C, Bi G-Q, Zhuang X: **Super-resolution fluorescence imaging of organelles in live cells with photoswitchable membrane probes.** *Proc Natl Acad Sci U S A* 2012, **109**:13978–13983.
4. Collot M, Ashokkumar P, Anton H, Boutant E, Faklaris O, Galli T, Mely Y, Danglot L, Klymchenko AS: **MemBright: A Family of Fluorescent Membrane Probes for Advanced Cellular Imaging and Neuroscience.** *Cell Chem Biol* 2019, **26**:600-614.
5. Collot M, Boutant E, Fam KT, Danglot L, Klymchenko AS: **Molecular Tuning of Styryl Dyes Leads to Versatile and Efficient Plasma Membrane Probes for Cell and Tissue Imaging.** *Bioconjug Chem* 2020, **31**:875–883
6. Thompson AD, Omar MH, Rivera-Molina F, Xi Z, Koleske AJ, Toomre DK, Schepartz A: **Long-Term Live-Cell STED Nanoscopy of Primary and Cultured Cells with the Plasma Membrane HIDE Probe Dil-SiR.** *Angew Chem Int Ed* 2017, **56**:10408–10412.
7. Chu L, Tyson J, Shaw JE, Rivera-Molina F, Koleske AJ, Schepartz A, Toomre DK: **Two-color nanoscopy of organelles for extended times with HIDE probes.** *Nat Commun* 2020, **11**:4271.
8. Wang C, Taki M, Sato Y, Tamura Y, Yaginuma H, Okada Y, Yamaguchi S: **A photostable fluorescent marker for the superresolution live imaging of the dynamic structure of the mitochondrial cristae.** *Proc Natl Acad Sci U S A* 2019, **116**:15817–15822.
9. Gupta A, Rivera-Molina F, Xi Z, Toomre D, Schepartz A: **Endosome motility defects revealed at super-resolution in live cells using HIDE probes.** *Nat Chem Biol* 2020, **16**:408-414.
10. Xu H, Zhang H, Liu G, Kong L, Zhu X, Tian X, Zhang Z, Zhang R, Wu Z, Tian Y, et al.: **Coumarin-Based Fluorescent Probes for Super-resolution and Dynamic Tracking of Lipid Droplets.** *Anal Chem* 2019, **91**:977–982

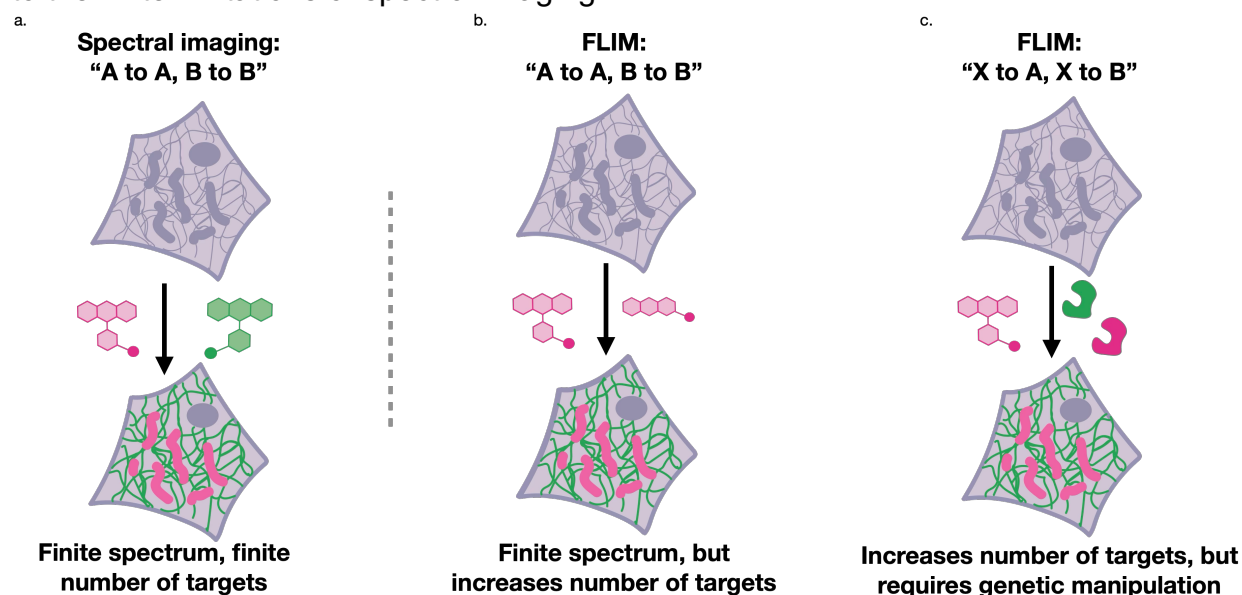
Addendum

Since the publication of this article, there have been updates and interesting developments in the field of small-molecule imaging tools. Here, we highlight two developments in the field that are relevant to this dissertation: multiplexing using fluorescence lifetime imaging microscopy (FLIM) and bioorthogonal turn-on dyes:

FLIM Multiplexing: a solution to the limitations of spectral imaging.

Spectral imaging remains the primary mode of visualizing multiple fluorescent targets in a cell. Typically, fluorophores are carefully selected before an experiment to ensure that their emission spectra do not overlap (**Addendum Fig. 1a**). Fundamentally, there are challenges: 1) the visible spectrum has a finite range, and 2) the emission spectra of fluorophores span a width of this range. So, a practical limitation exists—the number of targets is finite.

Fluorescence lifetime imaging (FLIM) is complementary to spectral imaging and operates in an orthogonal plane. Simply put, FLIM measures how many photons arrive from a pixel in an image and when they arrive. It is also independent of excitation wavelength and intensity. Traditionally, FLIM provides an optical readout of physiological properties (e.g., pH), as fluorescence lifetime changes with a fluorophore's local environment. However, every fluorophore—even spectrally overlapping—has an intrinsic fluorescence lifetime. As a result, FLIM can distinguish spectrally overlapping fluorophores—a potential solution to the finite limitations of spectral imaging.



Addendum Fig. 1 | Spectral imaging versus FLIM multiplexing: the ability to image more targets simultaneously. **a**, The traditional approach to spectral imaging: label different targets with spectrally distinct fluorophores. **b**, FLIM multiplexing with individual fluorophores. Using spectrally overlapping fluorophores to target different biomolecules, but utilizing FLIM to separate the targets by lifetime. **c**, Single molecule approach to FLIM multiplexing. In this approach, the target dictates the lifetime of the fluorophore. One can use a single fluorophore to label and distinguish multiple targets.

Broadly, there are two strategies for implementing FLIM multiplexing. In the first approach, one employs spectrally overlapping fluorophores with distinct enough lifetimes (Addendum Fig. 1 b). Frei et al.¹ discuss using this approach through a combination of commercially available small-molecule probes and genetically encoded self-labelling enzymes. They imaged up to 8 targets simultaneously, using four spectral channels. In another study, Pisfil et al.² used the same approach, but added a layer of complexity—the addition of stimulated emission depletion (STED) microscopy. They detailed imaging up to five targets simultaneously, at super-resolution.

The second strategy is—in the best way possible—slick. The fluorescence lifetime of a fluorophore is sensitive to its environment. Self-labelling enzymes such as HaloTag generate a microenvironment for fluorophores, often influencing their fluorescence intensity. SiR dyes, for example, undergo a fluorescence enhancement upon binding to HaloTag³. In another report, Frei et al.⁴ engineered HaloTag variants with different turn-on capabilities. The real genius lies in the fact that these turn-ons correlated directly with fluorescence lifetime. They designed a system where the targeting entity (a HaloTag mutant) modulates the lifetime of a single fluorophore, thereby generating multiple signatures for the same molecule (Addendum Fig. 1c). The authors were able to image multiple targets, simultaneously, with a single dye.

FLIM multiplexing is just getting its bearings. With technology developments such as phasor analysis^{5,6}, interpreting lifetime data and separation of lifetime species is simplified for the end-user. We anticipate that FLIM multiplexing will offer a realistic and adoptable solution to the limitations of spectral imaging.

Fluorogenic dyes: designing high-contrast imaging tools

Background fluorescence is a routine hurdle in fluorescence microscopy. When labeling with small-molecule fluorophores, unbound fluorophores—usually present from incomplete labeling—contribute to the overall signal in an image. This off-target fluorescence adds a layer of complexity to the analysis and quantification of microscopy images. For small-molecule tools, this disadvantage leads to reduced contrast and an additional layer of post-experimental processing.

One solution to background fluorescence is the development of an intrinsic molecular switch—a fluorophore that fluoresces when bound to its target. For example, SiR is a fluorophore that is environmentally sensitive⁷: in polar environments, it is fluorescent; in non-polar environments, it is non-fluorescent. Using SiR with HaloTag results in lower background fluorescence, as only SiR bound to HaloTag will fluoresce. Numerous intrinsically fluorogenic probes exist, some of which do not rely on protein targeting strategies^{7–10}. Designing and developing intrinsically fluorogenic dyes relies on altering the chromophore structure, i.e., the fluorophore, its target, and its fluorogenicity are intertwined. Changes to the fluorophore scaffold will change its fluorogenic properties but simultaneously affect its spectral and photophysical properties^{11–15}.

Bioorthogonal chemistry has revolutionized probe development and biological discovery. The inverse electron demand Diels-Alder reaction (iEDDA) is one of the quickest

bioorthogonal ligation reactions used in cellular imaging¹⁶; its speed makes it an excellent candidate for live-cell and temporally sensitive imaging experiments. One fortuitous side effect of the reaction is that it can be fluorogenic¹⁷. Tetrazines used in the iEDDA tend to be excellent fluorescence quenching agents. Multiple reports have detailed the fluorogenic effects of covalently tethering a tetrazine to a fluorophore to develop a turn-on probe^{18–23}. The tetrazine acts like a switch: unreacted, the fluorophore cannot fluoresce (OFF). Reacting the tetrazine with its bioorthogonal partner (usually tethered to the cellular target) disrupts the tetrazine's quenching properties, resulting in a fluorescence enhancement (ON). The strategy can be applied to a wide range of fluorophores, allowing one to decouple photophysical properties, cellular targets, and fluorogenicity from each other²⁴. One probe worth highlighting is HD555, a tetramethyl-rhodamine-tetrazine turn-on probe¹⁸. The probe has a 113-fold fluorescence turn-on upon reacting with its partner—a remarkable increase in fluorescence, which ensures target-specific fluorescence and minimal background fluorescence. The authors employ a modular synthetic strategy that would allow chemists to adopt the scaffold to other rhodamine-based dyes.

Collectively, these technologies open the door for the development of small-molecule tools. They result in better fluorophores for microscopists and better labeling systems for biologists. These advances minimize biological perturbations and maximize imaging output.

References

1. Frei, M. S., Koch, B., Hiblot, J. & Johnsson, K. Live-Cell Fluorescence Lifetime Multiplexing Using Synthetic Fluorescent Probes. *ACS Chem. Biol.* **17**, 1321–1327 (2022).
2. Pisfil, M. G. *et al.* Stimulated emission depletion microscopy with a single depletion laser using five fluorochromes and fluorescence lifetime phasor separation. *Scientific Reports* **12**, 14027 (2022).
3. Wang, L. *et al.* A general strategy to develop cell permeable and fluorogenic probes for multicolour nanoscopy. *Nature Chemistry* **12**, 165–172 (2019).
4. Frei, M. S. *et al.* Engineered HaloTag variants for fluorescence lifetime multiplexing. *Nat Methods* **19**, 65–70 (2022).
5. Digman, M. A., Caiolfa, V. R., Zamai, M. & Gratton, E. The Phasor Approach to Fluorescence Lifetime Imaging Analysis. *Biophysical Journal* **94**, L14–L16 (2008).
6. Malacrida, L., Ranjit, S., Jameson, D. M. & Gratton, E. The Phasor Plot: A Universal Circle to Advance Fluorescence Lifetime Analysis and Interpretation. *Annual Review of Biophysics* **50**, 575–593 (2021).
7. Lukinavičius, G. *et al.* A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins. *Nature Chemistry* **5**, 132–139 (2013).
8. Lukinavičius, G. *et al.* Fluorogenic probes for live-cell imaging of the cytoskeleton. *Nat Methods* **11**, 731–733 (2014).
9. Lukinavičius, G. *et al.* Fluorogenic Probes for Multicolor Imaging in Living Cells. *Journal of the American Chemical Society* **138**, 9365–9368 (2016).
10. Wang, L., Frei, M. S., Salim, A. & Johnsson, K. Small-Molecule Fluorescent Probes for Live-Cell Super-Resolution Microscopy. *Journal of the American Chemical Society* **141**, 2770–2781 (2018).
11. Grimm, J. B. *et al.* Carbofluoresceins and Carborhodamines as Scaffolds for High-Contrast Fluorogenic Probes. *ACS Chemical Biology* **8**, 1303–1310 (2013).
12. Grimm, J. B. *et al.* A general method to improve fluorophores for live-cell and single-molecule microscopy. *Nature Methods* **12**, 244–250 (2015).
13. Grimm, J. B. *et al.* Bright photoactivatable fluorophores for single-molecule imaging. *Nature Methods* **13**, nmeth.4034 (2016).
14. Zheng, Q. *et al.* Rational Design of Fluorogenic and Spontaneously Blinking Labels for Super-Resolution Imaging. *ACS central science* **5**, 1602–1613 (2019).
15. Grimm, J. B. *et al.* A general method to optimize and functionalize red-shifted rhodamine dyes. *Nature methods* **17**, 815–821 (2020).

16. Oliveira, B. L., Guo, Z. & Bernardes, G. J. L. Inverse electron demand Diels–Alder reactions in chemical biology. *Chemical Society Reviews* **46**, 4895–4950 (2017).
17. Pinto-Pacheco, B., Carbery, W. P., Khan, S., Turner, D. B. & Buccella, D. Fluorescence Quenching Effects of Tetrazines and Their Diels–Alder Products: Mechanistic Insight Toward Fluorogenic Efficiency. *Angewandte Chemie International Edition* **59**, 22140–22149 (2020).
18. Werther, P. *et al.* Bio-orthogonal Red and Far-Red Fluorogenic Probes for Wash-Free Live-Cell and Super-resolution Microscopy. *ACS Cent. Sci.* **7**, 1561–1571 (2021).
19. Wieczorek, A., Werther, P., Euchner, J. & Wombacher, R. Green- to far-red-emitting fluorogenic tetrazine probes – synthetic access and no-wash protein imaging inside living cells. *Chemical Science* **8**, 1506–1510 (2016).
20. Wieczorek, A., Buckup, T. & Wombacher, R. Rigid tetrazine fluorophore conjugates with fluorogenic properties in the inverse electron demand Diels–Alder reaction. *Organic & Biomolecular Chemistry* **12**, 4177–4185 (2014).
21. Kozma, E., Girona, G. E., Paci, G., Lemke, E. A. & Kele, P. Bioorthogonal double-fluorogenic siliconrhodamine probes for intracellular super-resolution microscopy. *Chemical Communications* **53**, 6696–6699 (2017).
22. Yang, J., Šečkutė, J., Cole, C. M. & Devaraj, N. K. Live-Cell Imaging of Cyclopropene Tags with Fluorogenic Tetrazine Cycloadditions. *Angewandte Chemie International Edition* **51**, 7476–7479 (2012).
23. Carlson, J. C. T., Meimetis, L. G., Hilderbrand, S. A. & Weissleder, R. BODIPY–Tetrazine Derivatives as Superbright Bioorthogonal Turn-on Probes. *Angewandte Chemie International Edition* **52**, 6917–6920 (2013).
24. Beliu, G. *et al.* Bioorthogonal labeling with tetrazine-dyes for super-resolution microscopy. *Communications biology* **2**, 261 (2019).

Chapter 2: Long-term, super-resolution HIDE imaging of the inner mitochondrial membrane in live cells with a cell-permeant lipid probe

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Abstract:

The inner mitochondrial membrane (IMM) generates power to drive cell function and its dynamics control mitochondrial health and cellular homeostasis. IMM structure is complex, however, and imaging its dynamics with high temporal and spatial resolution is complicated by the photosensitivity of IMM-resident enzymes. Here we describe the cell-permeant, lipid-like small molecule MAO-N₃ and use it to assemble high-density, environmentally sensitive (HIDE) probes that selectively label and image the IMM in live cells and multiple cell states. MAO-N₃ pairs with SPAAC-reactive fluorophores to support HIDE imaging using confocal, Structured Illumination (SIM), Single Molecule Localization (SMLM), and Stimulated Emission Depletion (STED) microscopy, all with significantly improved resistance against photobleaching. These probes generate images with excellent spatial and temporal resolution, require no genetic manipulations, are non-toxic in model cell lines and primary cardiomyocytes, even under conditions that amplify the effects of mitochondrial toxins, and can visualize mitochondrial dynamics for 12.5 hours.

Introduction

The mitochondrion is the powerhouse of the cell and contributes to numerous physiological processes including programmed cell death, innate immunity, autophagy, redox signaling, calcium homeostasis, and stem cell reprogramming. It powers the cell using oxidative phosphorylation, sustains the cell *via* multiple signaling activities, and kills the cell when necessary by initiating programmed cell death¹. Mitochondria are distinguished by multiple membrane structures that each perform distinct functions. The outer mitochondrial membrane (OMM) acts as a diffusion barrier, transduces signals in and out of the intermembrane space, and mediates multiple inter-organelle interactions². The inner mitochondrial membrane (IMM) consists of two discrete elements, the inner boundary membrane (IBM) and the cristae. The IBM parallels the OMM and contains transporters that shuttle ions and metabolites between the intermembrane space and the mitochondrial matrix, which houses enzymes responsible for the tricarboxylic acid cycle and heme biosynthesis. The cristae are densely packed membrane invaginations with diffraction-limited dimensions that support oxidative phosphorylation, mitochondrial DNA maintenance, and iron–sulfur cluster biogenesis¹. The IMM and OMM undergo continual fission and fusion, and individual crista remodel continuously in response to signals from other organelles². These dynamic events drive mitochondrial biogenesis and recycling and play a pivotal role in apoptosis signaling². Multiple pathological conditions, in both neurons (autosomal dominant optic atrophy)^{2,3} and energy-demanding myocytes (Barth's syndrome^{4,5} and Kearns-Sayre syndrome⁶) are associated with mutations in IMM-localized enzymes. The on-demand initiation of mitochondrial apoptosis is also desirable in the development of novel cancer therapies⁷.

Imaging the IMM is challenging due to its complex and diffraction-limited dimensions, especially if the goal is to study dynamics. Respiratory chain enzymes within cristae are sensitive to fluorophore-mediated or high-energy light-induced damage^{8,9}, which complicates use of super-resolution methods such as Stimulated Emission Depletion¹⁰ and Single Molecule Localization microscopies (STED and SMLM, respectively)^{11,12}.

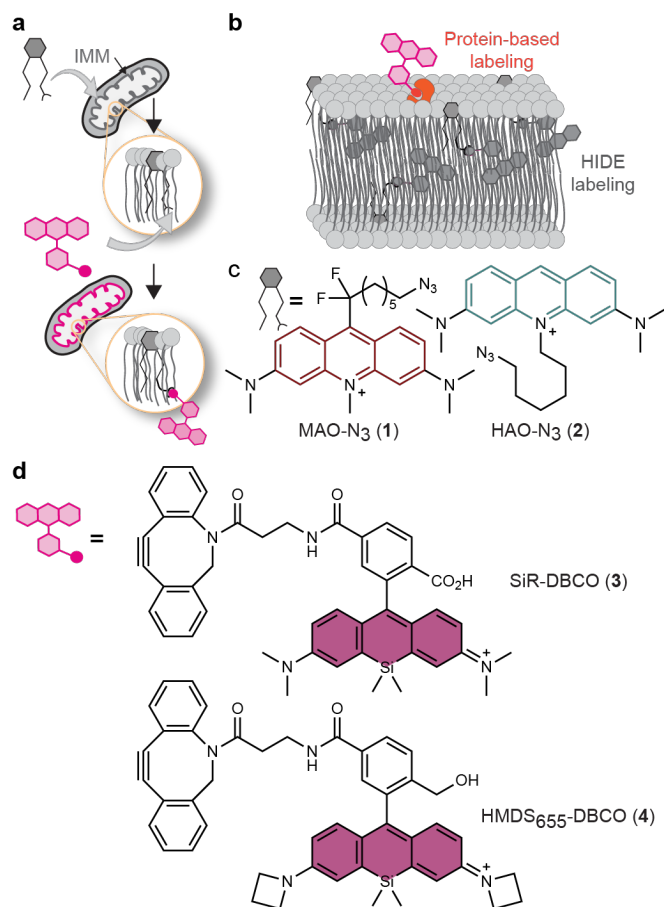


Fig. 1 | New HIDE probes for imaging the inner mitochondrial membrane. a, A HIDE probe is assembled in cellulo from two cell-permeant components. One component is a lipid-like small molecule appended to a reactive bioorthogonal functional group, such as an azide (N₃). The second is a modality-specific fluorophore equipped with the appropriate click reaction partner. b, HIDE probes extend imaging time because they localize within organelle membranes at higher density than possible with a self-labeling or fluorescent protein, and because in this hydrophobic environment only a small fraction of the molecules absorb light at the excitation wavelength. Since fewer molecules absorb light at any given time, fewer molecules bleach; what remains constitutes a reservoir of fresh fluorophores that replenish photobleached dye molecules during an imaging experiment. c, Derivatives of acridine orange, namely MAO-N₃ and HAO-N₃, used to generate the HIDE probes reported here. d, Structures of two silicon rhodamine dyes used in conjunction with MAO-N₃ and HAO-N₃. HMDS₆₅₅-DBCO was used for single molecule localization microscopy (SMLM), whereas SiR-DBCO was used for all other imaging modalities.

Some of these challenges are alleviated using small molecule dyes to image IMM dynamics at super-resolution. MitoPB Yellow, for example, is relatively photostable, localizes selectively to the IMM, and can be combined with STED to image IMM dynamics for extended time periods (300 frames over 390 s)¹³. However, MitoPB Yellow has limited spectral tuning potential for multi-color imaging and longer imaging times are precluded by the requisite high-energy (488 nm) excitation wavelength—mitochondrial swelling is induced after as few as 150 frames. Moreover, since MitoPB Yellow is not modular, it is not easily adapted to other imaging modalities. MitoEsq-635⁹ and PK Mito Orange (PKMO)¹⁴ also localize selectively to the IMM and are sufficiently photostable for STED imaging. MitoEsq-635 is excited by lower energy light (633 nm) than MitoPB Yellow, but is also less photostable, supporting the acquisition of only 40 STED frames over 50 min to visualize IMM dynamics⁹. PKMO is also excited by lower energy light (591 nm) and supports two-color STED imaging, but can support only 33 frames over 5 min¹⁴. These comparisons emphasize the need for new tools that support long time-lapse imaging of the mitochondrial inner membrane with high spatiotemporal-resolution. The ideal tools to study mitochondrial cristae remodeling and inter-organelle crosstalk should be bright, photostable, spectrally tunable, capable of supporting multiple imaging modalities, and derived from small molecules to enable studies in primary cell lines.

High-density, environmentally sensitive (HIDE) probes are small molecules that selectively label organelle membranes and resist photobleaching, leading to exceptionally long time-lapse images of organelles in live cells, even at super-resolution^{15–22}. Each HIDE probe consists of two parts (**Fig. 1a**). The first is an organelle-specific, lipid or lipid-like small molecule that is click-ready by virtue of a reactive functional group such as an azide (N_3). The second is a far-red fluorophore equipped with the appropriate reaction partner. These two parts, when added sequentially to live cells, undergo a bioorthogonal reaction that localizes the fluorophore at high density within the organelle membrane (**Fig. 1b**)¹⁸. The combination of dense localization and a hydrophobic environment results in a 10-50-fold enhancement in apparent photostability. HIDE probes have been used for long time-lapse imaging of the ER, Golgi, plasma membrane, mitochondrial matrix, and late endolysosomes^{15–22}. They label organelles in model cell lines, primary cells from patient samples²⁰, even neurons^{19,21}. And because they are assembled from two parts, the emission properties can be altered at will to support multi-color labeling and all established modalities²¹. One mitochondrial HIDE probe (RhoB-TCO) has been reported^{18,21}, but it labels mitochondrial subcompartments with minimal specificity; it is not selective for the IMM and cannot resolve IMM dynamics and fine structure^{18,21}.

Here we describe two new lipid-like small molecules, MAO- N_3 and HAO- N_3 (**Fig. 1c**) and use them to assemble HIDE probes selective for the inner mitochondrial membrane. These HIDE probes are benign to mitochondrial health, even under conditions that amplify mitochondrial toxicity. When paired with the appropriate fluorophore, MAO- N_3 supports long time-lapse imaging of the IMM using confocal, Structured Illumination (SIM), Single-Molecule Localization (SMLM), and Stimulated Emission Depletion (STED) microscopies. When paired with SiR-DBCO (**Fig. 1d**) and detected using confocal microscopy, MAO- N_3 visualizes the mitochondria for more than 12.5 h with minimal loss in signal intensity or cell viability, whereas the commercial dye MitoTracker DeepRed

loses > 50% signal intensity within 2h and the labeled cells proliferate slowly. When paired with SiR-DBCO and visualized using SIM, MAO-N3 visualizes the IMM for more than three times as long as the combination of SiR-CA and Halo-TOMM20, and for 16-times longer than the fluorescent protein marker mEmerald-TOMM20. When paired with HMDS655-DBCO (Fig. 1d) and visualized using SMLM, MAO-N3 distinguishes discrete IMM cristae structures that are not resolved using RhoB-HMSiR, a previously reported HIDE probe assembled using RhoB-TCO and HMSiR-Tz18. When paired with SiR-DBCO and visualized using STED, MAO-N3 again improved visualization of discrete IMM cristae structures relative to RhoB-SiR21, and supports the acquisition of a 125-frame STED movie showing the dynamics of cristae remodeling.

Results

HIDE probe design and synthesis. The design of inner mitochondrial membrane (IMM)-selective HIDE probes was inspired by the properties of nonyl acridine orange (NAO), a small molecule fluorophore that localizes selectively to the inner mitochondrial membrane (IMM)²³. Selectivity is believed to result from favorable, non-covalent interactions with cardiolipin, an unconventional lipid that is enriched within the IMM²⁴. Despite its selective localization, NAO is not photostable and cannot support long time-lapse imaging, especially using modalities that demand high-intensity irradiation^{23,25}. We designed two NAO analogs that could assemble HIDE probes suitable for long time-lapse imaging for the IMM: HAO-N₃ and MAO-N₃ (**Fig. 1c**). HAO-N₃ was synthesized from acridine orange *via* *N*-alkylation (**Extended Data Fig. 1a**)²⁵, while MAO-N₃ made use of a Minisci-type radical difluoroalkylation²⁶ reaction followed by methylation (**Extended Data Fig. 1b**).

In vitro characterization of HAO-N₃ and MAO-N₃. We first evaluated the *in vitro* photophysical properties of HAO-N₃ and MAO-N₃ in comparison with the parent fluorophore NAO. NAO exhibits an absorption maximum at 496 nm, an emission maximum at 519 nm, and a quantum yield of 0.16²⁷. The photophysical properties of HAO-N₃ are similar, with an absorption maximum at 496 nm, an emission maximum at 517 nm, and a quantum yield of 0.11 (**Fig. 2a** TOP and **Extended Data Fig. 2a-d**). In contrast, the absorption and emission curves for MAO-N₃ were red-shifted²⁸, with an absorption maximum at 520 nm and an emission maximum at 570 nm (**Fig. 2a** BOTTOM and **Extended Data Fig. 2e-h**). MAO-N₃ (QY = 0.01, $\epsilon = 3.0 \times 10^4 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) is also dimmer than HAO-N₃ (QY = 0.11, $\epsilon = 6.8 \times 10^4 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). Both the red shift and lower brightness of MAO-N₃ are favorable properties for the lipid-like component of a HIDE probe. These properties minimize cross-talk with GFP ($\lambda_{\text{ex}} = 490 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$) and RFP ($\lambda_{\text{ex}} = 555 \text{ nm}$, $\lambda_{\text{em}} = 583 \text{ nm}$) organelle markers as well as common small molecule fluorophores that would otherwise complicate multicolor imaging experiments. *In vitro* experiments verified that an aqueous solution of MAO-N₃ and HMDS₆₅₅-DBCO reacted within minutes *in vitro* at 37 °C to generate the expected SPAAC product, MAO-HMDS₆₅₅ (**Fig. 2b**, **Supplementary Fig. 1a**).

HIDE probes generated using HAO-N₃ and MAO-N₃ are non-toxic. Next we evaluated the effect of HAO-N₃, MAO-N₃, and HIDE probes derived thereof on the viability of HeLa cells under standard glucose-rich conditions (DMEM with 4.5 g/L glucose, supplemented with 10% FBS) and oxidative conditions supplemented with galactose (DMEM with 4.5 g/L galactose, supplemented with 10% FBS) (**Fig. 2c** and **Supplementary Fig. 2**). Cells grown in galactose-supplemented media are reliant on oxidative phosphorylation and thus sensitive to mitochondrial toxins²⁹. Cell viability was evaluated using a commercial assay that detects ATP (CellTiter Glo® 2.0). As expected, cells treated with the known mitochondrial toxin alexidine³⁰ displayed diminished ATP levels, and the effects were larger when cells were incubated in oxidative media containing galactose in place of glucose. The ATP levels of cells treated for 1 h with 100 nM MAO-N₃ or HAO-N₃, with or without co-incubation with 750 nM HMDS₆₅₅-DBCO (**Fig. 2c**) or 750 nM SiR-DBCO (**Supplementary Fig. 2**), did not differ significantly from the ATP levels of untreated cells. For HeLa cells treated with 2-deoxy-D-glucose (2-DG), a common glycolysis inhibitor,

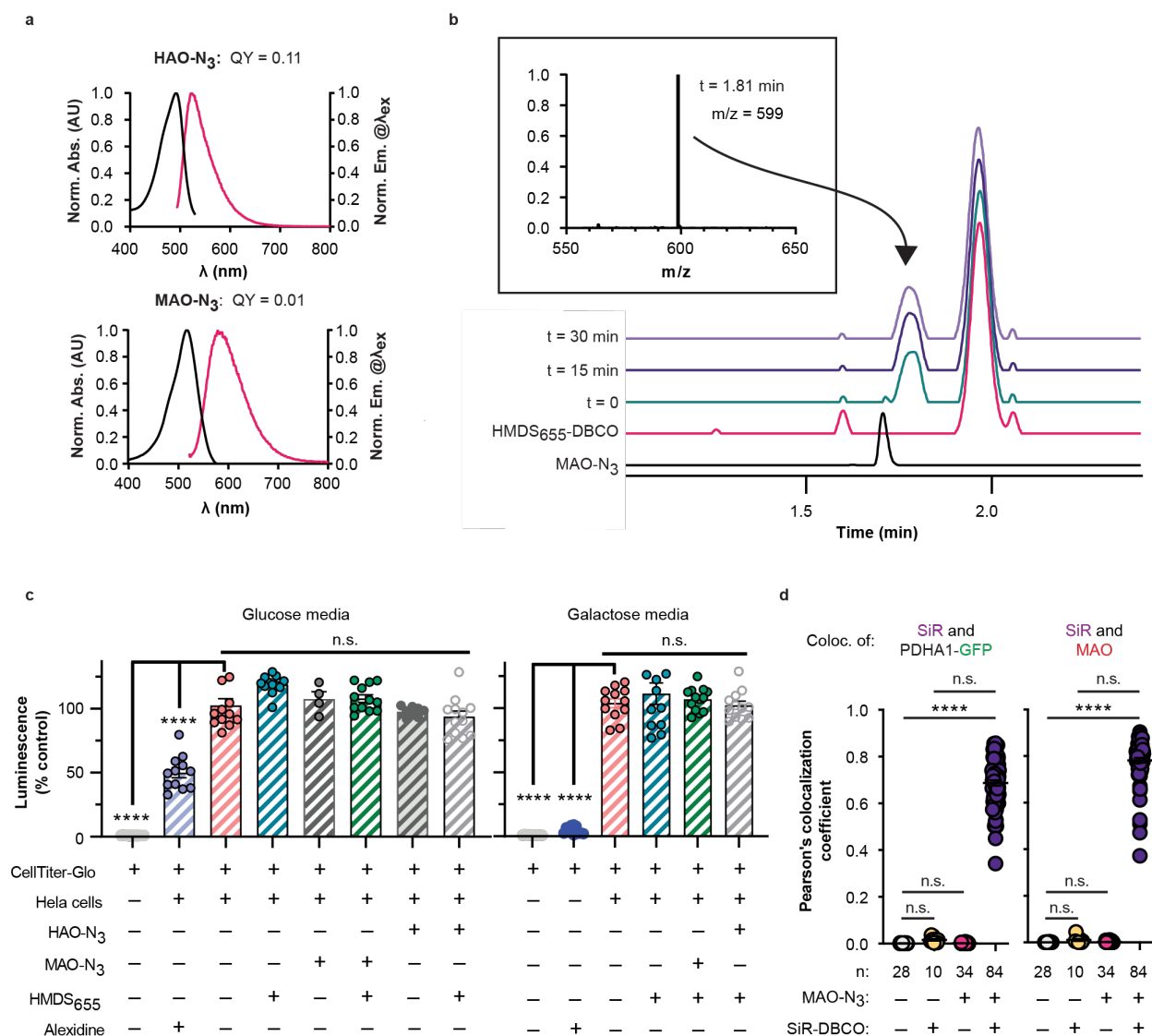


Fig. 2. Assembly and characterization of HIDE probes generated using HAO-N₃ and MAO-N₃. **a**, Absorption and emission spectra and quantum yield of HAO-N₃ and MAO-N₃ at 2 μ M concentration in Dulbecco's phosphate-buffered saline (DPBS, pH 7.4, room temperature). **b**, *In vitro* SPAAC reaction of 100 μ M MAO-N₃ and 400 μ M HMDS₆₅₅-DBCO at 37 °C in DI H₂O. At the times indicated, 1 μ L aliquots of the reaction mixture were withdrawn and analyzed by LC/MS as described in **Online Methods**. **Insert**: Mass spectrum confirming the formation of MAO-HMDS₆₅₅ (MW = 1197.6, m/z = 599), which elutes at 1.81 min. **c**, HeLa cells incubated in standard glucose-rich media (DMEM with 4.5 g/L glucose, supplemented with 10% FBS) or galactose-supplemented oxidative media (DMEM with 4.5 g/L galactose, supplemented with 10% FBS) were treated with MAO-N₃, HAO-N₃ and/or HMDS₆₅₅-DBCO and the ATP levels were measured immediately as described in **Online Methods**. Plots show the relative bioluminescence signals (% relative to untreated cells). Untreated cells serve as a negative control; cells treated with 5 μ M alexidine serve as a positive control. Each set of conditions include 12 biological replicates. **d**, Pearson's colocalization coefficients (PCC) representing the colocalization of 750 nM SiR-DBCO with 100 nM MAO-N₃ or PDHA1-GFP (a *bona fide* mitochondrial matrix marker). PCC (SiR/PDHA1-GFP) = 0.72 ± 0.01 , PCC (SiR/MAO) = 0.65 ± 0.01 . *n* sourced from at least two biological replicates. Error bars = s.e.m., *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$, n.s. = not significant, from one-way ANOVA with Dunnett's post-analysis accounting

comparison to the negative control where cells were incubated with cell culture media only (for **c**) or Tukey's multiple comparison test (for **d**).

even though a global decrease of ATP level was observed, no significant changes were present in MAO-N₃, MAO-HMDS₆₅₅ or MAO-SiR labeled cells compared to untreated control (**Extended Data Fig. 3a, b**). We also treated cardiomyocytes differentiated from human induced pluripotent stem cells (hiPSC) with 100 nM MAO-N₃ followed by 750 nM SiR-DBCO. No apparent effect on the rate of cell body contractions was observed over the course of 110 s (**Supplementary Movie 1**).

To evaluate more quantitatively whether HIDE probes prepared using MAO-N₃ affect mitochondrial function, we performed real-time respirometry assays (**Extended Data Fig. 3c-f**). HeLa cells were labeled with MAO-N₃, MAO-SiR or MAO-HMDS₆₅₅, and the oxygen consumption and extracellular acidification rates were measured under multiple energetic states and in both glucose-rich and galactose-supplemented media. MitoTracker Deep Red (MTDR), a commercial, carbocyanine-based mitochondria dye with an emission profile similar to SiR ($\lambda_{\text{ex}} = 644 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$) was also evaluated for comparison purposes. We measured the oxygen consumption rate (OCR) of each labeled cell population under basal conditions, as well as those in which ATP synthesis was inhibited (using oligomycin), maximized (using FCCP), and in which oxidative phosphorylation was inhibited (using Rotenone plus Antimycin A) (**Extended Data Fig 3 c,d**).

As anticipated, the measured basal OCR for unlabeled HeLa cells in glucose-rich media ($6.41 \pm 0.07 \text{ pmol/min/kcells}$) was slightly lower than the values measured in galactose-supplemented media ($8.67 \pm 0.08 \text{ pmol/min/kcells}$); both values compare favorably with literature reports²⁹. Regardless of media, OCR decreases when ATP synthesis is inhibited with oligomycin, maximized when the inner mitochondrial membrane is depolarized by FCCP, and decreases to virtually zero when oxidative phosphorylation is inhibited using Rotenone plus Antimycin A. In both glucose and galactose-supplemented media, OCR is diminished by the presence of MTDR but not by the presence of MAO-SiR or MAO-HMDS₆₅₅. The apparent decrease in maximal OCR in glucose-rich media after FCCP treatment when cells were labeled with only MAO-N₃ is not statistically significant, and regardless is not observed when the azide functional group was lost after bioconjugation. These studies provide confidence that MAO-SiR or MAO-HMDS₆₅₅ have no measurable effect on mitochondrial function in both standard (glucose-rich) or oxidative (galactose-supplemented) media.

To fully understand how MAO-generated HIDE probes affect the cellular bioenergy generation dynamics, we also calculated the rate of ATP generation via mitochondrial-related oxidative phosphorylation (MitoATP) as well as the rate generated by glycolysis (GlycoATP) using OCR and extracellular acidification rate (ECAR) values³¹. When using standard, glucose-rich media, no significant changes in OCR, mitoATP, or glycoATP were observed regardless of whether cells were treated with MAO-N₃, MAO-SiR or MAO-HMDS₆₅₅. In comparison, MTDR-labeled cells exhibited a 57% increase in glycoATP and 51% decrease in mitoATP, which indicated that this commonly used fluorophore is not

benign to oxidative phosphorylation (**Extended Data Fig. 3e**)³². When cells were incubated in galactose-supplemented media, although all the labeled cells showed an increase in glycoATP, only MTDR labeled cells showed a 36% decrease in mitoATP (**Extended Data Fig. 3f**). These data further support the conclusion that MAO-N₃ or MAO-derived HIDE probes are benign to mitochondrial respiration and ATP generation.

HIDE probes derived from MAO-N₃ and HAO-N₃ localize to mitochondria. Confocal microscopy was used to quantify the extent to which MAO-SiR, assembled *in cellula* using MAO-N₃ and SiR-DBCO (**Supplementary Fig. 1b**), colocalized with a *bona fide* mitochondrial marker in live HeLa cells. As a marker, we used a fusion of GFP with the leader sequence of pyruvate dehydrogenase (E1) alpha subunit-1 (PDHA1-GFP), which localizes to the mitochondrial matrix³³ and whose emission is separate from that of MAO and SiR. Mitochondria in HeLa cells expressing PDHA1-GFP appeared as multiple discontinuous tubular structures emblematic of healthy mitochondria^{2,34} in the presence or absence of 100 nM MAO-N₃ and/or 750 nM SiR-DBCO (**Supplementary Fig. 3b**). Cells treated with MAO-N₃ alone showed significant signal at 570 nm, near the MAO emission maximum, which colocalized with the emission associated with PDHA1-GFP (PCC = 0.77 ± 0.01 , **Supplementary Fig. 3c**). Cells treated with SiR-DBCO in the absence of MAO-N₃ showed negligible signal at 660 nm, near the SiR emission maximum. However, cells treated with both MAO-N₃ and SiR-DBCO showed evidence of signal from SiR, which colocalized well with that of PDHA1-GFP and MAO (PCC = 0.72 ± 0.01 and 0.65 ± 0.01 , respectively) (**Fig. 2d**). Although HeLa cells treated with 100 nM MAO and 750 nM SiR-DBCO showed significant signal at 520 nm, near the MAO emission maximum, negligible signal in the SiR channel was observed—illustrating that the azido group of MAO-N₃ and a subsequent SPAAC reaction^{35,36} is essential to recruit SiR-DBCO to the mitochondria (**Supplementary Fig. 4**). Additional experiments demonstrated minimal colocalization of MAO-SiR with either the ER or the Golgi (**Extended Data Fig. 4**), using corresponding *bona fide* markers (GALNT1-GFP³⁷ for Golgi and KDEL-GFP³⁸ for ER). Analogous experiments using HAO-N₃, SiR-DBCO, and RFP-tagged organelle markers (**Extended Data Fig. 5**), confirmed that HAO-N₃ is also capable of mitochondria-specific labeling. We conclude that HAO-N₃ and MAO-N₃ localize selectively to mitochondria in live HeLa cells, and formation of the corresponding HIDE probe upon SPAAC reaction with SiR-DBCO localizes SiR to mitochondria.

HIDE probe MAO-SiR supports confocal live-cell imaging of mitochondria for more than 12 h with no loss of signal intensity. We labeled HeLa cells with 200 nM MAO-N₃ and 100 nM SiR-DBCO and obtained a 220-frame confocal time-lapse series with a 670 x 670 μm^2 field of view over the course of 12.5 h (**Fig. 3a** and **Supplementary Movie 2**). Each frame in the time series was reconstructed by stitching 16 individual frames together. Over this time period, HeLa cells labeled with MAO-SiR exhibited negligible signal decay and increased in density by $27 \pm 6\%$, which compares well with an increase of $23 \pm 1\%$ for untreated cells (**Fig. 3c,d** and **Supplementary Movie 3**)³⁹. In comparison, cells labeled with 100 nM MitoTracker Deep Red, a commercial, carbocyanine-based mitochondria dye with a similar emission profile ($\lambda_{\text{ex}} = 644 \text{ nm}$, $\lambda_{\text{em}} = 665 \text{ nm}$), increased in density by only $9 \pm 4\%$, and the signal intensity decreased by 50% within 2 h (**Fig. 3b-d** and **Supplementary Movie 4**). Although previous work demonstrated HIDE probes

increase the length of time over which bright images can be acquired using SMLM and STED microscopy^{15,18–22}, these experiments reveal that comparable improvements are seen even during microscopy using lower intensity irradiation.

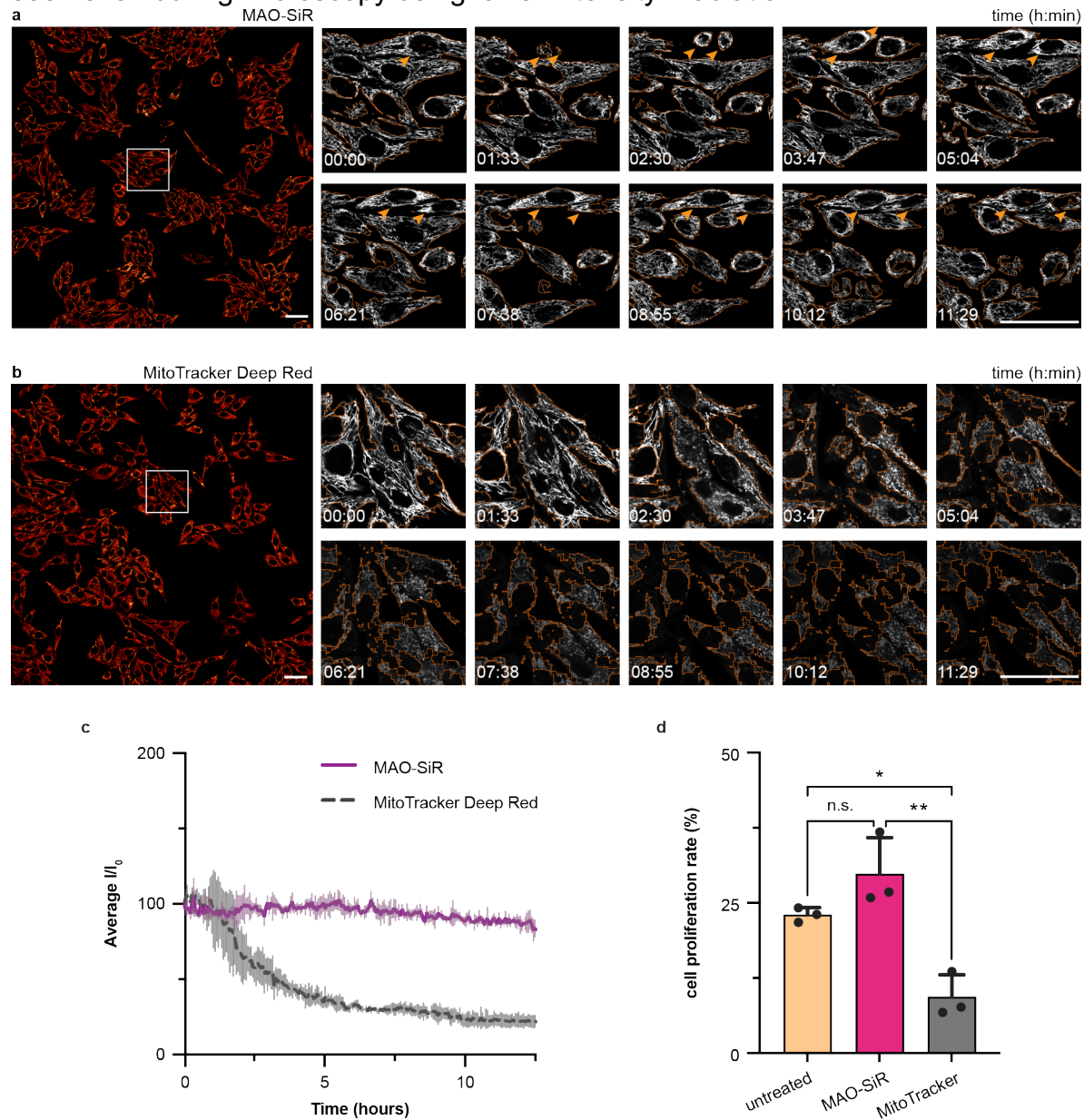


Fig. 3. Long time-lapse confocal image using MAO-SiR or MitoTracker Deep Red. **a-b**, HeLa cells were labeled with **a**, 200 nM MAO- N_3 and 100 nM SiR-DBCO or **b**, 100 nM MitoTracker Deep Red and imaged using a point-scanning confocal microscope. A total of 220 frames of $670 \times 670 \mu m^2$ images were obtained over 12.5 h. Individual frames from regions of interest (white rectangle) shown to the right. Orange outlines indicate segmentation boundaries used to isolate signals for intensity quantification. Orange arrows in panel **a** represent observable cell division events. Scale bar: 50 μm . **c**, Plots of average signal decay over time. Mean signals from segmented regions were averaged to determine the mean frame intensity, Mean frame intensities from each dataset were averaged and plotted for each time point along with the relative standard deviation. $n = 3$ biologically independent replicates. $t_{1/2}$ (MitoTracker) = 1.96 ± 0.05 h, $t_{1/2}$

(MAO-SiR) = N.A. Intensities for plots in **c** were scaled relative to the first frame's intensity in the data sets. **d**, Cell proliferation rates for untreated, MAO-SiR or MitoTracker Deep Red labeled HeLa cells over the entire FOV over 12.5 h. $n = 3$ biologically independent replicates. Error bars = s.e.m. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$, n.s. = not significant, from one-way ANOVA with Tukey's multiple comparison test (for **d**)

MAO-SiR labels the IMM even after membrane depolarization. Many IMM and mitochondria-targeting probes, such as PKMO¹⁴, TMRM⁴⁰, RhoB-TCO/SiR-Tz²¹, label mitochondria on the basis of membrane potential. As a result, none of these probes are suitable for imaging depolarized mitochondria. Because MAO-SiR is believed to label mitochondria via an interaction with specific lipids, a depolarization-independent mechanism, we hypothesized that it would be retained within depolarized mitochondria and not be lost as is observed for probes retained on the basis of membrane potential. To test this hypothesis, we labeled HeLa cells with MAO-SiR and depolarized the cells with FCCP, a well-known mitochondrial membrane uncoupler which rapidly shuttles protons across inner mitochondrial membrane (**Extended Data Fig. 6a,b**)⁴¹. Tetramethylrhodamine methyl ester (TMRM), which is retained in mitochondria on the basis of membrane potential⁴², was used as a control (**Extended Data Fig. 6c,d**). The fluorescent signal from MAO-SiR is retained within mitochondria for at least 30 min after FCCP treatment (**Extended Data Fig. 6b,e-g**). By contrast, the fluorescent signal due to TMRM dropped by 98% **within 30 min** after depolarization with FCCP (**Extended Data Fig 6d,f,g**).

SIM imaging of the mitochondria using SiR-DBCO and MAO-N₃. Having confirmed using confocal microscopy that the HIDE probe MAO-SiR is non-toxic, mitochondria-specific, and supports extended-time imaging, we turned to higher-resolution methods to evaluate its intra-organelle localization. HeLa cells expressing either mEmerald-TOMM20 (OMM marker) or PDHA1-GFP (mitochondrial matrix marker) were incubated with 75 nM MAO-N₃ and 600 nM SiR-DBCO and imaged using lattice SIM (**Fig. 4a**). Images of treated cells expressing mEmerald-TOMM20 show clear differentiation of the OMM (mEmerald) and internal mitochondrial fine structure (SiR) (**Fig. 4b,c**). Images of treated cells expressing PDHA1-GFP (mitochondrial matrix marker) also show differences in signal localization (**Fig. 4d-f**). While a profile of the signals due to PDHA1-GFP are relatively continuous, those due to MAO-SiR are not (**Fig. 4e,f**), revealing a discontinuous structure that resembles those seen in **Fig. 4b**. Due to the limited resolution of SIM (**Supplementary Fig. 5a-d**), the signals from the SiR channel (FWHM = 171 ± 9 nm, **Supplementary Fig. 5d**) cannot fully resolve the cristae topology of the IMM^{13,43}. Despite this limitation, these SIM experiments provide strong evidence that the HIDE probe MAO-SiR labels neither the OMM nor the mitochondrial matrix.

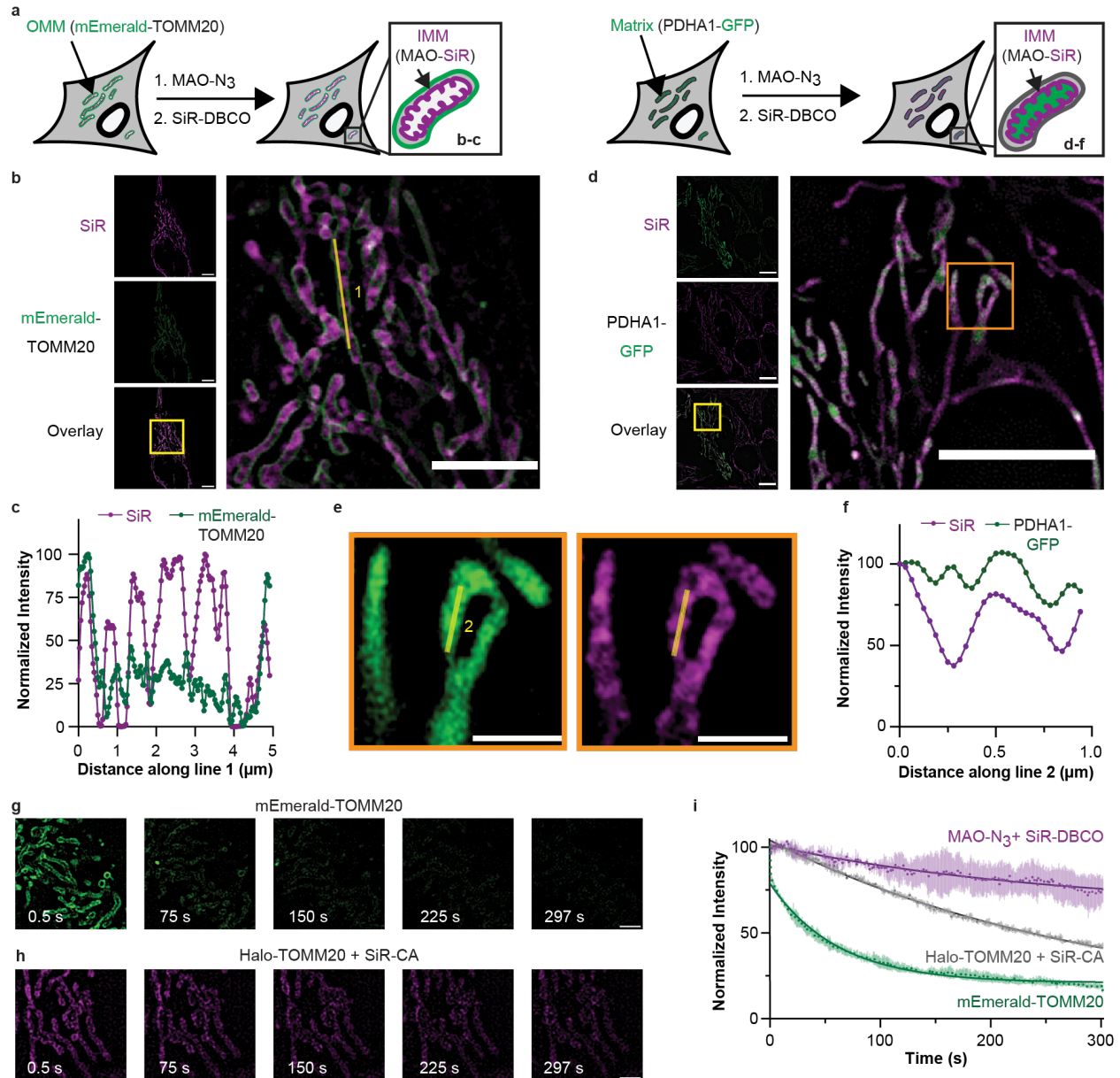


Fig. 4. SIM imaging of mitochondria using MAO-SiR. **a**, HeLa cells expressing PDHA1-GFP or mEmerald-TOMM20 were treated with 75 nM MAO-N₃ and 600 nM SiR-DBCO as described in **Online Methods** and imaged using lattice SIM. **b**, SIM images of cells expressing mEmerald-TOMM20 and labeled with MAO-SiR as described in **a**. Scale bar: 5 μm. **c**, Plots of signal intensity from SiR and mEmerald along line 1. **d**, SIM images of cells expressing PDHA1-GFP and labelled with MAO-SiR as described in **a**. Scale bar: 5 μm. **e**, Enlarged image within orange box in panel **d**. **f**, Plots of signals due to SiR and GFP along line 2. **g-j**, Long time-lapse SIM imaging of IMM dynamics using the HIDE probe MAO-SiR, Halo-TOMM20 (labeled with SiR-CA to generate SiR-TOMM20), or mEmerald-TOMM20 in HeLa cells. Panels **g-j** are representative images from a 500-frame SIM series obtained over 297 s (**Supplementary Movie 5**). **g**, SIM image at $t = 0.5$ s. Scale bar: 10 μm. **h**, Snapshots of the bracketed region indicated in **g** at the times indicated. Scale bar: 2 μm. **i**, Kymograph of the yellow dashed line in **g**. Scale bar: 1 μm. **j**, IMM fission (white arrow) and fusion (yellow arrow) events from $t = 9.5$ s to $t = 33.3$ s in the bracketed region in **g**. Scale bar: 2 μm. **k and l**, Snapshots of selected regions of interest from **Supplementary Movie 6 and 7**, obtained

from HeLa cells expressing **k**, mEmerald-TOMM20 (**Supplementary Movie 6**) or **l**, SiR-TOMM20 (**Supplementary Movie 7**), at times indicated. Scale bar: 2 μm . **m**, Plots illustrated normalized SiR fluorescence signals of SiR-MAO, SiR-TOMM20, and green fluorescent protein signal from mEmerald-TOMM20 over time. $n = 3$ regions of interest (ROI), $N = 1$ cell. Intensities for plot **m** were scaled relative to the first frame's intensity in the data sets

To evaluate the time over which interpretable images can be acquired using SIM, we treated HeLa cells with MAO- N_3 and SiR-DBCO as described above and imaged them for 297 s (500 frames) (**Fig. 4g-j**, **Supplementary Movie 5**). Over a field of view sufficient to visualize an entire cell (**Fig. 4g**), this lattice SIM movie captured both IMM fission (white arrow) and fusion (yellow arrow) events (**Fig. 4j**) with no change in mitochondrial morphology (**Fig. 4h**) and excellent signal retention (**Fig. 4i**). When cells were imaged using mEmerald-TOMM20, little or no signal was observed after 75 s (**Fig. 4k**, **Supplementary Movie 6**). When cells expressing Halo-TOMM20-N-10 were labeled with SiR-HaloTag-ligand (SiR-CA) to generate the fluorophore-protein conjugate SiR-TOMM20, significant signal loss was also evident (**Fig. 4l**, **Supplementary Movie 7**). Exponential decay analyses of intensity data revealed a signal half-life of 713.5 ± 8.0 s for MAO-SiR, which is 3.2 times as long as that of SiR-CA/Halo-TOMM20 ($t_{1/2} = 224.4 \pm 0.6$ s) and 16 times longer than mEmerald-TOMM20 ($t_{1/2} = 42.4 \pm 0.5$ s) (**Fig. 4m**). The signal half-life of HAO-SiR ($t_{1/2} = 248.2 \pm 1.2$ s) and the previously reported mitochondrial HIDE probe RhoB-SiR ($t_{1/2} = 233.0 \pm 1.3$ s) were both also significantly smaller than MAO-SiR (**Extended Data Fig. 7**).

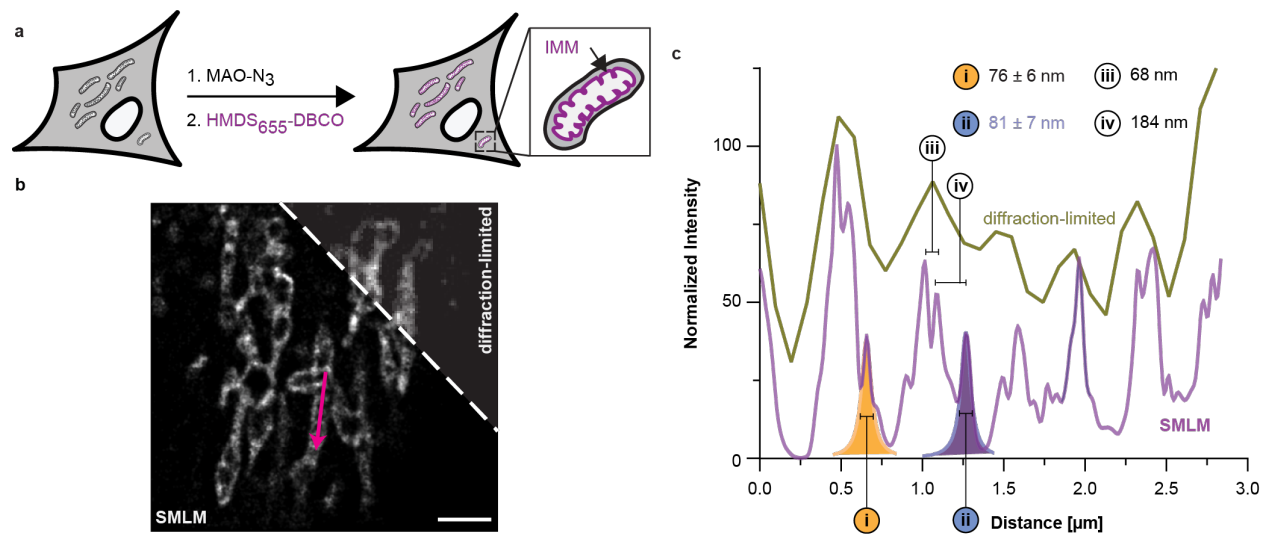


Fig. 5. SMLM imaging of the inner mitochondrial membrane using a HIDE probe assembled from MAO- N_3 and HMDS₆₅₅-DBCO (MAO-HMDS₆₅₅). **a**, HeLa cells were treated with 75 nM MAO- N_3 and 600 nM HMDS₆₅₅-DBCO and imaged using a widefield microscope with SMLM capability. **b**, Representative

SMLM image reconstructed with 800 frames ($t = 4$ s); the corresponding diffraction-limited image is shown for comparison. Scale bar: 2 μm . **c**, Plot of signal intensity along the magenta arrow in panel b in both the reconstructed SMLM and wide-field images. Two peaks in the SMLM image labeled i and ii were fitted into a Lorentzian function to obtain FWHM values of 76 ± 6 nm and 81 ± 7 nm, respectively. Distances (iii and iv) between two pairs of adjacent peaks were measured from centroid to centroid, giving values of 68 nm and 184 nm, respectively.

SMLM imaging of the mitochondria using HMDS₆₅₅-DBCO and MAO-N₃. We next used an MAO-derived HIDE probe and SMLM to visualize IMM fine structure. HeLa cells were treated with 75 nM MAO-N₃ for 40 min, washed, incubated for 20 min with 600 nM HMDS₆₅₅-DBCO⁴⁴ (**Fig. 5a**), and imaged using SMLM in an antioxidant-supplemented buffer¹⁸. A representative image constructed from 800 frames ($t = 4$ s) is shown in **Fig. 5b**. The low temporal resolution made it challenging to capture the fine structure of continuously moving mitochondria⁴⁵. Nevertheless, the images reveal multiple tubular cristae structures in a ladder-like pattern; these features were not resolved by a previously reported HIDE probe generated using RhoB-TCO and HMSiR-Tz¹⁸. Fourier Ring Correlation analysis was performed which yielded a global resolution of 161.7 nm⁴⁶. The resolution of the images using MAO-HMSiR is sufficiently high (FWHMs of 76 ± 6 nm and 81 ± 7 nm for representative peaks in the line profile) (**Fig. 5c**) to establish distances between adjacent cristae of 68 nm to 184 nm, comparable to previous reports^{13,43}.

STED imaging of IMM cristae enabled by SiR-DBCO and MAO-N₃. Having validated that HIDE probes assembled using MAO-N₃ offer tangible improvements in the time over which IMM images can be generated using both SIM and SMLM, we turned to the toughest test - STED. STED microscopy offers greater spatial resolution than SIM and greater temporal resolution than SMLM and is thus well-suited to reveal rapid dynamics in diffraction-limited cellular compartments such as the IMM. Previous IMM markers that support STED imaging, such as SiR-Cox8A⁴⁷, MitoPB Yellow¹³, PKMO¹⁴, and MitoEsq635⁹, are limited by photostability or photocytotoxicity.

We first examined different data acquisition modes in STED microscopy, using HeLa cells labeled with MAO-SiR. We acquired images of the cristae using gated STED and deconvolved the images using a theoretical point spread function (PSF) (FWHM up to 63 ± 2 nm, **Fig. 6a**, TOP). We then filtered the fluorescence signals on the basis of fluorescence lifetime in tauSTED mode⁴⁸ and acquired images of the IMM with excellent resolution (**Fig. 6a**, BOTTOM). These images clearly demark densely-packed, ladder-like mitochondrial cristae with regular dimensions (**Fig. 6b**) that are indiscernible using confocal microscopy (**Extended Data Fig. 8 a,b**). Moreover, the fluorescent signal from MAO-SiR (half-life = 112.7 ± 2.1 s) diminished 5-times more slowly than signal from SiR-Cox8A (half-life = 17.4 ± 0.4 s) and 10-times more slowly than SiR-TOMM20 (half-life = 10.4 ± 0.2 s) (**Fig. 6c**). This apparent photostability supported the acquisition of a 162.5 s movie of cristae dynamics containing 125 tauSTED frames (**Fig. 6d-f**). Distinct cristae are visible in images obtained with MAO-SiR until the last frame without the need for genetic manipulation (**Fig. 6d**, **Supplementary Movie 8**), whereas images obtained with SiR-Cox8A and SiR-TOMM20 are lost within 65 s (**Fig. 6e-f**, **Supplementary Movie 9, 10**).

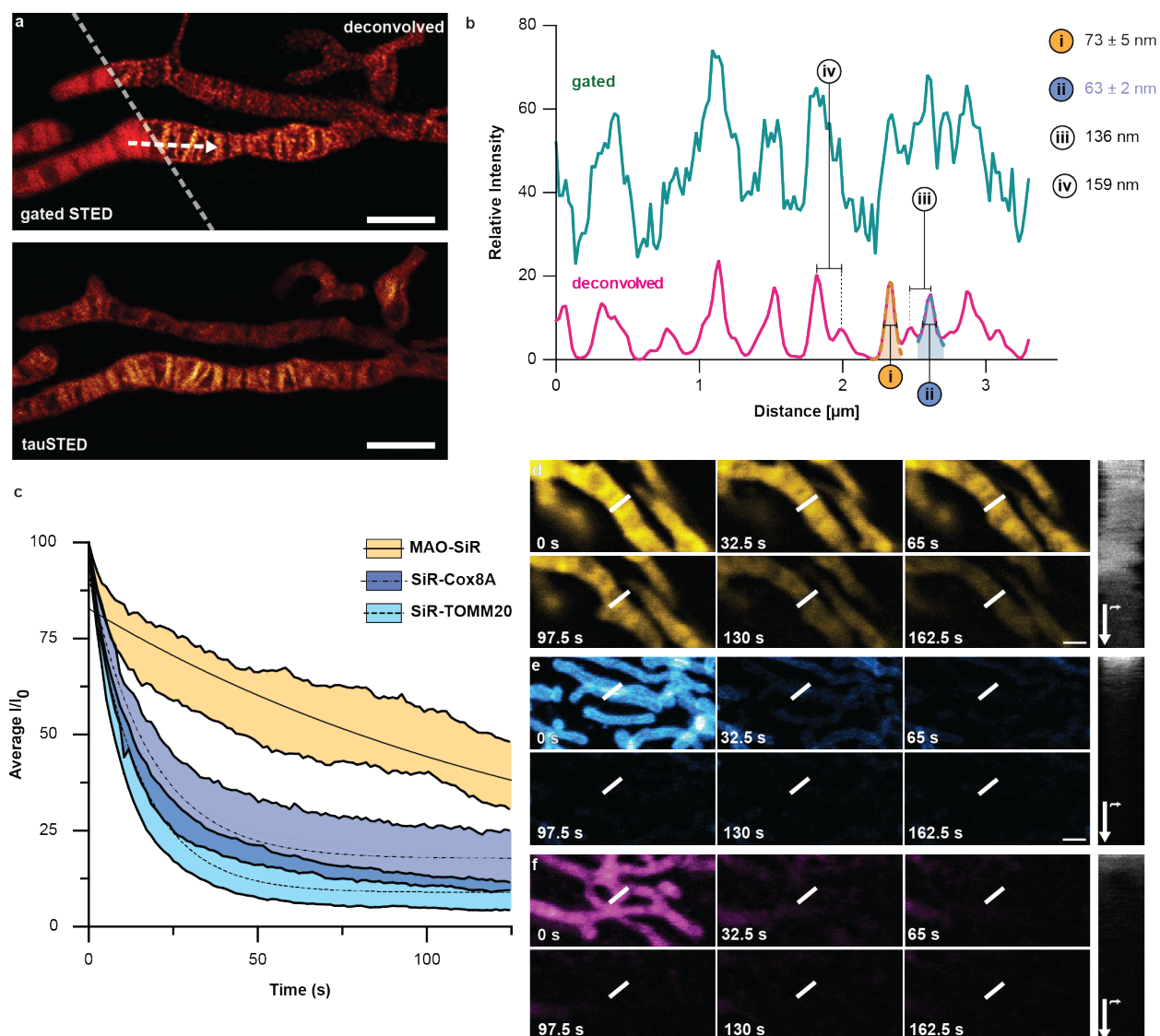


Fig. 6. STED imaging of mitochondrial IMM using MAO-SiR. **a**, Top: gated and deconvolved STED image; Bottom: tauSTED image generated using fluorescence lifetime filtering. **b**, Plot of SiR signal intensity along white arrow in **a** in the gated STED and deconvolved STED image. Two peaks in the STED image were fitted into a Lorentzian function to obtain an FWHM of (i) $73 \pm 5 \text{ nm}$ and (ii) $63 \pm 2 \text{ nm}$, respectively. Distances between pairs of adjacent peaks were also measured from centroid to centroid, giving values of (iii) 136 nm and (iv) 159 nm, respectively. Scale bar: 2 μm . **c**, Plots illustrated relative SiR fluorescence signals of SiR-MAO, SiR-SNAP/Cox8A-SNAP (SiR-Cox8A), and SiR-CA/Halo-TOMM20 (SiR-TOMM20), over 125 frames obtained in 162.5 s. $N = 7$ FOVs over 7 individual cells. Intensities for plot **c** were scaled relative to the first frame's intensity in the data sets. Error bars = s.e.m. **d-e**, Selected STED images using MAO-SiR (panel **d**, yellow, **Supplementary Movie 8**), SiR-CA/Halo-TOMM20 (panel **e**, blue, **Supplementary Movie 9**), or SiR-Cox8A (panel **f**, magenta, **Supplementary Movie 10**), each from a 125-frame, 162.5 s movie at indicated time points. White bar in each frame indicates the region of interest (ROI) for the kymograph in the right most panel. Kymograph plotted to the right of each frame set to track intensity of signal in ROI for each frame in the movie. Scale bar: 1 μm .

Discussion

The mitochondria is a highly dynamic organelle that undergoes coordinated and balanced cycles of fission and fusion to maintain its shape, content, transport ability, and size. These transient and rapid morphological changes allow mitochondria to meet metabolic demands, initiate the degradation of damaged organelles, and contribute to control of the cell cycle, immunity, and cellular apoptosis. Defects in mitochondrial dynamics cause disease⁴⁹. Decoupled fusion and fission and the resulting mitochondrial fragmentation occur in response to stress and during heart failure⁵⁰, neurodegenerative disease, cancer, and obesity. Recent studies have shown that the dynamics of the inner mitochondrial membrane are critical, for example, for the loss of mitochondrial DNA (mtDNA) from the matrix into the cytosol to trigger the innate immune cGAS/STING pathway⁵¹.

Imaging the mitochondria to detect dynamic morphological changes—especially those involving the complex inner membrane—is especially challenging, as the subcompartment is replete with enzymes that generate dye-inactivating reactive oxygen species. Moreover, the imaging process itself can damage essential macromolecules. These challenges are exacerbated when one seeks to evaluate changes in mitochondrial dynamics or interactions that occur over significant time frames, in primary or patient-derived cells, and/or using super-resolution imaging modalities that require intense irradiation. One proven strategy to overcome these challenges involves labeling the organelle membrane lipids as opposed to the proteins embedded within^{15–22,52}. Labeling the membrane lipids positions the dye at high density within a hydrophobic environment in which most dye molecules exist in a dark state and do not absorb light. Balancing the labeling density with the fraction of molecules in the dark state produces bright images that last because the dark state molecules act as a reservoir to replenish dyes that bleach.

Here we describe new HIDE probes that selectively image an organelle sub-compartment – the inner mitochondrial membrane – in live cells and over exceptional long time frames. These probes, all assembled from the lipid-like molecule MAO-N₃, are photostable, lack detectable toxicity, and support exceptional spatial resolution of the IMM using multiple modalities, all without genetic manipulations. Confocal images of the mitochondria generated using MAO-SiR remained stable for more than 12.5 h with little or no loss in signal intensity or cell viability, more than 6-times longer than possible using MitoTracker Deep Red. SIM images of the inner mitochondrial membrane using MAO-SiR lasted for 16-times longer than those obtained using mEmerald-TOMM20, and supported acquisition of 500 frames over the course of 5 min as opposed to 20 frames over 2.8 min possible with MitoTracker Green⁵³. When paired with HMDS₆₅₅-DBCO (**Fig. 1d**) and visualized using SMLM, MAO-N₃ distinguished discrete IMM cristae structures that were not resolved using a previously reported HIDE probe assembled using RhoB-TCO¹⁸. When paired with SiR-DBCO and visualized using STED (**Fig. 1d**), MAO-N₃ again improved visualization of discrete IMM cristae structures relative to RhoB-TCO²¹, and supported the acquisition of a 125-frame STED movie showing cristae dynamics. HIDE probes based on MAO-N₃ are versatile, non-toxic, photostable, and completely cell-permeant. These features should make them highly useful, alone or in combination with

other organelle markers, for long-term analysis of natural and pathological mechanisms related to IMM dynamics and inter-organelle interactions.

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Author Contributions

S.Z. and A.S. conceived the project; S.Z., N.D., D.M., and A.S. designed experiments. S.Z. designed and synthesized MAO-N₃ and HAO-N₃; S.Z. and L.L synthesized DBCO-conjugated fluorophores. S.Z., N.D., and D.M. prepared HeLa samples for microscopy and performed imaging experiments. K.M., S.Z. and E.M. prepared iPSC-CM samples for microscopy. S.Z., N.D., and D.M. analyzed the data with inputs from A.S. S.Z., N.D., L.L., and A.S. wrote the manuscript.

Competing Interests

Authors declare no existing competing interest.

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References

1. Spinelli, J. B. & Haigis, M. C. The multifaceted contributions of mitochondria to cellular metabolism. *Nat Cell Biol* 20, 745–754 (2018).
2. Giacomello, M., Pyakurel, A., Glytsou, C. & Scorrano, L. The cell biology of mitochondrial membrane dynamics. *Nat Rev Mol Cell Biol* 21, 204–224 (2020).
3. Lenaers, G. et al. Dominant optic atrophy. *Orphanet Journal of Rare Diseases* 7, 46 (2012).
4. Murphy, E. et al. Mitochondrial Function, Biology, and Role in Disease. *Circulation Research* 118, 1960–1991 (2016).
5. Peoples, J. N., Saraf, A., Ghazal, N., Pham, T. T. & Kwong, J. Q. Mitochondrial dysfunction and oxidative stress in heart disease. *Exp Mol Med* 51, 1–13 (2019).
6. Kabunga, P. et al. Systematic review of cardiac electrical disease in Kearns–Sayre syndrome and mitochondrial cytopathy. *International Journal of Cardiology* 181, 303–310 (2015).
7. Klein, K. et al. Role of Mitochondria in Cancer Immune Evasion and Potential Therapeutic Approaches. *Frontiers in Immunology* 11, 2622 (2020).
8. Jakobs, S., Stephan, T., Ilgen, P. & Bräser, C. Light Microscopy of Mitochondria at the Nanoscale. *Annu. Rev. Biophys.* 49, 289–308 (2020).
9. Yang, X. et al. Mitochondrial dynamics quantitatively revealed by STED nanoscopy with an enhanced squaraine variant probe. *Nat Commun* 11, 3699 (2020).
10. Klar, T. A., Jakobs, S., Dyba, M., Egner, A. & Hell, S. W. Fluorescence microscopy with diffraction resolution barrier broken by stimulated emission. *Proceedings of the National Academy of Sciences* 97, 8206–8210 (2000).
11. Betzig, E. et al. Imaging Intracellular Fluorescent Proteins at Nanometer Resolution. *Science* 313, 1642–1645 (2006).
12. Dickson, R. M., Cubitt, A. B., Tsien, R. Y. & Moerner, W. E. On/off blinking and switching behaviour of single molecules of green fluorescent protein. *Nature* 388, 355–358 (1997).
13. Wang, C. et al. A photostable fluorescent marker for the superresolution live imaging of the dynamic structure of the mitochondrial cristae. *PNAS* 116, 15817–15822 (2019).
14. Liu, T. et al. Multi-color live-cell STED nanoscopy of mitochondria with a gentle inner membrane stain. *Proceedings of the National Academy of Sciences* 119, e2215799119 (2022).
15. Erdmann, R. S. et al. Super-Resolution Imaging of the Golgi in Live Cells with a Bioorthogonal Ceramide Probe. *ANGEWANDTE CHEMIE-INTERNATIONAL EDITION* 53, 10242–10246 (2014).

16. Bottanelli, F. et al. Two-colour live-cell nanoscale imaging of intracellular targets. *Nature Communications* 7, 10778 (2016).
17. Thompson, A. D., Bewersdorf, J., Toomre, D. & Schepartz, A. HIDE Probes: A New Toolkit for Visualizing Organelle Dynamics, Longer and at Super-Resolution. *BIOCHEMISTRY* 56, 5194–5201 (2017).
18. Takakura, H. et al. Long time-lapse nanoscopy with spontaneously blinking membrane probes. *Nat Biotechnol* 35, 773–780 (2017).
19. Thompson, A. D. et al. Long-Term Live-Cell STED Nanoscopy of Primary and Cultured Cells with the Plasma Membrane HIDE Probe DiI-SiR. *ANGEWANDTE CHEMIE-INTERNATIONAL EDITION* 56, 10408–10412 (2017).
20. Gupta, A., Rivera-Molina, F., Xi, Z., Toomre, D. & Schepartz, A. Endosome motility defects revealed at super-resolution in live cells using HIDE probes. *NATURE CHEMICAL BIOLOGY* 16, 408+ (2020).
21. Chu, L. et al. Two-color nanoscopy of organelles for extended times with HIDE probes. *NATURE COMMUNICATIONS* 11, (2020).
22. Tyson, J. et al. Extremely Bright, Near-IR Emitting Spontaneously Blinking Fluorophores Enable Ratiometric Multicolor Nanoscopy in Live Cells. *ACS CENTRAL SCIENCE* 7, 1419–1426 (2021).
23. Individual cristae within the same mitochondrion display different membrane potentials and are functionally independent. *The EMBO Journal* 38, e101056 (2019).
24. Petit, J. M., Maftah, A., Ratinaud, M. H. & Julien, R. 10N-nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria. *Eur J Biochem* 209, 267–273 (1992).
25. Rodriguez, M. E. et al. Targeting of mitochondria by 10-N-alkyl acridine orange analogues: Role of alkyl chain length in determining cellular uptake and localization. *Mitochondrion* 8, 237–246 (2008).
26. Zhou, Q. et al. Bioconjugation by Native Chemical Tagging of C–H Bonds. *J. Am. Chem. Soc.* 135, 12994–12997 (2013).
27. Dimitrijevs, P., Domracheva, I. & Arsenyan, P. Improved method for the preparation of nonyl acridine orange analogues and utilization in detection of cardiolipin. *New J. Chem.* 44, 9626–9633 (2020).
28. Grimm, J. B. et al. A general method to optimize and functionalize red-shifted rhodamine dyes. *Nat Methods* 17, 815–821 (2020).
29. Mishra, P., Carelli, V., Manfredi, G. & Chan, D. C. Proteolytic Cleavage of Opa1 Stimulates Mitochondrial Inner Membrane Fusion and Couples Fusion to Oxidative Phosphorylation. *Cell Metabolism* 19, 630–641 (2014).
30. Doughty-Shenton, D. et al. Pharmacological Targeting of the Mitochondrial Phosphatase PTPMT1. *J Pharmacol Exp Ther* 333, 584–592 (2010).

31. Desousa, B. R. et al. Calculation of ATP production rates using the Seahorse XF Analyzer. 2022.04.16.488523 Preprint at <https://doi.org/10.1101/2022.04.16.488523> (2023).
32. Sargiacomo, C., Stonehouse, S., Moftakhar, Z., Sotgia, F. & Lisanti, M. P. MitoTracker Deep Red (MTDR) Is a Metabolic Inhibitor for Targeting Mitochondria and Eradicating Cancer Stem Cells (CSCs), With Anti-Tumor and Anti-Metastatic Activity In Vivo. *Frontiers in Oncology* 11, (2021).
33. Dahl, H. H., Hunt, S. M., Hutchison, W. M. & Brown, G. K. The human pyruvate dehydrogenase complex. Isolation of cDNA clones for the E1 alpha subunit, sequence analysis, and characterization of the mRNA. *J Biol Chem* 262, 7398–7403 (1987).
34. Karbowski, M. & Youle, R. J. Dynamics of mitochondrial morphology in healthy cells and during apoptosis. *Cell Death Differ* 10, 870–880 (2003).
35. Debets, M. F. et al. Aza-dibenzocyclooctynes for fast and efficient enzyme PEGylation via copper-free (3+2) cycloaddition. *Chem. Commun.* 46, 97–99 (2010).
36. Gordon, C. G. et al. Reactivity of Biarylazacyclooctynones in Copper-Free Click Chemistry. *J. Am. Chem. Soc.* 134, 9199–9208 (2012).
37. Paulson, J. C. & Colley, K. J. Glycosyltransferases: Structure, localization, and control of cell type-specific glycosylation. *Journal of Biological Chemistry* 264, 17615–17618 (1989).
38. Andres, D. A., Dickerson, I. M. & Dixon, J. E. Variants of the carboxyl-terminal KDEL sequence direct intracellular retention. *Journal of Biological Chemistry* 265, 5952–5955 (1990).
39. Kollmorgen, G. M. & Griffin, M. J. THE EFFECT OF HYDROCORTISONE ON HeLa CELL GROWTH. *Cell Proliferation* 2, 111–122 (1969).
40. Wolf, D. M. et al. Individual cristae within the same mitochondrion display different membrane potentials and are functionally independent. *The EMBO Journal* 38, e101056 (2019).
41. Heytler, P. G. [58] Uncouplers of oxidative phosphorylation. in *Methods in Enzymology* vol. 55 462–472 (Academic Press, 1979).
42. Creed, S. & McKenzie, M. Measurement of Mitochondrial Membrane Potential with the Fluorescent Dye Tetramethylrhodamine Methyl Ester (TMRM). in *Cancer Metabolism: Methods and Protocols* (ed. Haznadar, M.) 69–76 (Springer, New York, NY, 2019). doi:10.1007/978-1-4939-9027-6_5.
43. Huang, X. et al. Fast, long-term, super-resolution imaging with Hessian structured illumination microscopy. *Nat Biotechnol* 36, 451–459 (2018).
44. Chi, W. et al. Descriptor Δ GC-O Enables the Quantitative Design of Spontaneously Blinking Rhodamines for Live-Cell Super-Resolution Imaging. *Angewandte Chemie International Edition* 59, 20215–20223 (2020).

45. Shim, S.-H. et al. Super-resolution fluorescence imaging of organelles in live cells with photoswitchable membrane probes. *Proceedings of the National Academy of Sciences* 109, 13978–13983 (2012).
46. Nieuwenhuizen, R. P. J. et al. Measuring image resolution in optical nanoscopy. *Nat Methods* 10, 557–562 (2013).
47. Stephan, T., Roesch, A., Riedel, D. & Jakobs, S. Live-cell STED nanoscopy of mitochondrial cristae. *Sci Rep* 9, 12419 (2019).
48. Alvarez, L. A. J. et al. Application Note: Pushing STED beyond its limits with TauSTED. *Nature Methods* (2021).
49. Chan, D. C. Mitochondrial Dynamics and Its Involvement in Disease. *Annual Review of Pathology: Mechanisms of Disease* 15, 235–259 (2020).
50. Wai, T. et al. Imbalanced OPA1 processing and mitochondrial fragmentation cause heart failure in mice. *Science* 350, aad0116 (2015).
51. McArthur, K. et al. BAK/BAX macropores facilitate mitochondrial herniation and mtDNA efflux during apoptosis. *Science* 359, eaao6047 (2018).
52. Dadina, N., Tyson, J., Zheng, S., Lesiak, L. & Schepartz, A. Imaging organelle membranes in live cells at the nanoscale with lipid-based fluorescent probes. *Current Opinion in Chemical Biology* 65, 154–162 (2021).
53. Fiolka, R., Shao, L., Rego, E. H., Davidson, M. W. & Gustafsson, M. G. L. Time-lapse two-color 3D imaging of live cells with doubled resolution using structured illumination. *Proceedings of the National Academy of Sciences* 109, 5311–5315 (2012).

Online Methods

Materials.

SiR-CA was synthesized according to previously reported methods. BacMam® 2.0 reagent, CellLight Mitochondria-GFP (PDHA1-GFP) (C10600), CellLight Mitochondria-RFP (PDHA1-RFP) (C10505), CellLight Golgi-GFP (GALNT1-GFP) (C10592), CellLight Golgi-RFP (GALNT1-RFP) (C10593), CellLight ER-GFP (KDEL-GFP) (C10590), CellLight ER-RFP (KDEL-RFP) (C10591), culturing media Dulbecco's Modified Eagle medium (DMEM, 12430054, 21063029, and A1443001), Opti-MEM with no phenol red (11058021), and fetal bovine serum (FBS), and chemical reagents Hoechst33342 (62249), MitoTracker Deep Red FM (M22426) were all purchased from ThermoFisher. CellTiter-Glo® 2.0 (G9242) was purchased from Promega, and alexidine dihydrochloride (A8986) was purchased from Sigma Aldrich. Plasmid encoding mEmerald-TOMM20 was a gift from Michael Davidson (mEmerald-TOMM20-N-10, Addgene plasmid # 54282). Plasmid encoding Halo-TOMM20 was a gift from Kevin McGowan (Halo-TOMM20-N-10, Addgene plasmid # 123284).

Cell culture.

HeLa cells (UC Berkeley Cell Culture Facility) were cultured in DMEM (ThermoFisher) supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 µg/mL). All cells were cultured at 37 °C in a humidified CO₂/air (5/95%) incubator. All cells were bona fide lines obtained from UCB-CCF and periodically tested for mycoplasma with DNA methods. Cells for imaging were seeded in 4- or 8-well Lab-Tek chambers (Nunc, Thermo Fisher #177399 and 177402, No. 1.0, 0.8 cm² for 4-well and 1.8 cm² for 8-well. Used for confocal colocalization studies, SIM and SMLM) or 4-well glass bottom µslides (Ibidi #80427, No. 1.5H, , 2.5 cm², for STED and overnight confocal imaging) at indicated density.

Microscopy.

Colocalization studies was performed on a Zeiss LSM 880 confocal microscope equipped with a Plan-Apochromat ×63/1.4 NA (Zeiss) oil objective and a diode 405 nm laser, an Argon 458, 488, 514 nm laser, a diode pumped solid-state 561 nm laser, and a 633 nm HeNe laser with standard settings. The pinhole size was set to 1 airy unit.

Overnight time-lapse confocal and STED microscopy were performed on a Leica STELLARIS 8 microscope (Leica Microsystems) equipped with a Leica DMI8 CS scanhead; a HC Plan-Apo 100x/1.4NA STED White oil immersion objective (for STED), a HC Plan-Apo 63x/1.4NA oil immersion objective (for point-scanning confocal time-lapse), a HC Plan-Apo 63x/1.4NA water immersion objective (for point-scanning confocal hiPSC-CM imaging), a pulsed white-light laser (440 nm- 790 nm; 440 nm: > 1.1 mW; 488

nm: > 1.6 mW; 560 nm: > 2.0 mW; 630 nm: > 2.6 mW; 790 nm: > 3.5 mW, 78 MHz), and a pulsed 775nm STED laser. Confocal imaging was carried out using either HyD S or HyD X detectors in analog or digital mode, while STED was exclusively carried out on HyD X detectors in photon counting mode. Live cell imaging conditions were maintained using a blacked out cage enclosure from Okolab. Temperature was maintained by heating the enclosure and monitored using Oko-Touch. pH was maintained by supplying humidified 5% CO₂ to the sample chamber.

Lattice SIM and SMLM imaging was performed on an inverted Zeiss Elyra 7 microscope equipped with a Plan-Apo 63x/1.46 NA Oil immersion objective (Zeiss) and a 403 nm (200 mW), a Sapphire 488 nm (1 W), 561 nm (1.3W) and a Lasos 642 nm (550 mW) laser, a MBS 405/488/561/641 and EF LBF 405/488/561/641 filter set, a LP 560 and a BP 570-620 + LP 655 filter cube, and two p.co edge high speed sCMOS cameras, using Zeiss Zen Black software.

Confocal imaging to monitor cell body contraction of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM).

hiPSC-CM were prepared according to reported procedure⁵⁴, and cultured on Matrigel (1:100 dilution; Corning)-coated 24-well μ -plates (Ibidi®, cat no. 82426) (7.0×10^4 cells per well) in RPMI 1640 medium (Life Technologies) with B27 supplement (Life Technologies). On the day of imaging, the hiPSC-CMs were labeled with 600 μ L of 100 nM MAO-N₃ in RPMI 1640/B27 ph(-) for 1 h, washed (3 x 600 μ L RPMI 1640/B27 ph(-)), incubated with 600 μ L of 0.75 μ M SiR-DBCO in RPMI 1640/B27 ph(-) for 1 h, and washed again (RPMI 1640/B27 ph(-), 6 x 10 min). Finally, the cells were imaged in 700 μ L RPMI 1640/B27 ph(-) at 37°C and 5% CO₂. A total of 213 frames from a field of view of 72.5 μ m x 72.5 μ m was obtained with a time interval of 0.52 s, with a time-lapse of 110 s. Pixel size: 0.142 μ m, pixel dwell time: 0.95 μ s, scan speed: 1000 Hz, laser power: 25.7 % (652 nm).

Confocal imaging for colocalization with organelle markers.

Two days before imaging, HeLa cells were seeded in LabTek-I 8-well chambers (1.5×10^4 cells per well). After 24 hours, 6 μ L of a CellLight BacMam reagent (40 particles per cell) for organelles of interest (ER, Golgi or mitochondria) was added to each well and incubation was continued for 16–20 h. The cells were subsequently treated with 300 μ L of 100 nM HAO-N3 or MAO-N3 (diluted from 0.2 mM DMSO stock solution into DMEM ph(-) buffer) for 1 hour. The cells were washed 3X with DPBS and treated with 300 μ L of a 750 nM solution of SiR-DBCO in DMEM ph(-) for 1 h. Each well was then washed with 300 μ L DMEM supplemented with 10% FBS and incubated for 10 min. The

wash/incubation was repeated 6 times to minimize non-specific dye labeling. Cells were then incubated with 300 μ L of 0.5 μ g/mL Hoechst33342 (ThermoFisher, #62249) solution for 1 min to label nuclei and help identify the plane of view. Cells were washed with warm DPBS and imaged in DMEM ph(-) at room temperature. Pairwise co-localization of HAO/MAO/SiR/GFP markers was evaluated by measuring the overlap of fluorescent signals from two spectrally separated channels using the PCC for individual cells using the JaCOP plugin on the FIJI software. PCC values for each condition obtained from multiple cells collected over at least 2 biological replicates were pooled and represented as Mean with S.E.M using Prism 8.4.3.

Overnight confocal imaging for cell proliferation monitoring.

48 hours before imaging, HeLa cells (P3-P10) were seeded in a 4-well chamber (2.2×10^4 cells per well). On the day of the experiment, the cells were treated with 700 μ L of a 200 nM solution of MAO- N_3 (diluted from 0.2 mM DMSO stock solution in OptiMEM ph(-) buffer) for 1 hour. The cells were washed 1X with DMEM ph (-) and treated with 700 μ L of a 100 nM solution of SiR-DBCO (diluted from 2 mM DMSO stock solution into OptiMEM ph(-) buffer) for 1 h. The cells were then washed with 700 μ L DMEM supplemented with 10% FBS and incubated in the same buffer for 1h. For experiment with MitoTracker Deep Red, the cells were treated with 700 μ L of a 100 nM solution of MitoTracker Deep Red (diluted from 0.2 mM DMSO stock solution into OptiMEM ph(-) buffer) for 30 min, and washed 3x with 700 μ L of DPBS. For untreated cells, the cells were washed 3x with 700 μ L of DPBS. Finally, the cells were imaged in 700 μ L DMEM ph(-) with 10% FBS, at 37°C and 5% CO₂.

A region of 4 x 4 frames was imaged every 2 minutes, and each time-point in the time-lapse was derived by stitching the 16 frames together. Each time-point captured a field of view of 670 μ m x 670 μ m. For each frame within the time-point composite, the field of view was 184.52 x 184.52 μ m. Focal plane was maintained using Leica's Adaptive Focus Control system. Frame size: 1024 x 1024, pixel size: 0.18 μ m, pixel dwell time: 2.8375 μ s, detection window: 662 - 749 nm. laser power: 10% (652 nm) for MAO-SiR and untreated cells, 2% for MitoTracker DeepRed-treated cells. Cell counts of frame #1 and frame #220 were performed manually using the Cell Counter plugin in FIJI. Segmentation was run to isolate cell bodies from background, and a mask was generated to identify ROIs for intensity quantification. Mean signal for each ROI was averaged for one time point to give the mean frame intensity. Each mean frame intensity was scaled relative to the first frame of the time series. Then the mean for each frame was averaged over three data sets to determine the half-life. Half-life of signal intensity was obtained via performing a nonlinear regression of the exponential decay function using OriginLab OriginPro 2022b.

Confocal imaging and flow cytometry of cells with depolarized mitochondria.

48 hours before imaging, HeLa cells (P3-P10) were seeded in a 4-well chamber (2.2×10^4 cells per well). After 24 hours, 10 μL of the CellLight BacMam Mito-GFP reagent (40 particles per cell) for mitochondria matrix labeling (PDHA1-GFP) was added to each well and incubation was continued for 16–20 h. On the day of the experiment, the cells were treated with 700 μL of a 200 nM solution of MAO- N_3 (diluted from 0.2 mM DMSO stock solution in OptiMEM ph(-) buffer) for 1 hour. The cells were washed 1X with OptiMEM ph(-) and treated with 700 μL of a 100 nM solution of SiR-DBCO (diluted from 2 mM DMSO stock solution into OptiMEM ph(-) buffer) for 1 h. The cells were then washed with 700 μL DMEM supplemented with 10% FBS and incubated in the same buffer for 1h. For experiment with tetramethyl rhodamine methyl ester (TMRM), the cells were treated with 700 μL of a 100 nM solution of TMRM (diluted from 0.1 mM DMSO stock solution into OptiMEM ph(-) buffer) for 30 min, washed 3x with 700 μL of DMEM supplemented with 10% FBS and incubated in the same buffer for 1h. Finally, the cells were washed again with 700 μL DMEM ph(-) with 10% FBS and imaged in the same buffer, at 37°C and 5% CO_2 . For depolarized cells, after labeling with MAO-SiR or TMRM, the cells were incubated with 700 μL of 10 μM FCCP in DMEM ph(-) with 10% FBS for 30 min, then washed 3x with 700 μL DMEM ph(-) with 10% FBS imaged in the same buffer, at 37°C and 5% CO_2 .

After imaging, cells were washed, trypsinized, and pelleted at 200 g for 3 min as described above. The pellet was resuspended in 100 μL DPBS and transferred to a 1.5 mL microcentrifuge tube. Flow cytometry measurements were performed at room temperature with an Attune NxT flow cytometer. Hoechst 33342 excited with a laser at 405 nm, and the emission filter was set at 440 ± 50 nm. The fluorophore tetramethylrhodamine methyl ester (TMRM) was excited with a laser at 561 nm, and the emission filter was set at 585 ± 16 nm. MAO-SiR was excited with a laser at 638 nm, and the emission filter was set at 652 ± 10 nm. The collected data were analyzed using GraphPad Prism 8.4.3.

Two color SIM imaging of OMM and IMM using mEmerald-TOMM20 and MAO-SiR.

Two days before imaging, HeLa cells were plated (2.5×10^4 cells per well) in a 4-well chamber and incubated overnight. After 16 hours, the cells were transfected with plasmids encoding mEmerald-TOMM20 using FuGENE HD transfection reagent (Promega) according to manufacturer's protocol, and incubated overnight. On the following day, the HeLa cells were labeled with 600 μL of 75 nM MAO- N_3 in DMEM ph(-) for 1 h, washed (3 x 600 μL DMEM ph(-)), incubated with 600 μL of 0.6 μM SiR-DBCO in DMEM ph(-) for 1 h, and washed again (DMEM ph(-) 10% FBS, 6 x 10 min). The sample was subsequently visualized via Lattice SIM imaging on a Zeiss Elyra 7 microscope.

Laser power: 6% (642 nm, estimated at 0.04 kw/cm²) and 3% (488 nm, estimated at 0.04 kw/cm²), field of view: 80.14 µm x 80.14 µm, camera exposure time: 40 ms, 13 phases were acquired for SIM reconstruction. Beam splitter: LBF 405/488/561/642, filter set: BP 495-550 + LP 655. Incubation conditions: DMEM ph(-) with 10% FBS, 37°C, 5% CO₂. SIM reconstruction was performed on the Zeiss Zen Black software. Nonlinear regression for Gaussian and Lorentzian fitting of peaks from line profiles was performed using OriginLab OriginPro 2022b and plotted using Graphpad Prism 8.4.3.

Two color SIM imaging of mitochondrial matrix and IMM using BacMam 2.0 MitoGFP and MAO-SiR.

Two days before imaging, HeLa cells were plated (2.5×10^4 cells per well) in a 4-well chamber and incubated overnight. After 16 hours, the cells were transfected with 10 µL Celllight BacMam 2.0 MitoGFP for 8 hrs. The cells were then washed (3x600 µL DMEM ph(-) with 10% FBS), and incubated overnight. On the following day, the HeLa cells were labeled with 600 µL of 75 nM HAO-N₃ in DMEM ph(-) for 1 h at 37 °C with 5% CO₂, washed (3x 600 µL DMEM ph(-)), incubated with 600 µL of 0.6 µM SiR-DBCO in DMEM ph(-) for 1 h at 37 °C with 5% CO₂, and washed again (DMEM ph(-) 10% FBS) for 6x10 min. The sample was subsequently visualized via Lattice SIM imaging. Laser power: 6% (642 nm, estimated at 0.04 kw/cm²) and 3% (488 nm, estimated at 0.04 kw/cm²), field of view: 80.14 µm x 80.14 µm, camera exposure time: 40 ms, 13 phases were acquired for SIM reconstruction. Beam splitter: LBF 405/488/561/642, filter set: BP 495-550 + LP 655. Incubation conditions: DMEM ph(-) with 10% FBS, 37°C, 5% CO₂. SIM reconstruction was performed on the Zeiss Zen Black software. Nonlinear regression for Gaussian and Lorentzian fitting of peaks from line profiles was performed using OriginLab OriginPro 2022b and plotted using Graphpad Prism 8.4.3.

Time-lapse SIM imaging of IMM using MAO-SiR .

HeLa cells were plated (3.0×10^4 cells per well) in 4-well LabTekII chambers (Nunc, Thermo Fisher Scientific) and incubated overnight. On the following day, the HeLa cells were labeled with 600 µL of 75 nM HAO-N₃ in DMEM ph(-) for 1 h at 37 °C, washed (3x 600 µL DMEM ph(-)), incubated with 600 µL of 0.6 µM SiR-DBCO in DMEM ph(-) for 1 h at 37 °C with 5% CO₂, and washed again (DMEM ph(-) 10% FBS) for 6x10 min. The time-lapse SIM imaging of labeled cells was performed on a Zeiss Elyra 7 microscope using Zeiss Zen Black software. Laser power: 0.4 % (642 nm, estimated at 0.0028 kw/cm²), field of view: 80.14 µm x 80.14 µm, camera exposure time: 40 ms, 13 phases per frame were acquired for SIM reconstruction. 100 ms interval between each frame, 500 frames in total over an acquisition time of 297 s. Beam splitter: LBF 405/488/561/642; filter set: SBS LP 560. SIM reconstruction was performed on the Zeiss Zen Black software. Imaging buffer and conditions: DMEM ph(-) with 10% FBS, 37°C, 5% CO₂). Kymograph of line profile (**Fig. 4g**) was obtained using the KymographBuilder plugin on the FIJI software.

Fluorescence intensity was obtained from randomly picked 3 μm x 3 μm regions using ImageJ and plotted using Graphpad Prism 8.4.3. Half-life of signal intensity was obtained via performing a nonlinear regression of the exponential decay function using OriginLab OriginPro 2022b.

Time-lapse SIM imaging of OMM using mEmerald-TOMM20.

Two days before imaging, HeLa cells were plated (2.5×10^4 cells per well) in a 4-well chamber and incubated overnight. After 16 hours, the cells were transfected with plasmids encoding mEmerald-TOMM20 using FuGENE HD transfection reagent (Promega) according to manufacturer's protocol, and incubated overnight. On the following day, the HeLa cells were visualized via Lattice SIM imaging on a Zeiss Elyra 7 microscope. Laser power: 0.4 % (488 nm, estimated at 0.005 kw/cm²), field of view: 80.14 μm x 80.14 μm , camera exposure time: 40 ms, 13 phases per frame were acquired for SIM reconstruction. 100 ms interval between each frame, 500 frames in total over an acquisition time of 297 s. Beam splitter: LBF 405/488/561/642; filter set: SBS LP 560. Imaging buffer and conditions: DMEM ph(-) with 10% FBS, 37°C, 5% CO₂). SIM reconstruction was performed on the Zeiss Zen Black software. Fluorescence intensity was obtained from randomly picked 3 μm x 3 μm regions using ImageJ and plotted using Graphpad Prism 8.4.3. Half-life of signal intensity was obtained via performing a nonlinear regression of the exponential decay function using OriginLab OriginPro 2022b.

Time-lapse SIM imaging of OMM using Halo-TOMM20 and SiR-CA.

Two days before imaging, HeLa cells were plated (2.5×10^4 cells per well) in a 4-well chamber and incubated overnight. After 16 hours, the cells were transfected with plasmids encoding Halo-TOMM20 using FuGENE HD transfection reagent (Promega) according to manufacturer's protocol, and incubated overnight. On the following day, the HeLa cells were incubated with 600 μL of 2 μM SiR-CA in DMEM ph(-) for 1 h at 37 °C with 5% CO₂, and washed 3x (600 μL of DMEM ph(-) supplemented with 10% FBS). visualized via Lattice SIM imaging on a Zeiss Elyra 7 microscope. Laser power: 0.4 % (488 nm, estimated at 0.005 kw/cm²), field of view: 80.14 μm x 80.14 μm , camera exposure time: 40 ms, 13 phases per frame were acquired for SIM reconstruction. 100 ms interval between each frame, 500 frames in total over an acquisition time of 297 s. Beam splitter: LBF 405/488/561/642; filter set: SBS LP 560. Imaging buffer and conditions: DMEM ph(-) with 10% FBS, 37°C, 5% CO₂. SIM reconstruction was performed on the Zeiss Zen Black software. Fluorescence intensity was obtained from randomly picked 3 μm x 3 μm regions using ImageJ and plotted using Graphpad Prism 8.4.3. Half-

life of signal intensity was obtained via performing a nonlinear regression of the exponential decay function using OriginLab OriginPro 2022b.

SMLM imaging of IMM structure using HMDS₆₅₅-DBCO and MAO-N₃.

Prior to seeding, the 4-well Lab-Tek® II (Nunc) chamber was treated by sonication for 15 min in 1M KOH, rinsed three times with deionized H₂O, sterilized with 100% EtOH, and air dried overnight in a biological safety cabinet. HeLa cells were seeded onto pre-treated plates at a density of 3.0×10^4 cells per well and incubated overnight at 37 °C with 5% CO₂. On the following day, the cells were labeled with 600 µL of 75 nM MAO-N₃ in DMEM ph(-) for 40 min at 37 °C with 5% CO₂, washed (3x 600 µL DMEM ph(-)), incubated with 600 µL of 0.6 µM HMDS₆₅₅-DBCO in DMEM ph(-) for 20 min at 37 °C with 5% CO₂, and washed again (DMEM ph(-) with 10% FBS, 6x10 min, 37 °C with 5% CO₂). Finally, the media was exchanged with 600 µL of DMEM ph(-) supplemented with 1% FBS, 100 µM Trolox and 500 µM sodium ascorbate. The sample was subsequently visualized via SMLM imaging on an inverted Zeiss Elyra 7 microscope equipped with a Plan-Apo 63x/1.4NA Oil immersion lens (40 % laser power for 642 nm laser, 60.57° TIRF mirror angle, TIRF-uHP mode, 2.5 ms exposure time for each frame, 6000 frames obtained in 36 s).

STED imaging of cristae structures and their dynamics using SiR-DBCO and MAO-N₃.

HeLa cells were plated (2.2×10^4 cells per well) in a 4-well #1.5 glass bottom chamber (Ibidi, #80827) and incubated for 42 h. On the day of imaging, the cells were labeled with 700 µL of 200 nM MAO-N₃ in Opti-MEM ph(-) for 1 h, washed (1 x 700 µL DMEM ph(-)), incubated with 700 µL of 100 nM SiR-DBCO in Opti-MEM ph(-) for 1 h, washed with 700 µL DMEM supplemented with 10% FBS) and incubated for 1h. Finally the media was exchanged with DMEM ph(-), and the sample was subsequently visualized via STED imaging (Imaging conditions: 20% excitation laser power, depleted by 24% STED laser power, DMEM ph(-), 37°C, 5% CO₂). Deconvolution of gated STED images was performed based on the theoretical point spread function (PSF) and the classic maximum likelihood estimation (CMLE)⁵⁵ method using a commercial software package SVI Huygens Pro. Imaging parameters for time-lapse: 256 x 128, 1.3 s time interval, total of 125 frames, scan rate 100 Hz, no line accumulation/average, pixel size: 30 nm.

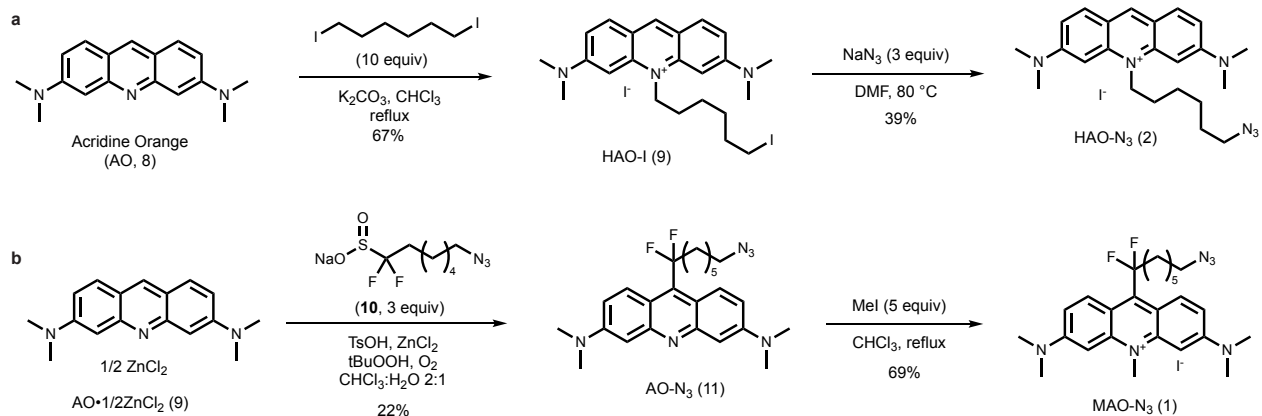
Data Availability

The materials and data reported in this study are available upon reasonable request from the corresponding author.

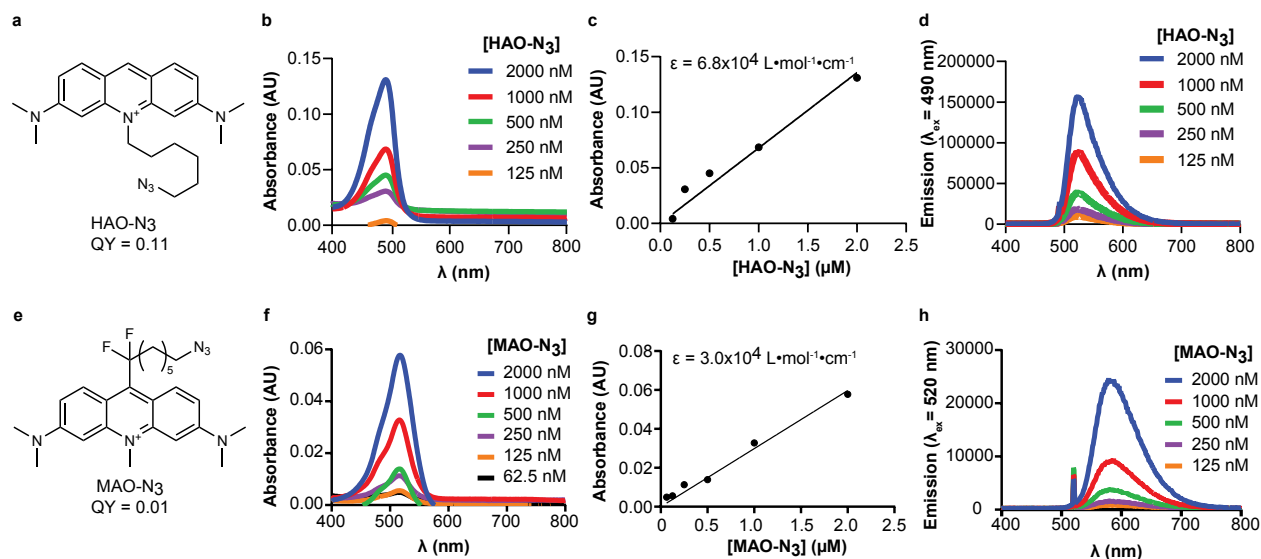
Method References

- (54) Grenier, V.; Martinez, K. N.; Benlian, B. R.; García-Almedina, D. M.; Raliski, B. K.; Boggess, S. C.; Maza, J. C.; Yang, S. J.; Gest, A. M. M.; Miller, E. W. Molecular Prosthetics for Long-Term Functional Imaging with Fluorescent Reporters. *ACS Cent. Sci.* **2022**, 8 (1), 118–121. <https://doi.org/10.1021/acscentsci.1c01153>.
- (55) Holmes, T. J.; Bhattacharyya, S.; Cooper, J. A.; Hanzel, D.; Krishnamurthi, V.; Lin, W.; Roysam, B.; Szarowski, D. H.; Turner, J. N. Light Microscopic Images Reconstructed by Maximum Likelihood Deconvolution. In *Handbook of Biological Confocal Microscopy*; Pawley, J. B., Ed.; Springer US: Boston, MA, 1995; pp 389–402. https://doi.org/10.1007/978-1-4757-5348-6_24.

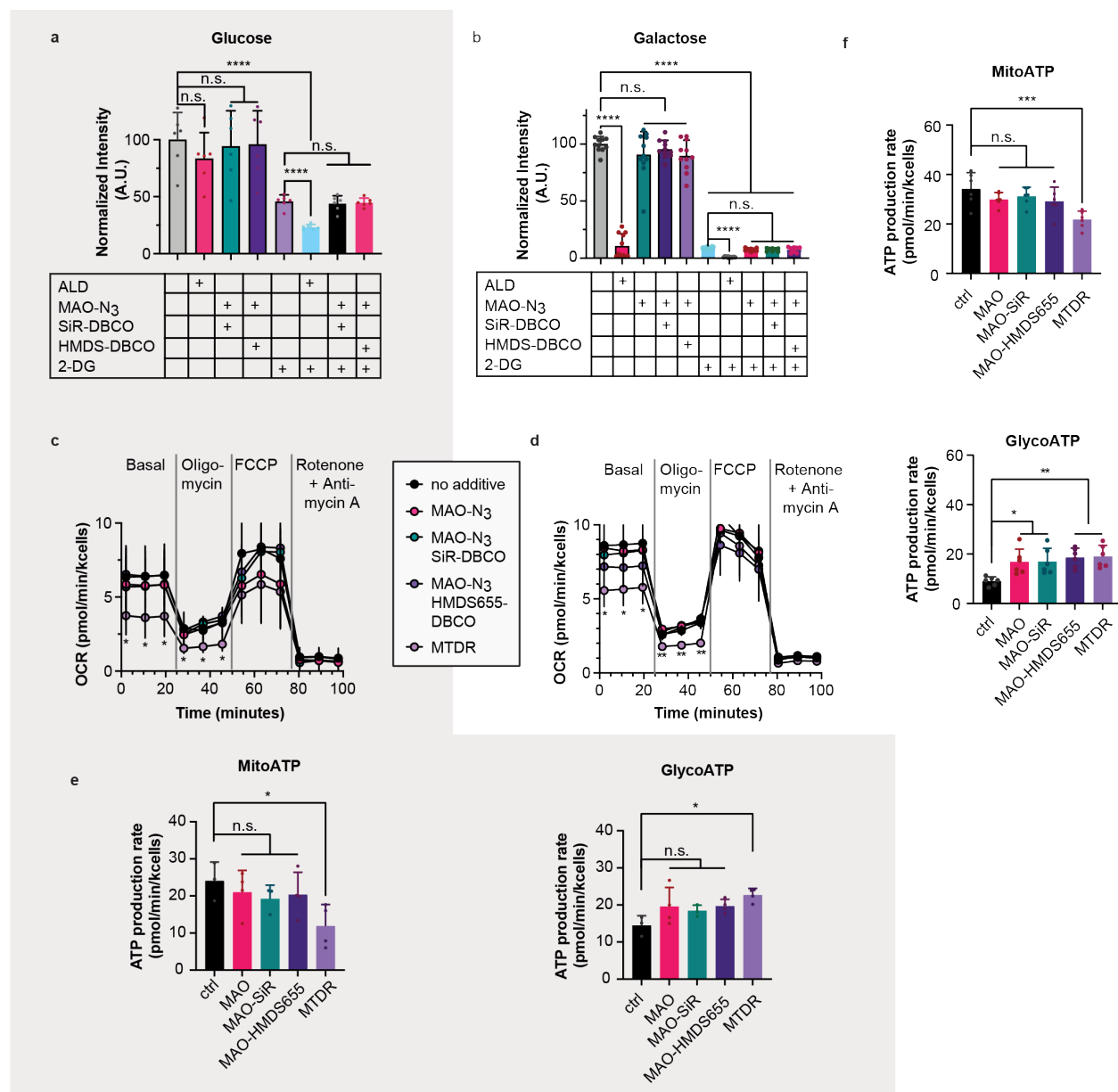
Extended Data Figures



Extended Data Fig. 1. Scheme illustrating the synthetic steps used to prepare HAO-N₃ and MAO-N₃. Synthetic details are available in **Supplementary Information**.

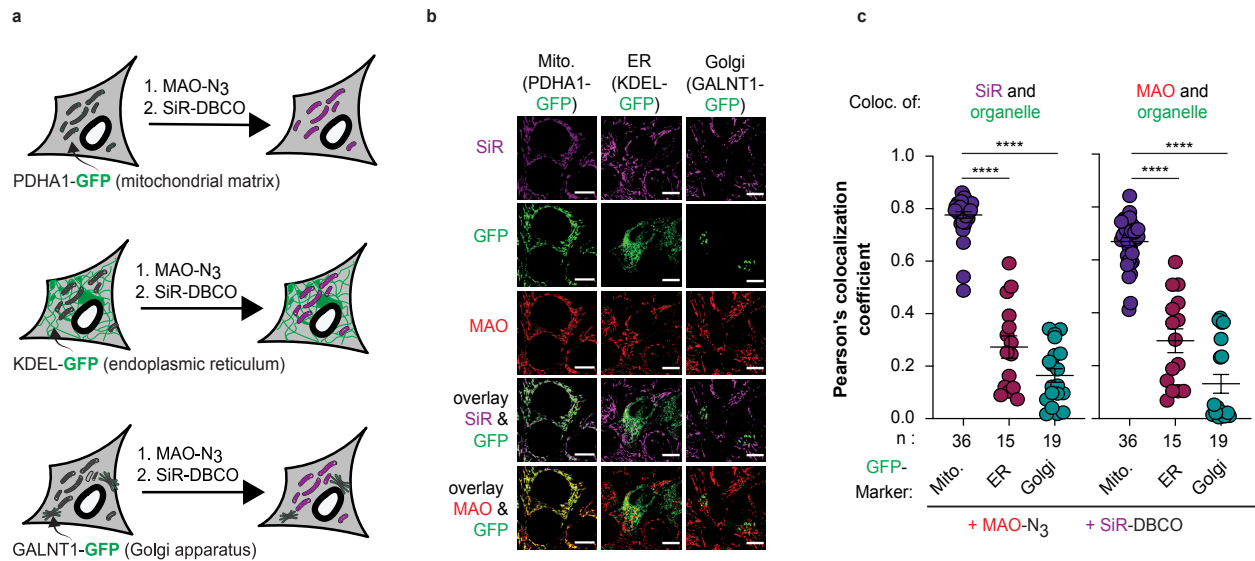


Extended Data Fig. 2. Photophysical properties of MAO-N₃ and HAO-N₃. **a**, Structure and quantum yield of HAO-N₃. **b**, Concentration-dependent UV-Vis absorption curve of HAO-N₃ shows a λ_{max} of 496 nm. **c**, Plot of absorbance @ λ_{max} against concentration of HAO-N₃ and the calculated extinction coefficient. **d**, Concentration dependent fluorescence emission of HAO-N₃ reveals a λ_{em} of 517 nm. **e**, Structure and quantum yield of MAO-N₃. **f**, Concentration-dependent UV-Vis absorption curve of MAO-N₃ shows a λ_{max} of 520 nm. **g**, Plot of absorbance @ λ_{max} against concentration of MAO-N₃ and its extinction coefficient. **h**, Concentration dependent fluorescence emission of MAO-N₃ reveals a λ_{em} of 570 nm.

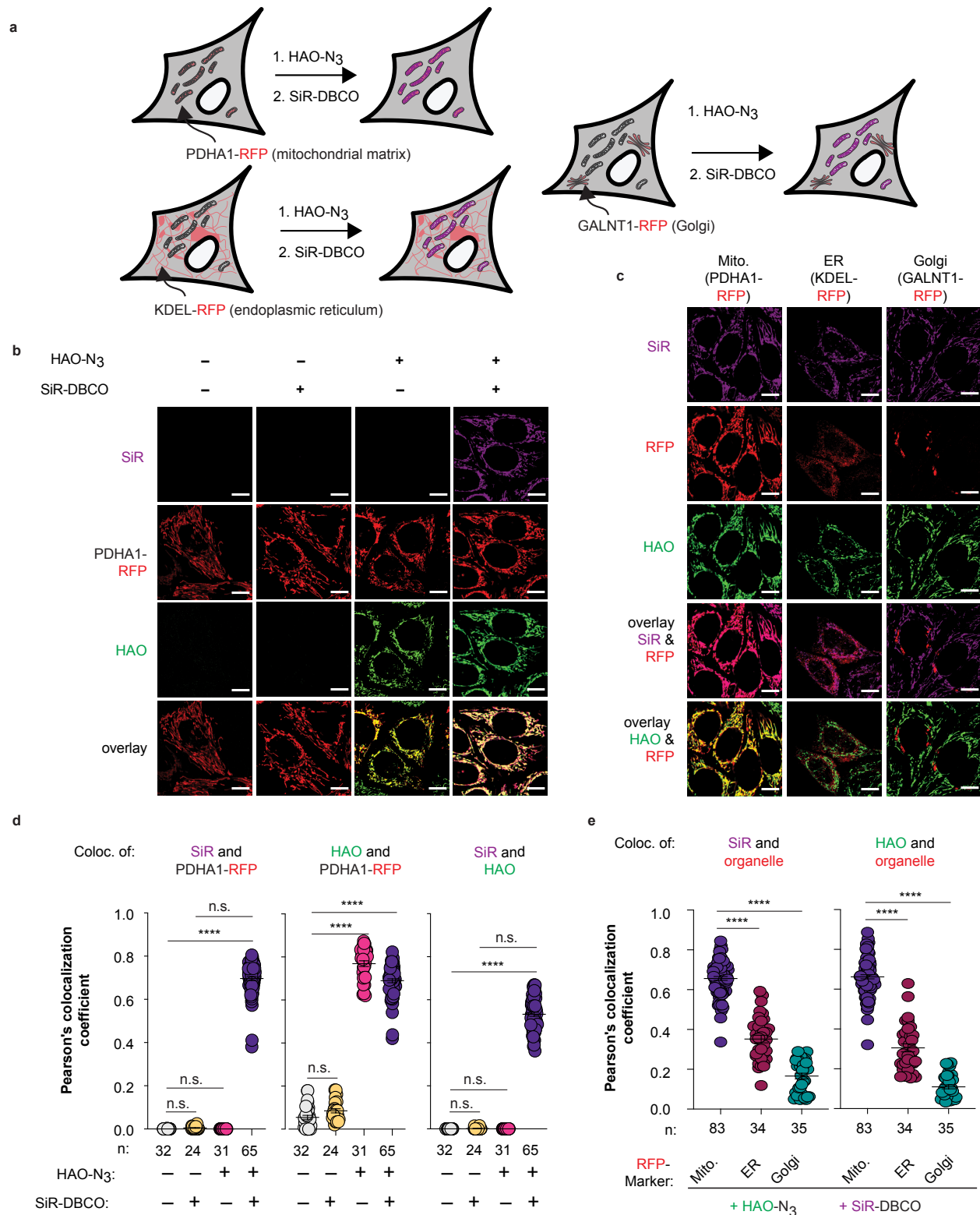


Extended Data Fig. 3. MAO-fluorophore conjugates are benign with respect to multiple metrics of mitochondrial function. Plots showing the cellular ATP levels of HeLa cells cultured in **a**, glucose-rich or **b**, galactose-supplemented media in the presence or absence of the mitochondrial toxin alexidine (ALD) or the glycolysis inhibitor 2-deoxy-D-glucose (2-DG). HeLa cells were cultured and treated with 2-DG, MAO-N₃, SiR-DBCO and/or HMDS655-DBCO following protocols in **Online Methods**. ATP levels were measured using CellTiter Glo 2.0 bioluminescent assay. $n = 6$ (for **a**) or 12 (for **b**) biologically independent replicates. **** $p < 0.0001$, n.s. (not significant) $0.5317 < p < 0.9994$. Normalized oxygen consumption rate (OCR) of HeLa cells incubated with MAO-HMDS655, MAO-SiR, MAO-N₃ or MitoTracker Deep Red (MTDR) in **c**, glucose-rich or **d**, galactose-supplemented media under basal conditions and after addition of oligomycin (1 μ M) which inhibits ATP synthase, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 10 μ M), which disrupts mitochondrial membrane potential, and the combination of rotenone and Antimycin A (Rot + AA, 0.1 μ M), which inhibits oxidative phosphorylation. ATP production rates of cells incubated in **e**, glucose-rich and **f**, galactose-supplemented media were calculated based on the measured OCR and extracellular

acidification rate (ECAR) using Seahorse Analytics software. $n = 4$ (for **c-e**) or 6 (for **f**) biologically independent replicates. For **e**, * $p = 0.0114$ or 0.0391 , n.s. (not significant) $0.1184 < p < 0.8650$; for **f**, *** $p = 0.0004$, ** $p = 0.0031$ or 0.0020 , * $p = 0.0170$ or 0.0145 , n.s. $0.2182 < p < 0.6294$. Error bars = s.e.m. Statistics generated using one-way ANOVA with Dunnett's post-analysis accounting comparison to the negative control where cells were incubated with cell culture media only.

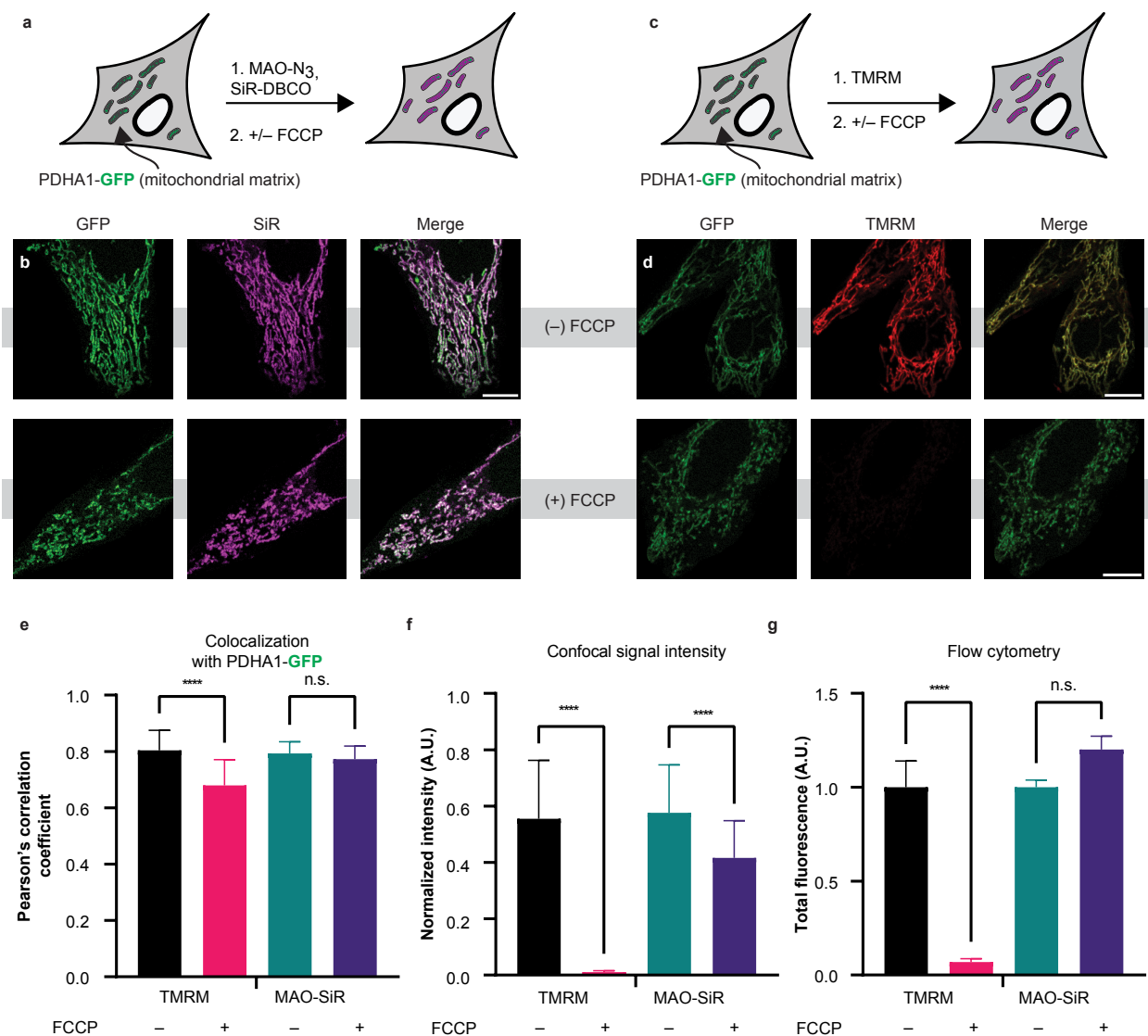


Extended Data Fig. 4. MAO-SiR, the HIDE probe generated from MAO-N₃ and SiR-DBCO, does not localize significantly to the ER or Golgi. **a**, HeLa cells expressing the mitochondrial matrix marker PDHA1-GFP (CellLight™ Mitochondria-GFP, BacMam 2.0), the endoplasmic reticulum (ER) marker KDEL-GFP (CellLight™ ER-GFP, BacMam 2.0), or the Golgi marker GALNT1-GFP (CellLight™ Golgi-GFP, BacMam 2.0) were treated as described in Online Methods and imaged on a point-scanning confocal microscope. **b**, Representative images and **c**, Pearson's colocalization coefficients (PCC) quantifying the colocalization between signals from MAO and SiR with PDHA1-GFP, KDEL-GFP, and GALNT1-GFP, respectively. PCC (MAO/PDHA1-GFP) = 0.66 ± 0.01 , PCC(SiR/PDHA1-GFP) = 0.66 ± 0.01 ; PCC (MAO/GALNT1-GFP) = 0.16 ± 0.03 ; PCC (SiR/GALNT1-GFP) = 0.13 ± 0.03 ; PCC (MAO/KDEL-GFP) = 0.27 ± 0.04 ; PCC (SiR/KDEL-GFP) = 0.29 ± 0.04 . Scale bar: 10 μ m. $n = 36, 15$, or 19 cells for each condition indicated in the graph, sourced from at least two biological replicates. Error bars = s.e.m. **** $p < 0.0001$, from one-way ANOVA with Tukey's multiple comparison test.

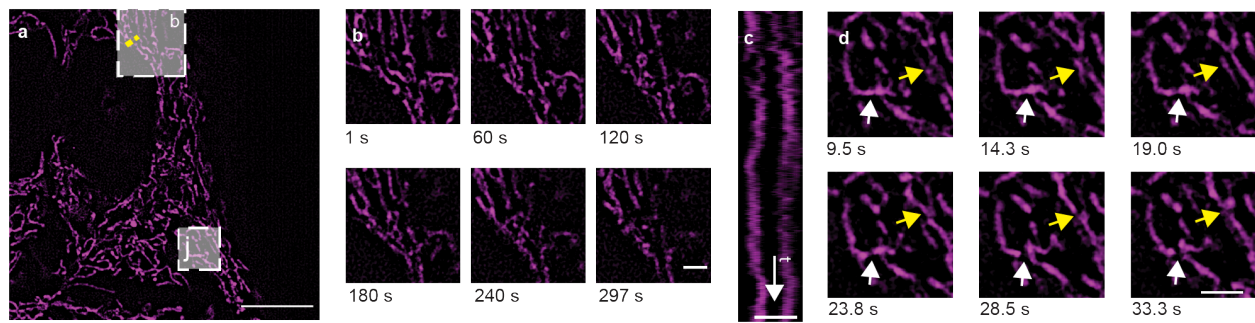


Extended Data Fig. 5. HAO-SiR, the HIDE probe generated from HAO-N₃ and SiR-DBCO, localizes to mitochondria. **a**, HeLa cells expressing the mitochondrial matrix marker PDHA1-RFP (CellLight™ Mitochondria-RFP, BacMam 2.0), the endoplasmic reticulum (ER) marker KDEL-RFP (CellLight™ ER-RFP,

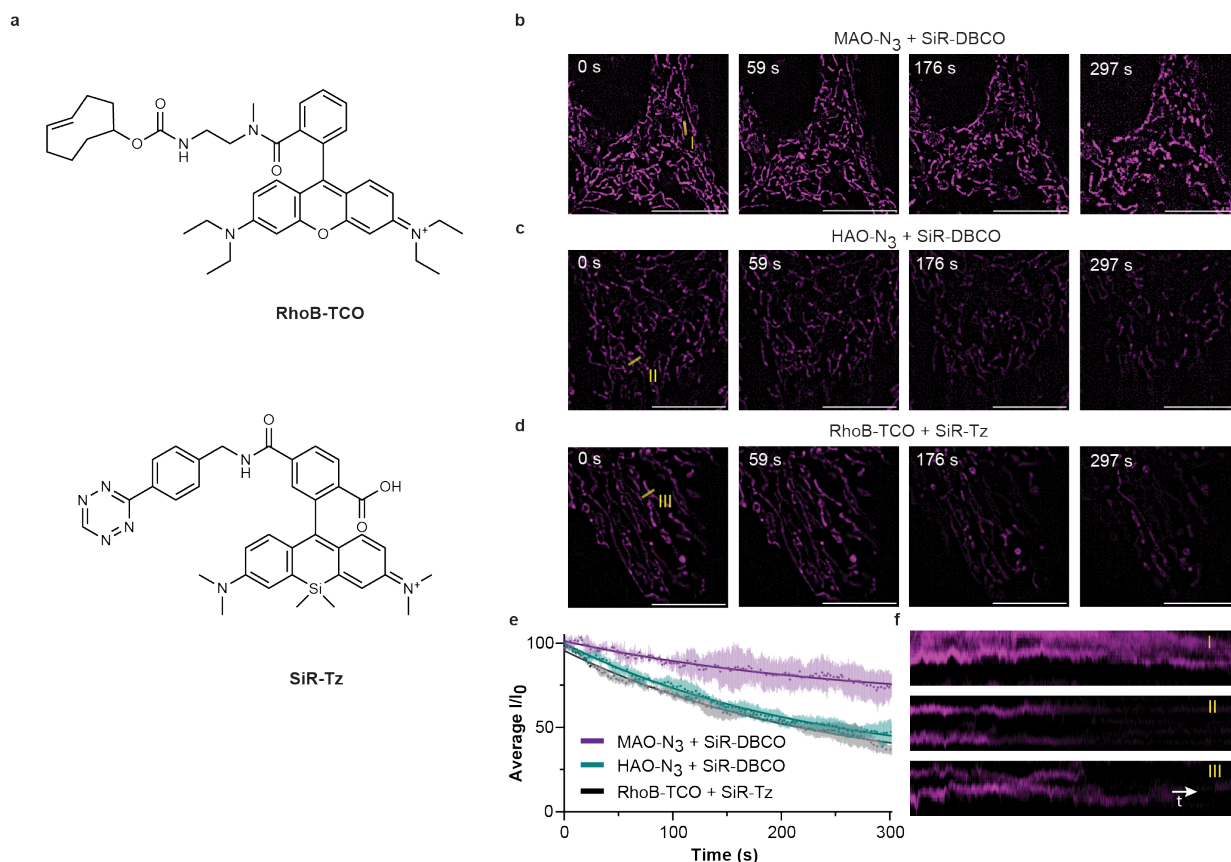
BacMam 2.0), or the Golgi marker GALNT1-RFP (CellLight™ Golgi-RFP, BacMam 2.0) were treated as described in **Online Methods** and imaged on a point-scanning confocal microscope. **b-c**, Confocal images representing the colocalization signal from HAO-N₃ or SiR-DBCO with **b**, mitochondria marker PDHA1-RFP or **c**, ER marker KDEL-RFP and Golgi marker GALNT1-RFP in HeLa cells. **d-e**, Pearson's colocalization coefficients (PCC) quantifying the colocalization of signal between HAO or SiR and mitochondria, ER or Golgi markers in HeLa cells. PCC(SiR/PDHA1-RFP) = 0.70 ± 0.01 , PCC (HAO/PDHA1-RFP) = 0.69 ± 0.01 , PCC (SiR/HAO) = 0.53 ± 0.01 ; PCC (HAO/GALNT1-RFP) = 0.11 ± 0.01 ; PCC (SiR/GALNT1-RFP) = 0.17 ± 0.01 ; PCC(HAO/KDEL-RFP) = 0.31 ± 0.02 ; PCC(SiR/KDEL-RFP) = 0.35 ± 0.02 . Scale bar: 10 μ m. $n = 32, 34, 31, 65, 83, 34$, or 35 cells for each condition indicated in the graph, sourced from at least two biological replicates. Error bars = s.e.m. **** $p < 0.0001$, n.s. (not significant) $0.3428 < p < 0.9999$, from one-way ANOVA with Tukey's multiple comparison test.



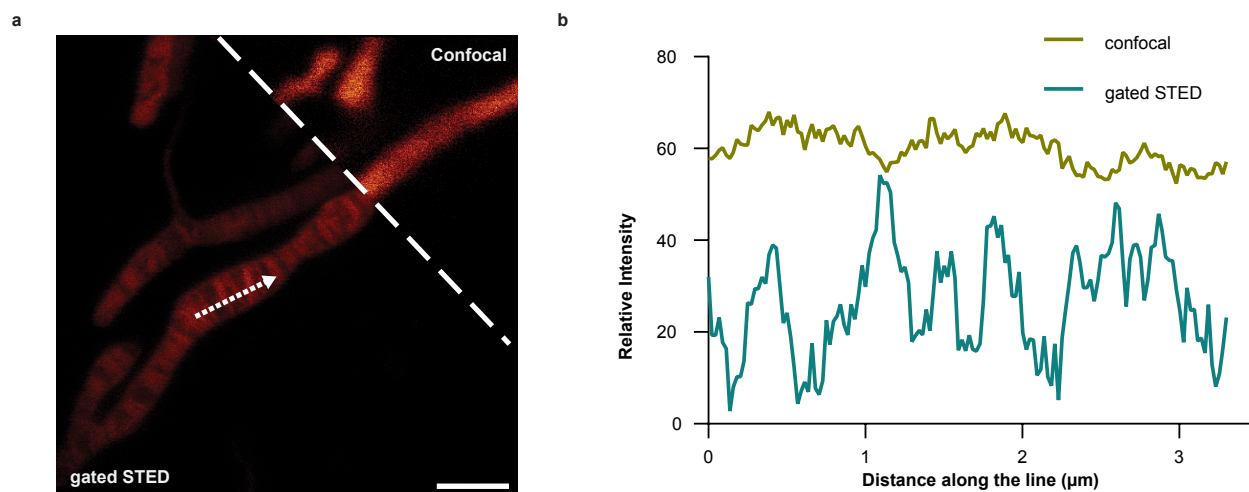
Extended Data Fig. 6. MAO-SiR visualizes mitochondria under depolarizing conditions induced by FCCP. **a**, HeLa cells expressing PDHA1-GFP were labeled with MAO-SiR (200 nM MAO-N₃ + 100 nM SiR-DBCO), treated with or without FCCP (10 μ M, 30min), and imaged using confocal microscopy; **b**, Representative confocal images of MAO-SiR-labeled cells with or without FCCP treatment; **c**, HeLa cells expressing PDHA1-GFP were labeled with tetramethylrhodamine methyl ester (TMRM, 100 nM), treated with or without 10 μ M FCCP, and imaged using confocal microscopy; **d**, Representative confocal images of TMRM-labeled HeLa cells with or without FCCP treatment; Effect of FCCP on the **e**, colocalization of PDHA1-GFP with TMRM or SiR; **f**, normalized signal intensity from confocal images; or **g**, total fluorescence as determined by flow cytometry. $n = 3$ biologically independent replicates for experiments shown in **e-g**. Scale bar: 10 μ m. Error bars = s.e.m. **** $p < 0.0001$, n.s. (not significant) $p = 0.3590$ (for **e**) or 0.0634 (for **g**), from one-way ANOVA with Šídák's multiple comparisons test.



Extended Data Fig. 7. Long time-lapse SIM imaging of IMM dynamics using the HIDE probe MAO-SiR in HeLa cells. Panels a-d are representative images from a 500-frame SIM series obtained over 297 s (Supplementary Movie 5). **a**, SIM image at $t = 0.5$ s. Scale bar: 10 μm . **b**, Snapshots of the bracketed region indicated in a at the times indicated. Scale bar: 2 μm . **c**, Kymograph of the yellow dashed line in a. Scale bar: 1 μm . **d**, IMM fission (white arrow) and fusion (yellow arrow) events from $t = 9.5$ s to $t = 33.3$ s in the bracketed region in a. **Scale bar: 2 μm .**

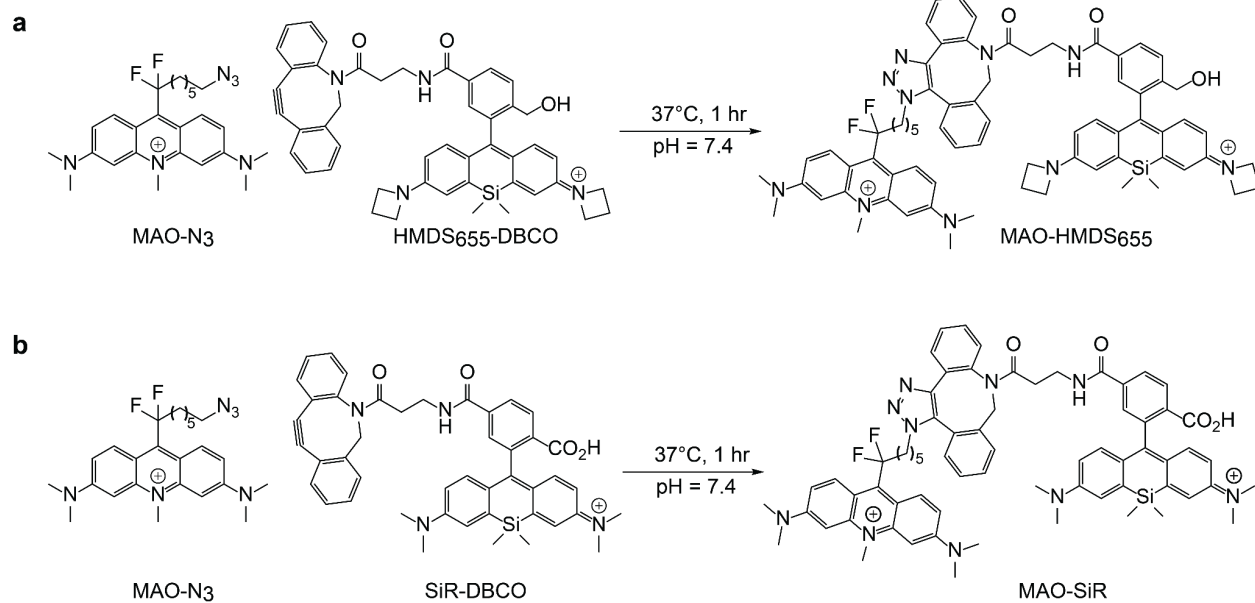


Extended Data Fig. 8. The IMM-selective HIDE probe supports extended-time SIM imaging in live cells. **a**, Structures of RhoB-TCO and SiR-Tz. HeLa cells were treated with **b**, MAO-N₃, **c**, HAO-N₃ or **d**, RhoB-TCO probes followed by SiR-DBCO (**b&c**) or SiR-Tz (**d**) as described in **Online Methods**, and imaged on a widefield microscope equipped with lattice SIM. Scale bar: 10 μ m. **e**, Plot illustrating normalized SiR fluorescence signals of MAO-SiR, HAO-SiR and RhoB-SiR over time. $t_{1/2}$ (HAO-SiR) = 248.2 ± 1.2 s; $t_{1/2}$ (RhoB-SiR) = 233.0 ± 1.3 s. $n = 3$ regions of interest (ROI), $N = 1$ cell. Error bar = s.e.m. **f**, kymograph of line I (MAO-SiR), II (HAO-SiR), and III (RhoB-SiR). Scale bar: 1 μ m

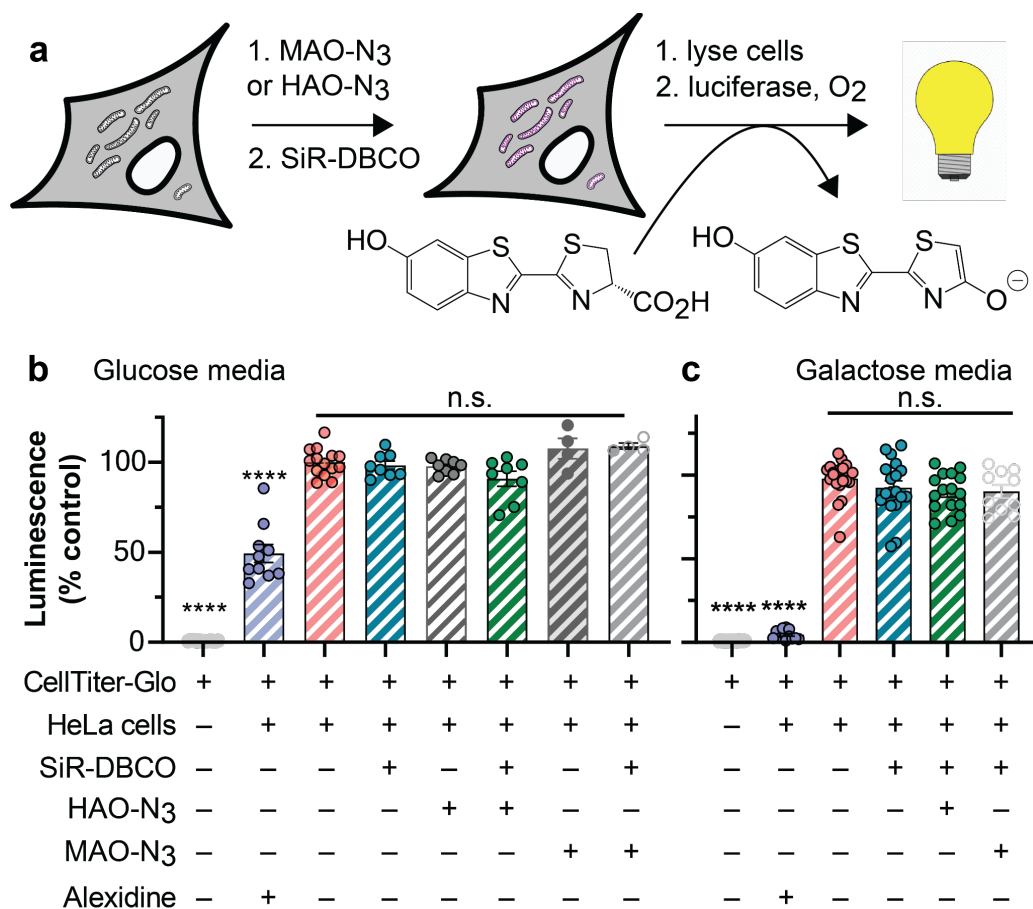


Extended Data Fig. 9. Comparison of diffraction limited confocal microscopy vs. time-gated STED microscopy when imaging the IMM using MAO-SiR. a, gated STED and confocal images of signals due to MAO-SiR, representative from 3 biologically independent replicates. Scale bar: 2 μm . **b,** Plots of signals due to SiR in confocal and STED channels along the dotted arrow in **a**.

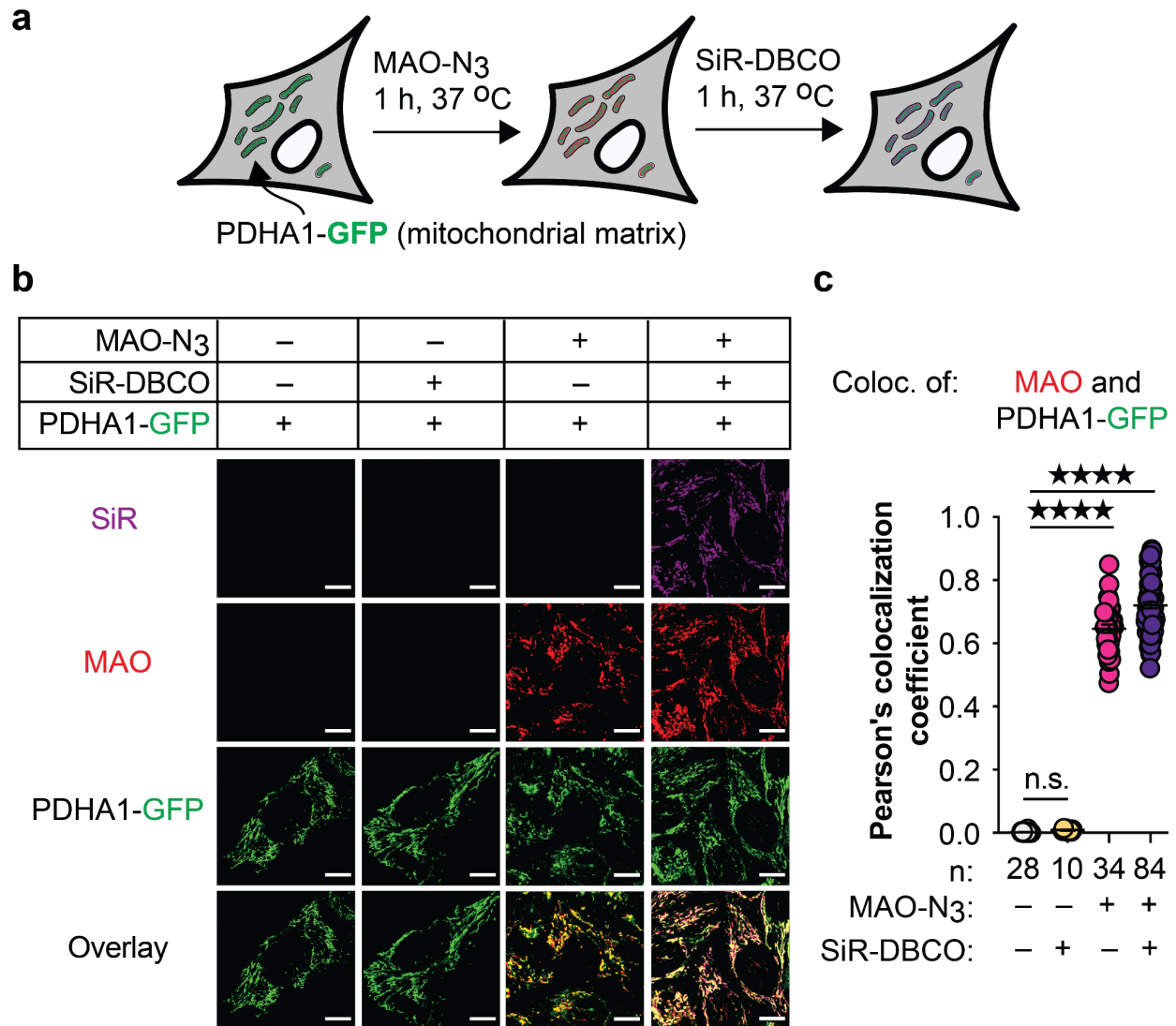
Supplementary Figures



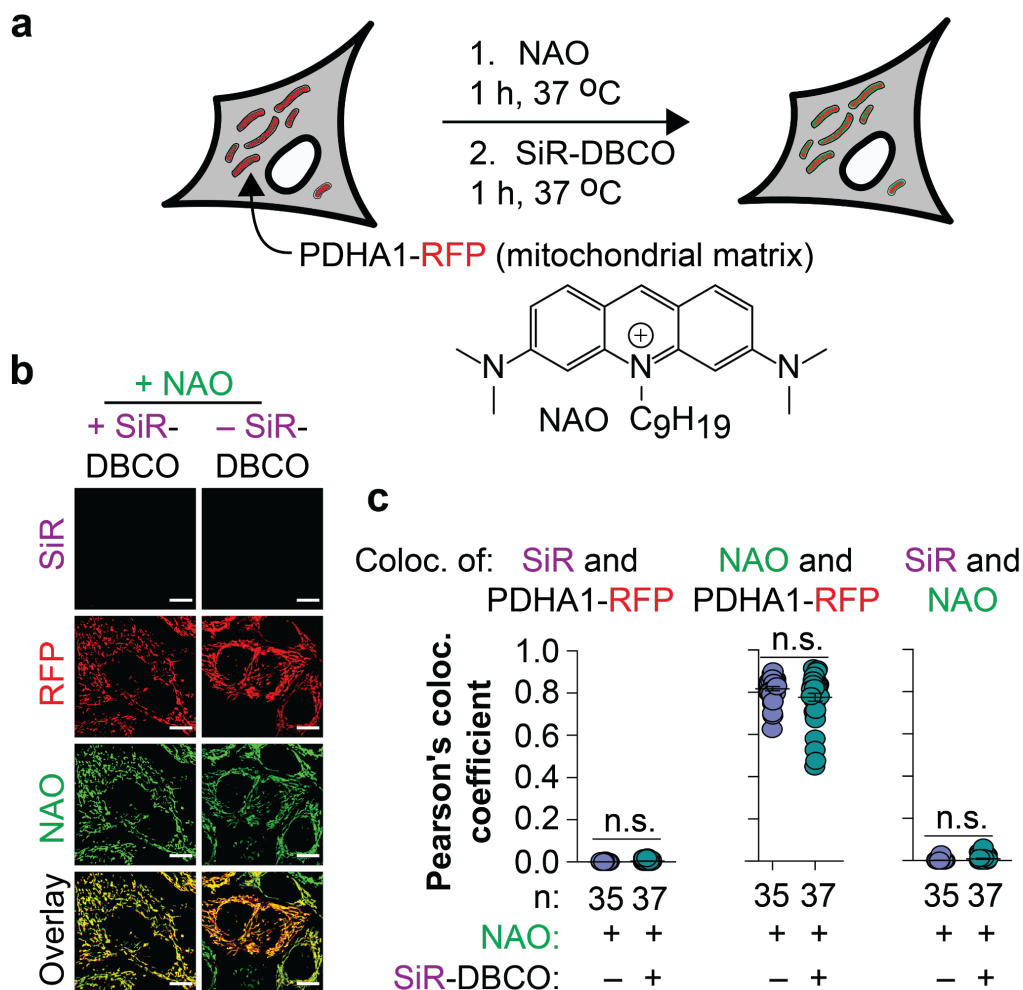
Supplementary Fig 1. Inner mitochondrial membrane-specific HIDE probes MAO-HMDS₆₅₅ and MAO-SiR assembled using SPAAC reaction.



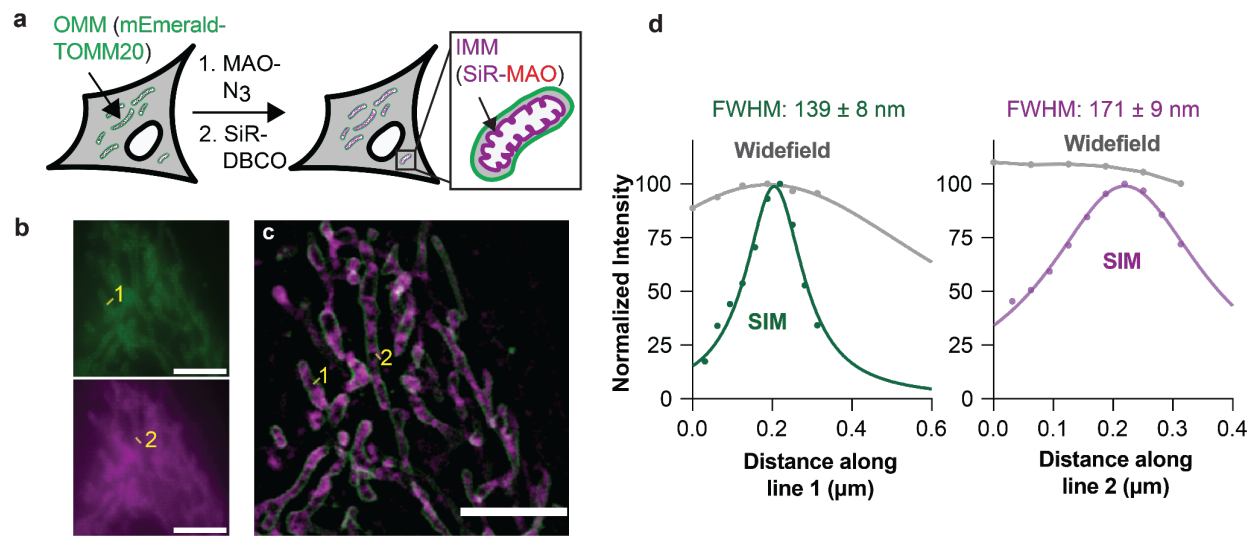
Supplementary Fig. 2. The HIDE probes HAO-SiR and MAO-SiR generated using HAO-N₃ and MAO-N₃ are non-toxic, even in galactose supplemented media. **a**, HeLa cells incubated in standard glucose (DMEM with 4.5 g/L glucose, supplemented with 10% FBS) or oxidative galactose-rich media (DMEM with 4.5 g/L galactose, supplemented with 10% FBS) were treated with HAO-N₃ or MAO-N₃ and SiR-DBCO and the ATP levels measured immediately as described in **Online Methods**. Plots show the relative bioluminescence signals (% relative to untreated cells). **b&c**, Plots show the relative bioluminescence signals of HeLa cell lysates incubated in **b**, standard or **c**, oxidative media. Untreated cells serve as a negative control; cells treated with 5 μ M alexidine serve as a positive control. Alexidine inhibits the PTEN-like mitochondrial phosphatase PTPMT1 and disrupts mitochondrial integrity²⁹. Error bars = s.e.m.; **** $p < 0.0001$, n.s. (not significant) $0.4180 < p < 1$ (for **b**) or $0.2082 < p < 1$ (for **c**) from one-way ANOVA with Dunnett's post-analysis accounting comparison to the negative control where the cells were incubated with cell culture media only. Each bar represents the average of 12 biological replicates.



Supplementary Fig. 3. MAO-SiR, the HIDE probe generated from MAO-N₃ and SiR-DBCO, localizes to mitochondria. **a**, HeLa cells expressing the mitochondrial matrix marker PDHA1-GFP (CellLight™ Mitochondria-GFP, BacMam 2.0) were treated as described in **Online Methods**. Cells were visualized using a point-scanning confocal microscope. **b**, Confocal images representing the colocalization of signal from MAO or SiR with PDHA1-GFP. **c**, Pearson's colocalization coefficients PCC (MAO/PDHA1-GFP) = 0.77 ± 0.01 . $n = 28, 10, 34$ or 84 cells for the conditions indicated in the graph, sourced from at least two biological replicates. Scale bar: $10 \mu\text{m}$. Error bars = s.e.m. **** $p < 0.0001$, n.s. (not significant) $p = 0.9890$, from one-way ANOVA with Tukey's multiple comparison test.



Supplementary Fig. 4. The azido group of MAO-N₃ and HAO-N₃ recruits SiR-DBCO to the mitochondria. **a**, HeLa cells expressing the mitochondrial matrix marker PDHA1-RFP (CellLight™ Mitochondria-RFP, BacMam 2.0) were treated with NAO and SiR-DBCO as described in **Online Methods** and visualized using a point-scanning confocal microscope. **b**, Representative confocal images used for quantification of Pearson's colocalization coefficients. **c**, Pearson's colocalization coefficients (PCC) representing the colocalization of signal from NAO or SiR with mitochondria marker PDHA1-RFP in HeLa cells. PCC (NAO/PDHA1-RFP) = 0.81 ± 0.01; no significant signal was observed in the SiR channel. Scale bar: 10 μm. n = # of cells. Error bars = s.e.m. n.s. (not significant) 0.2027 < p < 0.9991, from one-way ANOVA with Tukey's multiple comparison test.



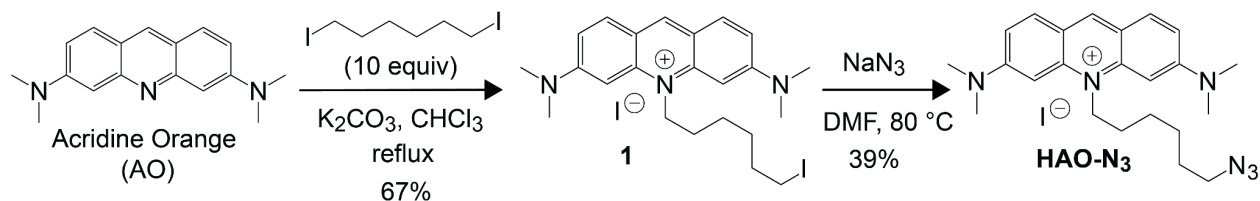
Supplementary Fig. 5. Two-color SIM imaging of mitochondria using the HIDE probe MAO-SiR and the OMM marker mEmerald-TOMM20. **a**, HeLa cells expressing mEmerald-TOMM20 were treated with MAO-N₃ and SiR-DBCO as described in Online Methods and imaged using lattice SIM. **b**, Widefield images of HeLa cells treated as described in **a**; mEmerald-TOMM20 (TOP, green, 510 nm) and SiR (BOTTOM, magenta, 660 nm) channels. Scale bar: 5 μ m. **c**, The reconstructed SIM image showing the merger of SiR and mEmerald signals. **d**, Plot illustrating the fit of signals along line 1 (LEFT) in panels **b** and **c** to a Gaussian function to establish FWHM values of 139 ± 8 nm for the SIM image. Plot illustrating the fit of signals along line 2 (RIGHT) in panels **b** and **c** to a Gaussian function to establish a FWHM of 171 ± 9 nm for the SIM image. Images in **b-c** are representative for at least 3 biologically independent replicates. Same dataset as Fig. 4b was reused here to demonstrate resolution for consistency and ease of reading.

Supplementary Note

Synthesis of HAO-N3, MAO-N3 and HMDS655-DBCO

General considerations. Unless otherwise noticed, all reactions were performed in a nitrogen atmosphere. Chemicals used for synthesis were either prepared according to literature reports or purchased from commercial sources and used without further purification. Flash chromatography was performed using a Teledyne Isco CombiFlash Rf system using pre-packed columns with RediSep Rf silica (40–60 μm). ^1H NMR and ^{13}C NMR spectra were recorded on Bruker Neo-500 (500 MHz for ^1H , 126 MHz for ^{13}C and 471 MHz for ^{19}F) or AV-600 (600 MHz for ^1H and 151 MHz for ^{13}C) NMR spectrometers using Bruker Topspin 3.6 and analyzed by Mestrelab Mestrenova 14.2. The values of chemical shifts (δ) are reported in ppm relative to the solvent residual signals of CD_3OD (3.31 ppm for ^1H , 49.0 ppm for ^{13}C), CDCl_3 (7.26 ppm for ^1H , 77.2 ppm for ^{13}C) or CD_3CN (1.94 ppm for ^1H , 1.32, 118.3 ppm for ^{13}C). Coupling constants (J) are reported in Hz. High-resolution mass spectra (HRMS) were recorded on an Agilent QTOF LCMS with ESI connected to an Agilent UHPLC. HPLC purifications were performed on a reverse-phase column (YMC-Triart C18, 150 mm $\times$ 10 mm S-5 μm , 12 nm) using an Agilent HPLC system.

Synthesis of HAO-N3



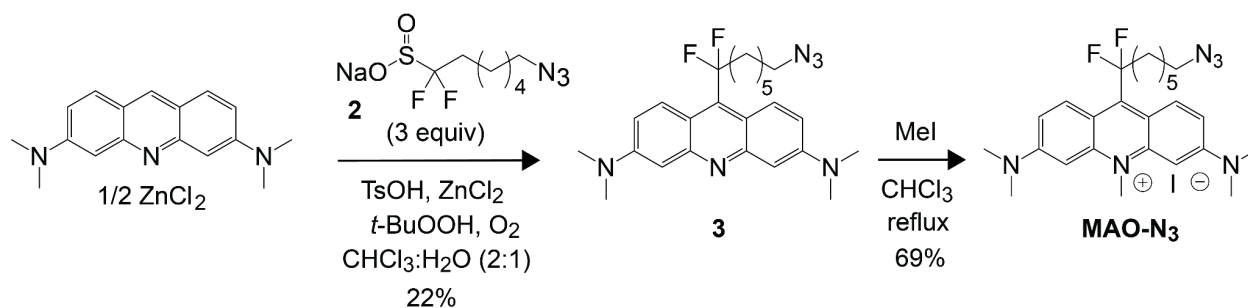
Acridine orange “free base” was obtained according to literature¹. Briefly, 4 g acridine orange $\frac{1}{2}$ ZnCl_2 (Chem-Impex) was dissolved 100 mL H_2O and then treated with excess NaOH solution (4 N) for 30 min. The free acridine orange was extracted with CH_2Cl_2 , washed with water, and dried over sodium sulfate. Removal of solvent under reduced pressure and drying under vacuum afforded 1.85 g of free acridine orange as a dark red-brown powder, which was used in the next step without further purification.

Preparation of 3,6-bis(dimethylamino)-10-(6-iodohexyl)acridin-10-ium (**1**). To a 20 mL microwave vial charged with K_2CO_3 (17.25 mg, 0.125 mmol, 0.5 equiv) was added acridine orange solution (92.5 mg, 0.25 mmol, 1 equiv in 5 mL CHCl_3), followed by addition of 1,6-diiodohexane (0.41 mL, 2.5 mmol, 10 equiv). The reaction mixture was heated at reflux for 16h, cooled, filtered, and concentrated *in vacuo*. The product **1** was obtained as a dark-red solid (100 mg, 67%), upon purification by flash column chromatography (neutral Al_2O_3 , 0 to 5% $\text{MeOH}:\text{CH}_2\text{Cl}_2$). ^1H NMR (500 MHz, CDCl_3) δ 8.68 (s, 1H), 7.89 (d, J = 9.3 Hz, 2H), 7.05 (dd, J = 9.3, 2.1 Hz, 2H), 6.67 (d, J = 2.2 Hz, 2H), 4.99 – 4.89 (m, 2H), 3.25 (t, J = 6.8 Hz, 2H), 2.01 (p, J = 7.8 Hz, 2H), 1.93 – 1.82 (m, 2H), 1.80 – 1.70 (m, 2H), 1.63 – 1.58 (m, 2H). ^{13}C NMR (126 MHz, CDCl_3) δ 155.7,

143.1, 142.8, 133.4, 117.3, 114.2, 93.2, 48.5, 41.5, 33.1, 30.5, 26.3, 26.2, 7.9. HRMS (ES⁺) calcd for (C₂₃H₃₁IN₃) [M⁺] 476.1557, found 476.1549.

Preparation of HAO-N₃. Compound **1** (30.2 mg, 0.05 mmol, 1.0 equiv) and NaN₃ (9.8 mg, 3 equiv) were dissolved in DMF (1 mL) in an 8 mL microwave vial. The reaction was heated at 80 °C for 16 h and monitored by TLC. Upon completion, the reaction mixture was diluted with 30 mL CH₂Cl₂ and washed 3X with brine (30 mL), dried (Na₂SO₄) and evaporated. The corresponding product HAO-N₃ was obtained as a dark-red solid (10.2 mg, 39%), upon purification by flash column chromatography (neutral Al₂O₃, 0 to 10% MeOH:CH₂Cl₂). ¹H NMR (500 MHz, Chloroform-*d*) δ 8.64 (s, 1H), 7.90 (d, *J* = 9.3 Hz, 2H), 7.10 (dd, *J* = 9.2, 2.2 Hz, 2H), 6.78 (d, *J* = 2.3 Hz, 2H), 5.10 – 4.94 (m, 2H), 3.36 (s, 12H), 3.31 (t, *J* = 6.7 Hz, 2H), 2.03 (tt, *J* = 7.9 Hz, 2H), 1.76 (tt, *J* = 7.6 Hz, 2H), 1.71 – 1.64 (m, 2H), 1.59 – 1.52 (m, 1H). ¹³C NMR (126 MHz, CDCl₃) δ 155.8, 143.3, 142.8, 133.5, 117.3, 114.2, 93.0, 51.5, 47.9, 41.1, 29.7, 28.9, 26.8, 26.3. HRMS (ES⁺) calcd for (C₂₃H₃₁N₆) [M⁺] 391.2605, found 391.2594.

Synthesis of MAO-N₃

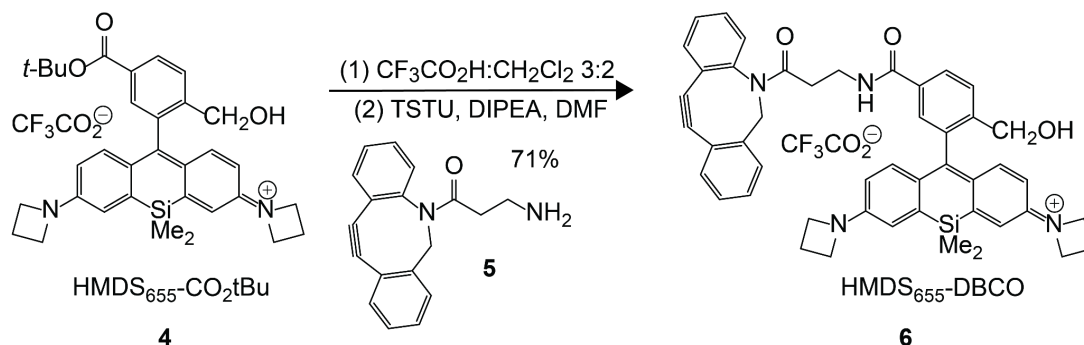


Preparation of 9-(7-azido-1,1-difluoroheptyl)-N³,N³,N⁶,N⁶-tetramethylacridine-3,6-diamine (3). Azide **3** was synthesized as reported². Difluoride **2** (90.0 mg, 0.34 mmol, 4.0 equiv)², acridine orange hemi ZnCl₂ salt (34.8 mg, 0.09 mmol, 1.0 equiv) and ZnCl₂ (17.5 mg, 0.13 mmol, 1.5 equiv) was dissolved in CHCl₃ (1 mL) and H₂O (0.5 mL) in a 4 mL vial equipped with septa screw cap, followed by addition of TsOH·H₂O (32.5 mg, 0.17 mmol, 2 equiv). The reaction mixture was cooled to 0 °C and *tert*-butyl hydroperoxide (TBHP, 70% in water, 0.07 mL, 60.5 mg, 0.47 mmol, 5.5 equiv) was added dropwise with vigorous stirring over 5 min. The reaction was then heated at 50 °C for 1 h, cooled to RT, and stirred for 24 h under continuous air flow. The mixture was then diluted with CH₂Cl₂ (2 mL), separated, and the aqueous layer was washed with CH₂Cl₂ (2 x 2 mL). The combined organic layers were washed with NaHCO₃, brine, dried (Na₂SO₄), and concentrated *in vacuo*. Azide **3** was obtained as a deep-red sticky solid (10 mg, 22%), upon purification by flash column chromatography (neutral Al₂O₃, 0 to 5% MeOH:CH₂Cl₂). The NMR spectrum of the obtained product matched the literature report.

Preparation of 9-(7-azido-1,1-difluoroheptyl)-3,6-bis(dimethylamino)-10-methylacridin-10-ium (MAO-N₃). To an 8 mL microwave vial was added a CHCl₃ (3 mL) solution of azide **3** (10 mg, 0.02 mmol, 1.0 equiv) and then CH₃I (32.2 mg, 0.23 mmol, 10 equiv). The reaction mixture was heated to reflux overnight before cooling and purification

via flash column chromatography (neutral Al_2O_3 , 0 to 5% $\text{MeOH}:\text{CH}_2\text{Cl}_2$). The corresponding product **MAO-N₃** was obtained as a deep red solid (9.1 mg, 69%). ^1H NMR (600 MHz, CHCl_3 - d) δ 8.20 (d, J = 9.9 Hz, 2H), 7.12 (dd, J = 10.0, 2.5 Hz, 2H), 6.89 (d, J = 2.5 Hz, 2H), 4.54 (s, 3H), 3.41 (s, 12H), 3.30 (t, J = 6.8 Hz, 2H), 2.52 (tt, J = 17.1, 8.2 Hz, 2H), 1.83 – 1.74 (m, 2H), 1.63 (tt, J = 7.1, 6.8 Hz, 2H), 1.53 – 1.42 (m, 4H). ^{13}C NMR (151 MHz, CHCl_3 - d) δ 154.4, 145.3 (t, J = 24.3 Hz), 144.6, 129.6 (t, J = 10.2 Hz), 125.1 (t, J = 247.4 Hz), 115.0, 114.5, 94.7, 51.4, 41.4, 41.3, 40.6 (t, J = 24.6 Hz), 28.8, 28.7, 26.6, 22.1. ^{19}F NMR (376 MHz, CHCl_3 - d) δ -81.48 (t, J = 17.4 Hz). HRMS (ES+) calcd for $(\text{C}_{23}\text{H}_{33}\text{F}_2\text{N}_6)$ $[\text{M}^+]$ 455.2729, found 455.2719.

Synthesis of HMDS₆₅₅-DBCO



Preparation of HMDS₆₅₅-CO₂tBu 4. Compound **4** was synthesized according to a previous report³.

Preparation of HMDS₆₅₅-DBCO. To a 4 mL vial equipped with a septa screw cap was added **5** (11.6 mg, 0.02 mmol, 1 equiv) and 2 mL of a 3:2 mixture of $\text{CF}_3\text{CO}_2\text{H}$ and CH_2Cl_2 . The resulting reaction mixture was stirred for 2 h and monitored by TLC. Upon completion, the mixture was concentrated *in vacuo* and thoroughly dried under high vacuum. The product was redissolved in DMF (2 mL), DIPEA (13.9 mg, 0.11 mmol, 5.0 equiv) was added, and the reaction mixture stirred for 5 mins. A solution of TSTU (8.1 mg, 0.03 mmol, 1.25 equiv) and DIPEA (13.9 mg, 0.11 mmol, 5.0 equiv) in DMF (1 mL) was then added. The reaction was stirred overnight and purified using an Agilent semi-prep HPLC system on a reverse-phase column (YMC-Triart C18, 150 mm $\times$ 10 mm S-5 μm , 12 nm) with eluents A (H_2O with 1% TFA) and B(CH_3CN with 1% TFA). The corresponding product HMDS₆₅₅-DBCO (**6**) was obtained as a blue solid (10.9 mg, 71%). ^1H NMR (500 MHz, MeOD) δ 7.64 (d, J = 7.8 Hz, 1H), 7.58 (s, 1H), 7.49 – 7.39 (m, 4H), 7.32 – 7.26 (m, 2H), 7.12 – 7.09 (m, 1H), 7.08 – 7.04 (m, 2H), 6.82 – 6.77 (m, 2H), 6.71 (dd, J = 8.1, 2.7 Hz, 2H), 6.44 (ddd, J = 8.6, 5.8, 2.6 Hz, 2H), 5.42 (s, 2H), 5.14 (d, J = 14.0 Hz, 2H), 3.92 – 3.79 (m, 8H), 3.69 (s, 2H), 2.48 (dq, J = 17.2, 5.9 Hz, 2H), 2.40 – 2.27 (m, 4H), 0.60 (s, 3H), 0.48 (s, 3H). ^{13}C NMR (151 MHz, MeOD) δ 173.48, 169.46, 152.59, 152.41, 152.30, 152.25, 149.33, 140.71, 140.61, 139.24, 135.19, 134.66, 133.34, 130.35, 129.92, 129.68, 129.62, 129.02, 128.82, 128.16, 127.77, 126.56, 124.53, 124.20, 123.65, 121.76, 119.23, 117.29, 116.49, 115.61, 115.34, 114.55, 114.50, 108.88, 94.23, 74.10, 56.51, 53.69,

53.66, 37.43, 35.36, 17.91, 0.15, -0.18. HRMS (ES⁺) calcd for (C₄₇H₄₅N₄O₃Si⁺) [M⁺] 741.3255, found 741.3261.

In vitro characterization of MAO-N₃ and HAO-N₃

In vitro spectroscopic characterization.

Solutions used for absorption and emission spectra and quantum yield were obtained by diluting a 2 mM DMSO stock solution of each fluorophore with Dulbecco's phosphate-buffered saline (DPBS, pH 7.4) to the desired concentration (2 μ M to 125 nM). Absorption spectra were measured at room temperature using a Perkin-Elmer Lambda365 spectrometer in 1 cm pathlength, 1 mL cuvettes. Extinction coefficients were determined by performing a linear fitting of absorbance maxima (490 nm for HAO-N₃, 520 nm for MAO-N₃) against concentration of MAO-N₃ or HAO-N₃. Fluorescence spectra were measured at room temperature using a Jobin Yvon Fluoromax fluorimeter using 1 cm pathlength, 3.5 mL cuvettes. Absolute quantum yield was measured on Hamamatsu Quantaaurus-QY (model No. C13534) using 1 cm pathlength, 3.5 mL cuvettes.

In vitro SPAAC reaction.

In a 300 μ L LCMS vial (equipped with high recovery insert and pre-slit rubber septum), 100 μ L MAO-N₃ (100 μ M) and 100 μ L of HMDS₆₅₅-DBCO (400 μ M) solutions in DI H₂O were mixed and incubated at 37 °C. As is indicated in **Fig. 2d**, 1 μ L aliquots of the reaction mixture were withdrawn upon mixing, after 15 min and 30 min of incubation, respectively. The aliquots were analyzed by a Waters Acquity SQD2 UPLC-MS system equipped with a 2.1 mm x 30 mm, CSH C18 column with eluents A (H₂O with 0.1% HCO₂H) and B (CH₃CN with 0.1% HCO₂H).

Media exchange for HeLa cells in galactose supplemented media.

Five days before the experiment, HeLa cells were seeded in a T-75 tissue culture treated flask at a density of 1.0×10^6 cells and incubated in DMEM supplemented with 10% FBS and 4.5 g/L glucose at 37 °C, 5% CO₂ for 24 h. On day 2, the media was exchanged with DMEM supplemented with 10% FBS, 2.25 g/L glucose and 2.25 g/L galactose and incubated for another 24h. On day 3, the media was exchanged to DMEM, 10% FBS, 1.125 g/L glucose and 3.375 g/L galactose and to media supplemented with 4.5 g/L galactose on day 4. 24 h after the final media exchange step, the HeLa cells were plated for cell viability test.

Cell viability assays.

HeLa cells were plated into the wells of a flat-bottom 96-well plate (2×10^4 cells per well in 300 μ L of media) and each well was incubated overnight. On the following day, the cells in each well were treated with 300 μ L DMEM (negative control) or DMEM solutions of 100 nM HAO-N₃, MAO-N₃, or 5 μ M alexidine (cytotoxicity positive control) for 1 h. Each well was then washed twice with 300 μ L DMEM ph(-), followed by a 1 h incubation with 300 μ L of DMEM ph(-) solutions of either 750 nM HMDS₆₅₅-DBCO or 750 nM SiR-DBCO.

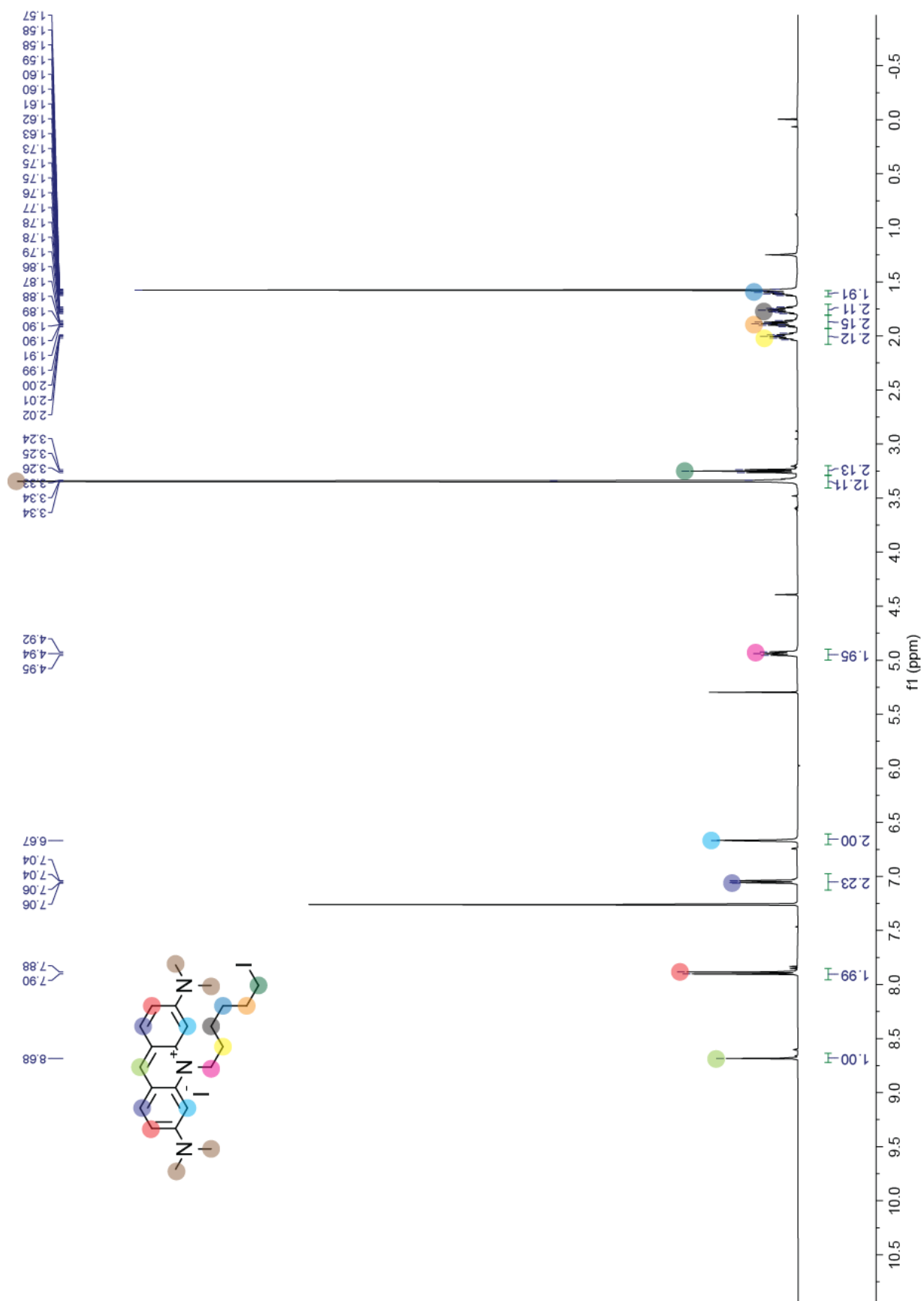
The media in each well was then exchanged with 300 μ L DMEM supplemented with 10% FBS and incubation continued for 20 min. The buffer exchange and incubation was repeated for a total of 3 times. The media in each well was discarded, replaced with 100 μ L of DPBS, and 100 μ L CellTiterGlo 2.0 (Promega) solution was added. The plate was incubated at room temperature for 15 min in the dark. The bioluminescent level of each well was measured using a Synergy H1 plate reader (96-well opaque-walled tissue culture plate, 22 °C). Each of the relative bioluminescence signals (% relative to untreated cells) shown represents the average of 12 replicates.

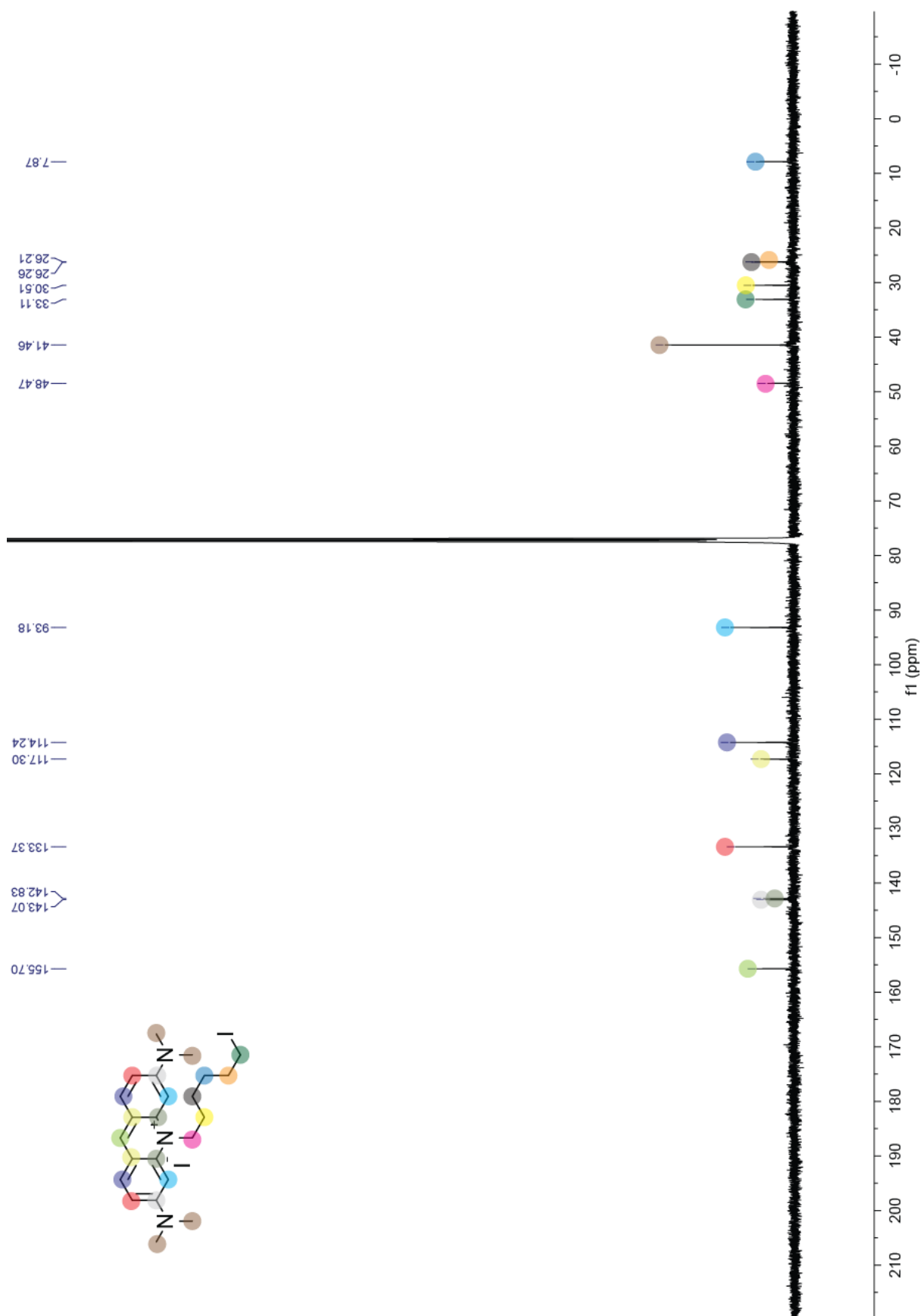
Real-time cell respirometry.

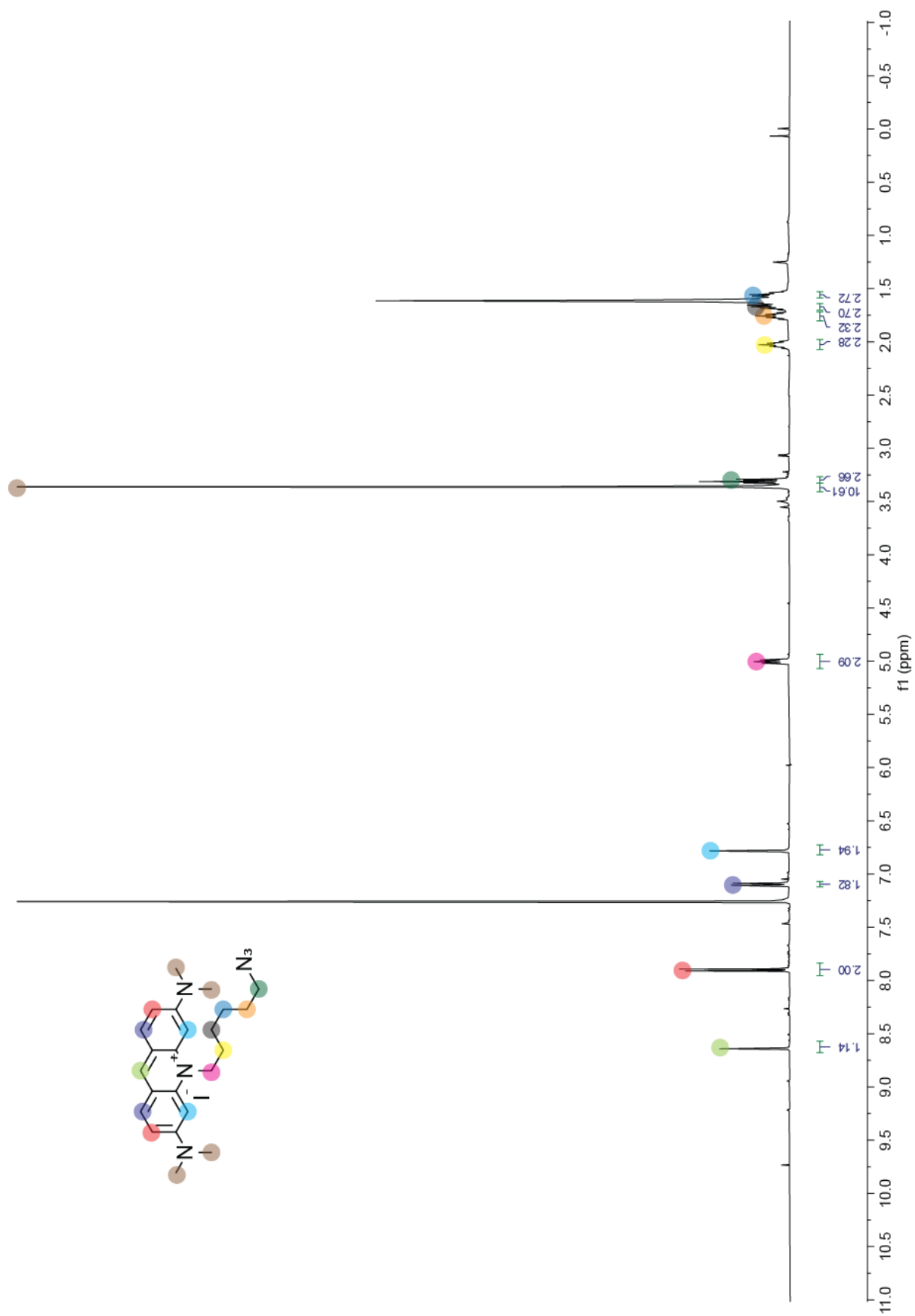
Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using a Seahorse XFe24 Extracellular Flux Analyzer (Agilent). HeLa cells ($6.5\text{--}8.5 \times 10^4$ cells per well) were seeded on the cell culture microplate (Agilent Technologies, 100777-004) and incubated overnight. On the following day, the cells in each well were treated with 500 μ L DMEM (basal conditions) or DMEM solutions of 200 nM MAO-N₃, or 100 nM MitoTracker Deep Red (control) for 1 h. Each well was then washed twice with 500 μ L DMEM ph(-), followed by a 1 h incubation with 500 μ L of DMEM ph(-) media or DMEM ph(-) solutions of either 100 nM HMDS₆₅₅-DBCO or 100 nM SiR-DBCO. The media in each well was then exchanged with 500 μ L DMEM supplemented with 10% FBS and incubation continued for 30 min. The buffer exchange and incubation was repeated for a total of 2 times. The media in each well was then exchanged 3 times with 500 μ L DMEM NaHCO₃(-) supplemented with 10% FBS, 4.5 g/L glucose or galactose, 2 mM glutamine, and 1 mM sodium pyruvate, and equilibrated in a CO₂-free incubator at 37 °C for 1 h before measuring OCR and ECAR values at different metabolic states. Each well was treated with oligomycin (final concentration 1 μ M), FCCP (final concentration 10 μ M), antimycin A (final concentration 1 μ M) and rotenone (final concentration 100 nM) to assess the electron transport chain function. OCR and ECAR values were normalized to cell number. Basal mitochondrial ATP and glycolytic ATP generation rates were calculated based on OCR and ECAR values using the Seahorse Analytics software.

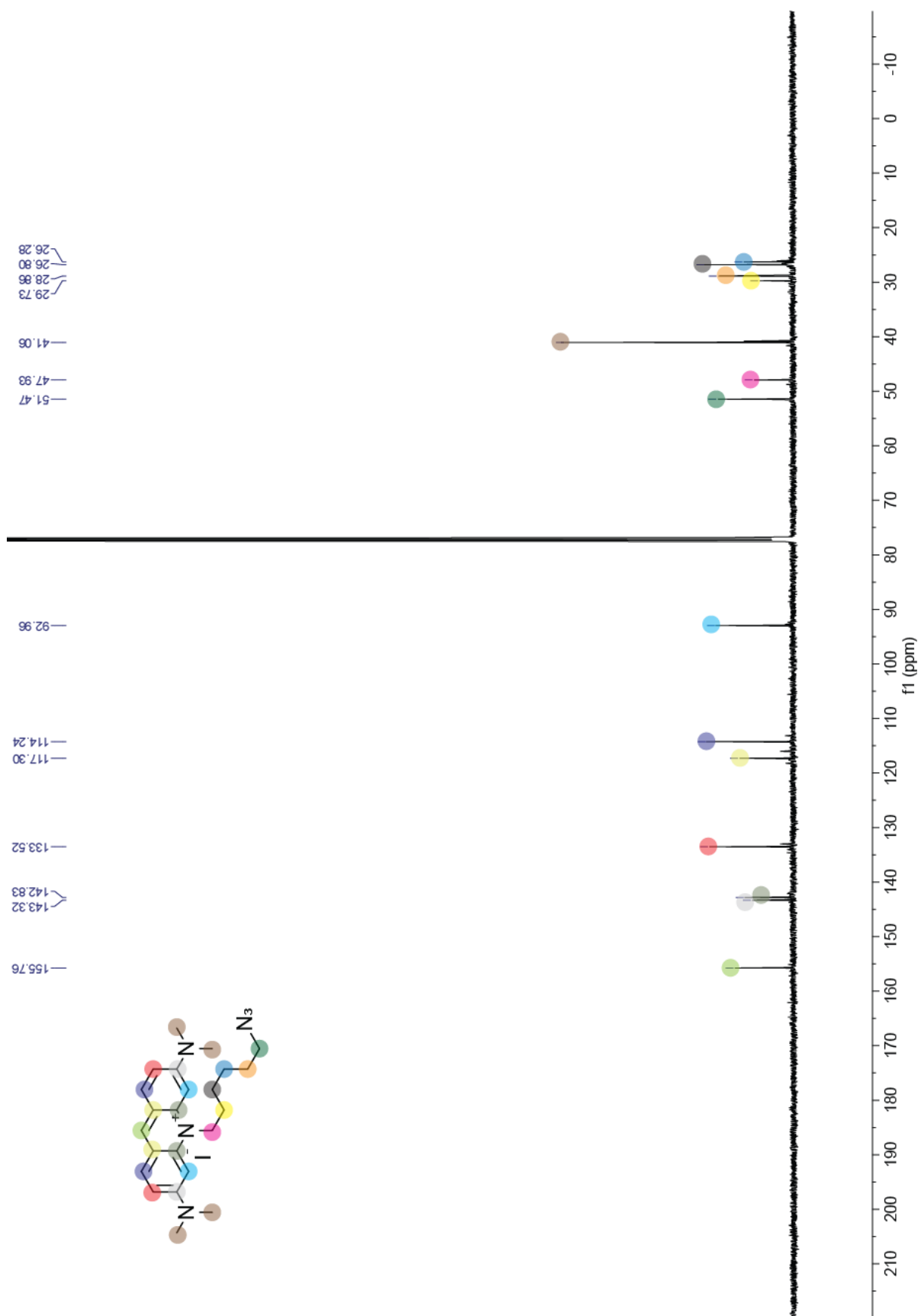
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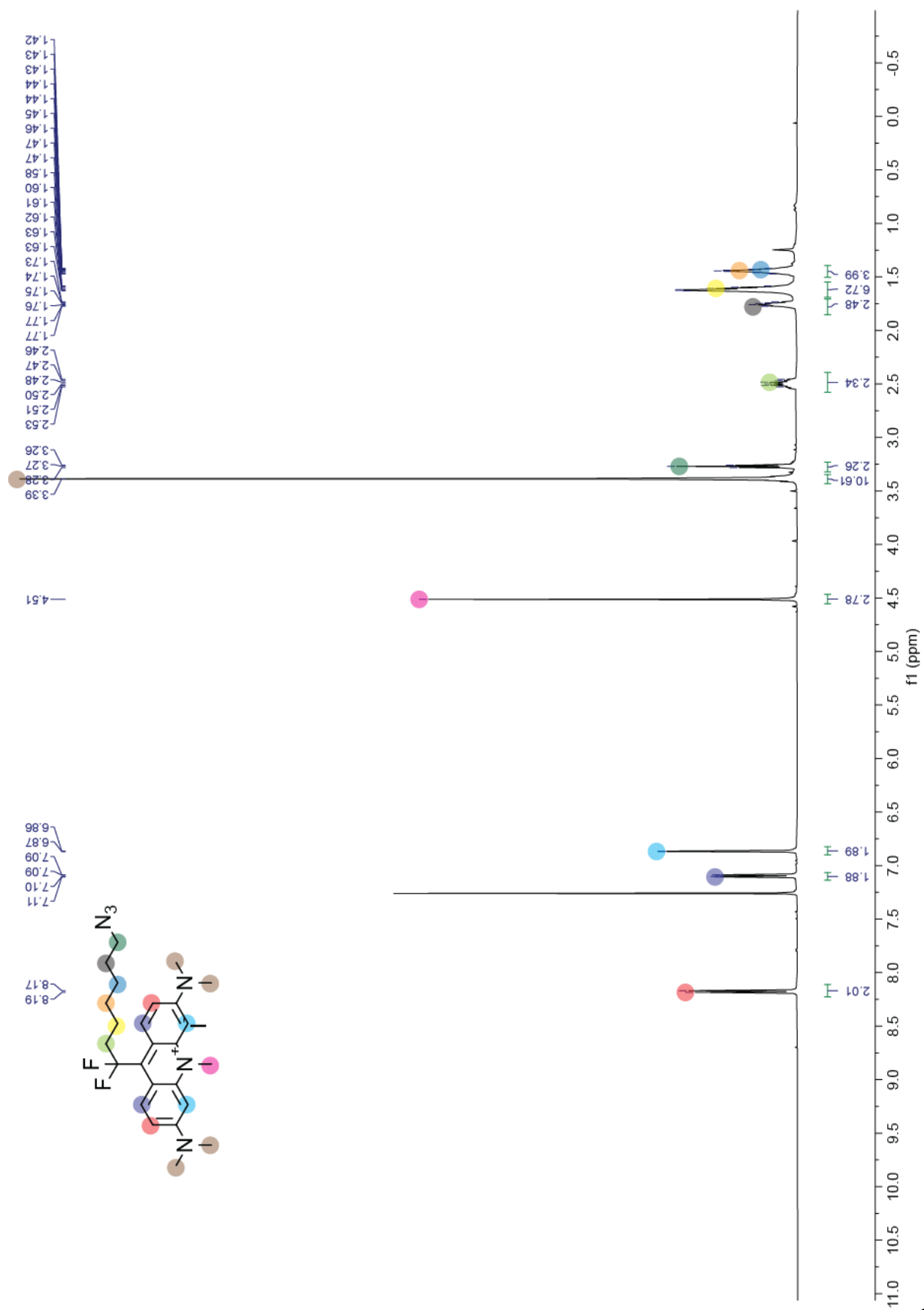
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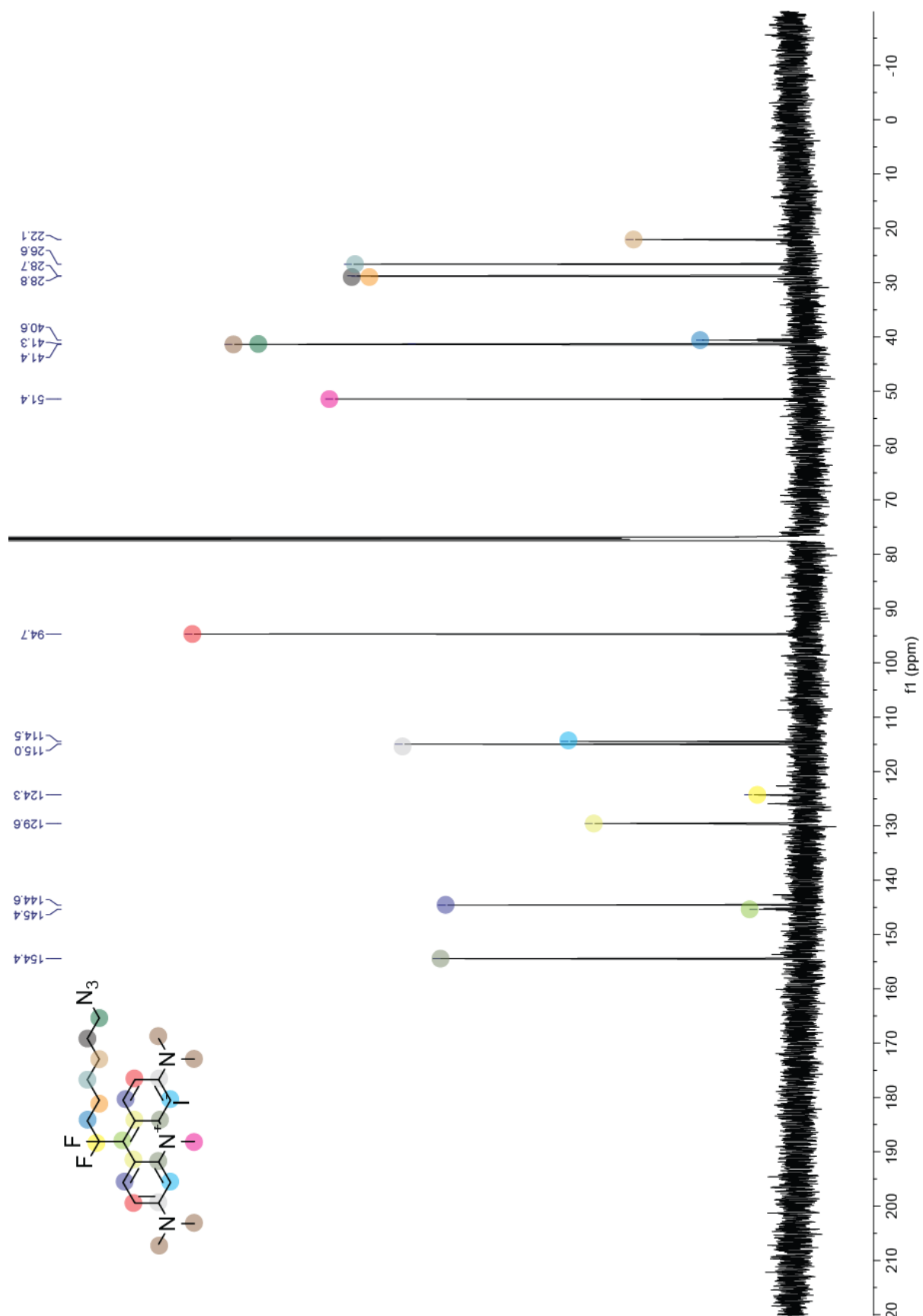


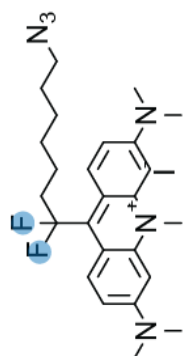




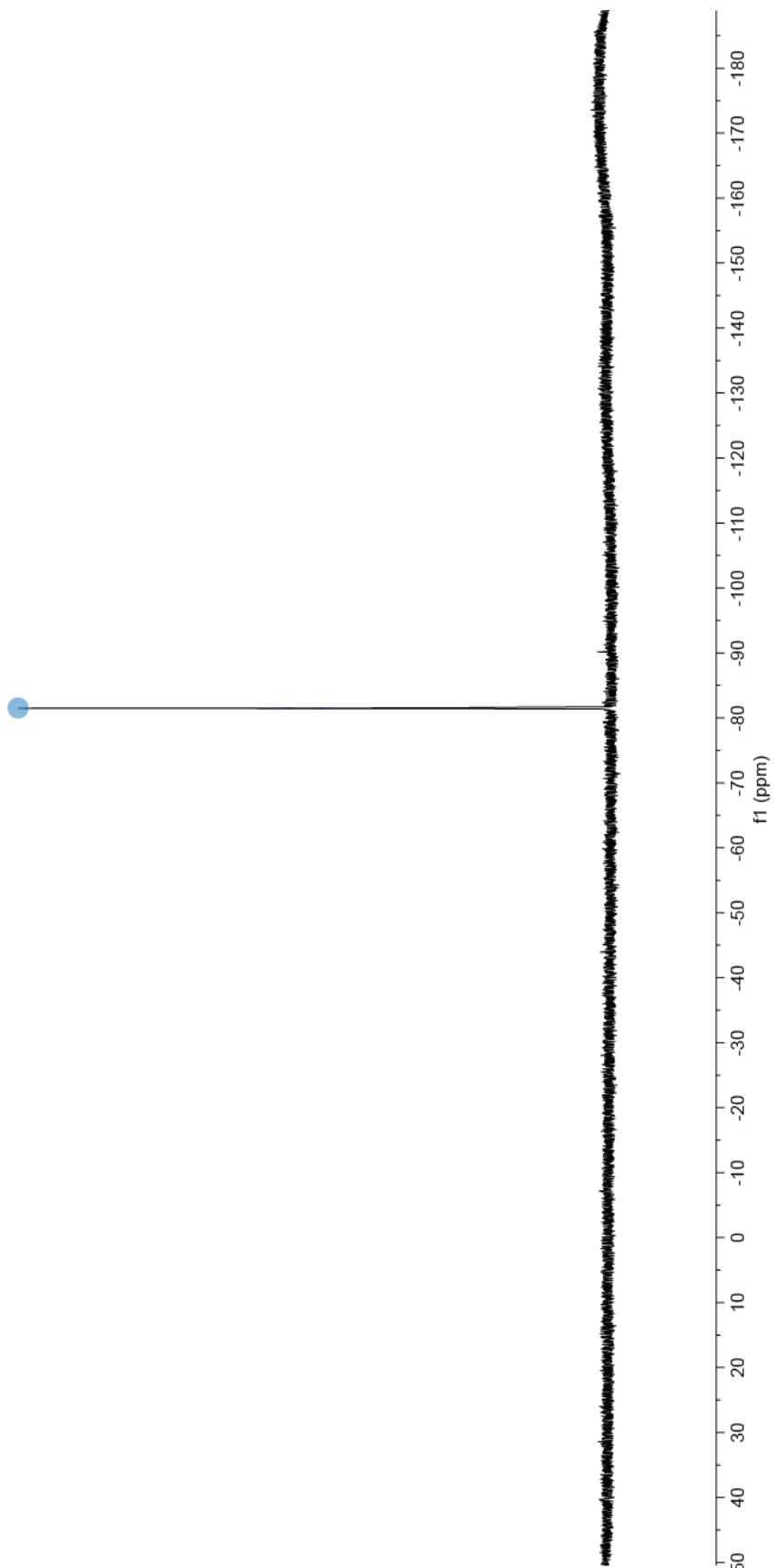


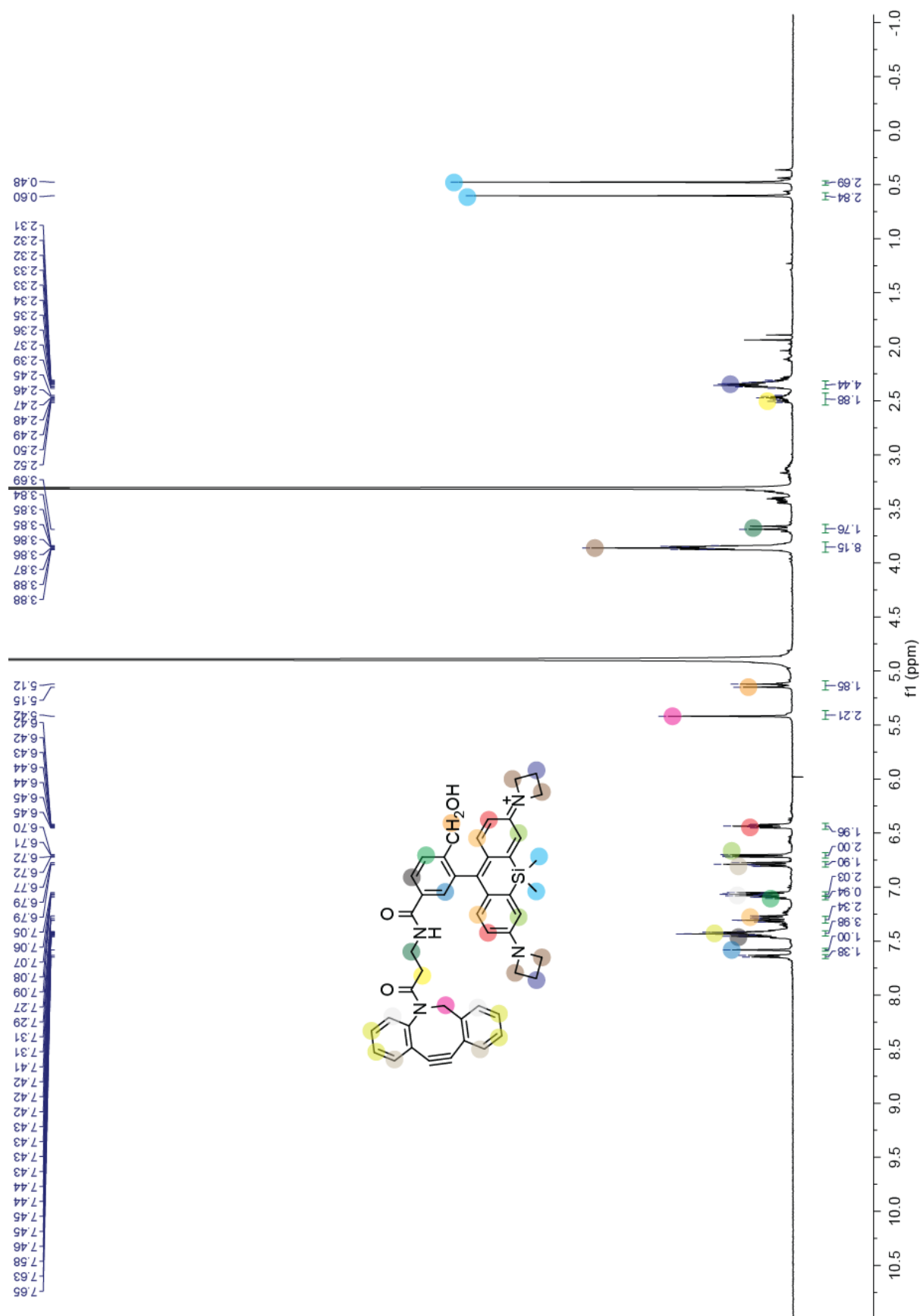


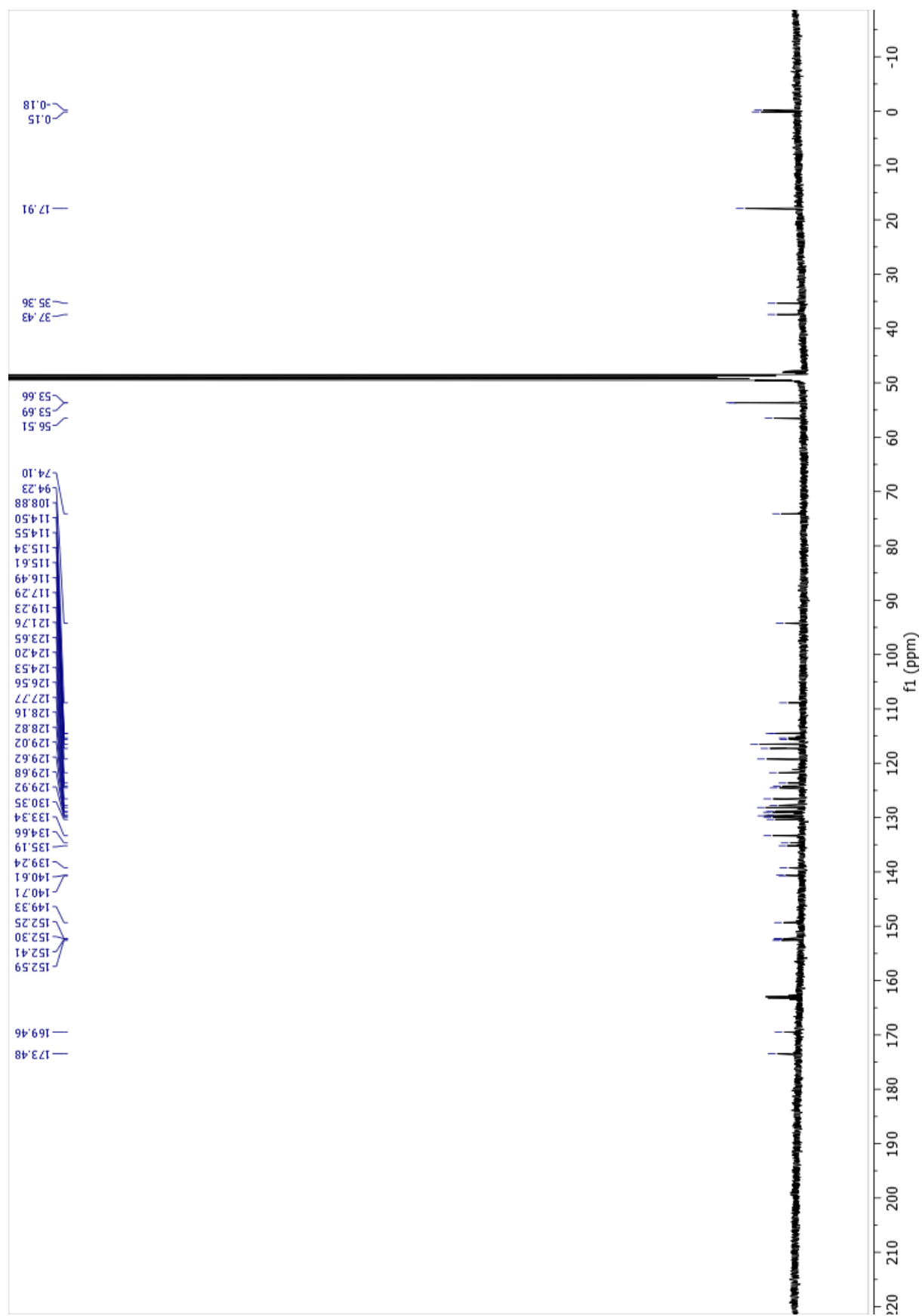




81.44
81.48
81.53







SI References

1. Acridine Orange Conjugated Polymersomes for Simultaneous Nuclear Delivery of Gemcitabine and Doxorubicin to Pancreatic Cancer Cells | Bioconjugate Chemistry. <https://pubs.acs.org/doi/10.1021/acs.bioconjchem.5b00694>.
2. Zhou, Q. *et al.* Bioconjugation by Native Chemical Tagging of C–H Bonds. *J. Am. Chem. Soc.* **135**, 12994–12997 (2013).
3. Chi, W. *et al.* Descriptor $\Delta\text{GC-O}$ Enables the Quantitative Design of Spontaneously Blinking Rhodamines for Live-Cell Super-Resolution Imaging. *Angew. Chem. Int. Ed.* **59**, 20215–20223 (2020).

Chapter 3: Selectively imaging multiple organelles in live cells with no transfection and only one dye

This chapter is based in part on a manuscript in preparation “Selectively imaging multiple organelles in live cells with no transfection and only one dye” and has been adapted with permission from all co-authors.

Abstract

Fluorescence microscopy is foundational to biological studies. However, it suffers from a fundamental challenge: The number of targets one can visualize simultaneously in a living cell. Due to spectral overlap, biologists are limited to a finite number of cellular targets, making dynamic, interaction-based studies challenging. Fluorescence lifetime imaging microscopy (FLIM) provides a long-sought-after solution to this challenge. FLIM adds an orthogonal dimension to spectral imaging— fluorescence lifetime—which allows a microscopist to separate two spectrally overlapping fluorophores. However, current approaches utilize genetic tags or individual fluorophores to label organelles, restricting the biological context or complicating the targeting of fluorophores to organelles. Here, we describe a new approach to FLIM multiplexing: using a tetrazine-triggered fluorogenic dye (HD555) and complementary bioorthogonal handles to develop a transfection-free multiplexing platform. We detail experiments that establish differences in fluorescence lifetime controlled by bioorthogonal chemistry and the ability to image three targets simultaneously in a single cell, using a single dye, without transfection.

Introduction

Fluorescence microscopy is a foundational tool in the arsenal of a cell biologist. Developments in the field, and breakthroughs in hardware have led to innovations such as super-resolution microscopy^{1–9}, light-sheet microscopy^{10–14} and fluorescence lifetime imaging^{15–24}. Each development offers more insight into the underpinnings of biology in a dynamic setting. The ability to visualize biology with high-spatial and temporal fidelity has, and will, open the doors for fundamental biological discovery. Understanding cellular dynamics demands simultaneous imaging of multiple subcellular regions or molecular species. However, multi-target dynamic imaging has a persistent hurdle—the spectral overlap of fluorophore labels.

Typically, multi-target imaging uses spectrally distinct fluorophores^{13,25}. However, the visible spectrum has a finite number of spectrally separable fluorophores that can visualize targets simultaneously. Advances in dye chemistry^{26–29,29–43} allow us to tune the spectra and properties of these fluorophores, tightening their emission profiles. Even so, the emission profiles of many fluorophores overlap, limiting simultaneous imaging.

Fluorescence lifetime imaging microscopy (FLIM), offers an orthogonal dimension to spectral separation. FLIM measures the average time a fluorophore remains excited before emitting a photon, a parameter known as fluorescence lifetime. Crucially, fluorescence lifetime is mainly independent of fluorophore concentration and excitation intensity but is sensitive to the fluorophore's microenvironment. A fluorophore's lifetime sensitivity to its environment makes FLIM an extraordinary tool to measure cellular properties—e.g., membrane viscosity^{18,44}, pH⁴⁵, ion concentration⁴⁶, membrane potential^{47,48}—in living cells. Additionally, tools utilizing FLIM to identify biologically relevant molecules, such as RNA aptamers⁴⁹, also exist. The ability to detect fluorescence lifetime for two spectrally identical species led to a new wave of multiplexed imaging: using FLIM to separate two spectrally identical, but structurally distinct, fluorophores.

Frei et al.²¹ demonstrated that spectrally overlapping fluorophores could be separated into distinct channels using FLIM, allowing one to double the number of cellular targets. Additionally, FLIM multiplexing has been used with stimulated-emission depletion (STED) microscopy to visualize up to five targets simultaneously²⁴, at super-resolution. These reports demonstrate that FLIM offers a solution to spectral overlap. However, the number of targets and fluorophores was identical in each case. This approach introduces a practical limitation: labelling targets for biological experiments.

Labelling adds complexity to a biological experiment. It adds time, stresses the cell, and introduces perturbations to the biological system. Frei et al. report²², an alternative—let the target dictate the lifetime. They engineered HaloTag variants to have a range of fluorescence turn-ons with chloroalkane-bound fluorophores. Differences in fluorescence turn-ons translate to differences in fluorescence lifetime. Using this approach, one fluorophore can label multiple targets, and the target's identity alters the fluorophore's lifetime. In this method, a single plasmid encodes multiple HaloTag protein fusions for cellular targets, and a single cell-permeable chloroalkane (CA) dye is used for labeling,

streamlining the workflow while achieving robust spatial multiplexing via FLIM. However, a critical limitation of the approach is its reliance on genetic transfection, which restricts its applicability in many biologically relevant systems.

To address these limitations, we devised an approach to modulate the lifetime of a fluorophore using bioorthogonal chemistry without the need for transfection or any genetic manipulation. In this work, we show that bioorthogonal chemistry can modulate fluorescence lifetime. Our approach employs a single synthetic dye⁵⁰, HD555, whose fluorescence lifetime can be modulated by its bioorthogonal reaction partner and cellular target. We demonstrate that it is possible to target HD555 to organelles specifically and use FLIM to separate signals from each organelle. This approach expands the applicability of lifetime-based multiplexing to a broader range of biological systems and provides a powerful platform for non-genetic, high-content cell imaging.

Background and concept

Our experiments began with HD555, a rhodamine fluorophore whose pendant aryl ring carries a bioorthogonal tetrazine moiety (**Fig. 1a**). The fluorescent emission of HD555 itself is internally quenched by interactions between the tetrazine and the extended rhodamine chromophore⁵⁰ (**Supplementary Fig. 1**). However, reaction of HD555 with a tetrazine reaction partner eliminates the quenching, resulting in fluorescent enhancements of up to 123-fold⁵⁰. Notably, the magnitude of the turn-on efficiency depends on the identity of the reaction partner and, hence, the final product's structure⁵¹. For example, with *trans*-cyclooctene (**TCO**) as the reaction partner, the turn-on efficiency is **33-fold**, whereas with bicyclo[6.1.0]non-4-yne (**BCN**) the efficiency is **123-fold** (**Fig. 1a**). We hypothesized that different turn-on efficiencies of HD555 reaction products would translate into different fluorescence lifetimes (**Supplemental Note**).

If these reaction products could be localized to a single organelle in a live cell, then one could imagine selectively visualizing different organelles with HD555 alone: after reaction, each organelle-specific product would be characterized by a different fluorescence lifetime (**Fig. 1a**). If HD555 was localized to a single organelle using a small molecule, then organelle structure and dynamics could be visualized with only small molecule reagents and without transfection or other genetic engineering using multi-channel fluorescence lifetime imaging (**Fig. 1b**). Such a tool would complement the use of organelle-specific HaloTag variants that can be self-labeled to generate products with distinct fluorescence lifetimes²².

In vitro studies

To test these ideas, we reacted HD555 with three different inverse electron-demand Diels-Alder tetrazine reaction partners, TCO, BCN, and Spiro[2.3]hex-1-ene (SpH)⁵², *in vitro* and evaluated the fluorescent intensity and lifetimes of the reaction products (**Extended Data Fig. 1a**). The intensity-weighted fluorescence lifetimes (**Extended Data Fig. 1b, Supplementary Table 1**) ranged from a high of 2.440 ± 0.011 ns for the BCN reaction product to a low of 1.935 ± 0.002 ns for the SpH reaction product. The intensity-weighted fluorescence lifetime of the TCO product was 2.211 ± 0.015 ns. Amplitude-

weighted lifetimes of the three products showed a similar trend (**Extended Data Fig. 1c**). Furthermore, when the lifetime data was subjected to phasor analysis, each reaction product formed a distinct population (**Extended Data Fig. 1d**). The relative lifetimes of the TCO and BCN reaction products are fully consistent with the previously observed higher turn-on⁵⁰ of the BCN product. Given rates of reaction of BCN and TCO with tetrazines^{53–56} and their wide use in live-cell experiments, we determined these two dienophiles to be the best candidates for live-cell experiments.

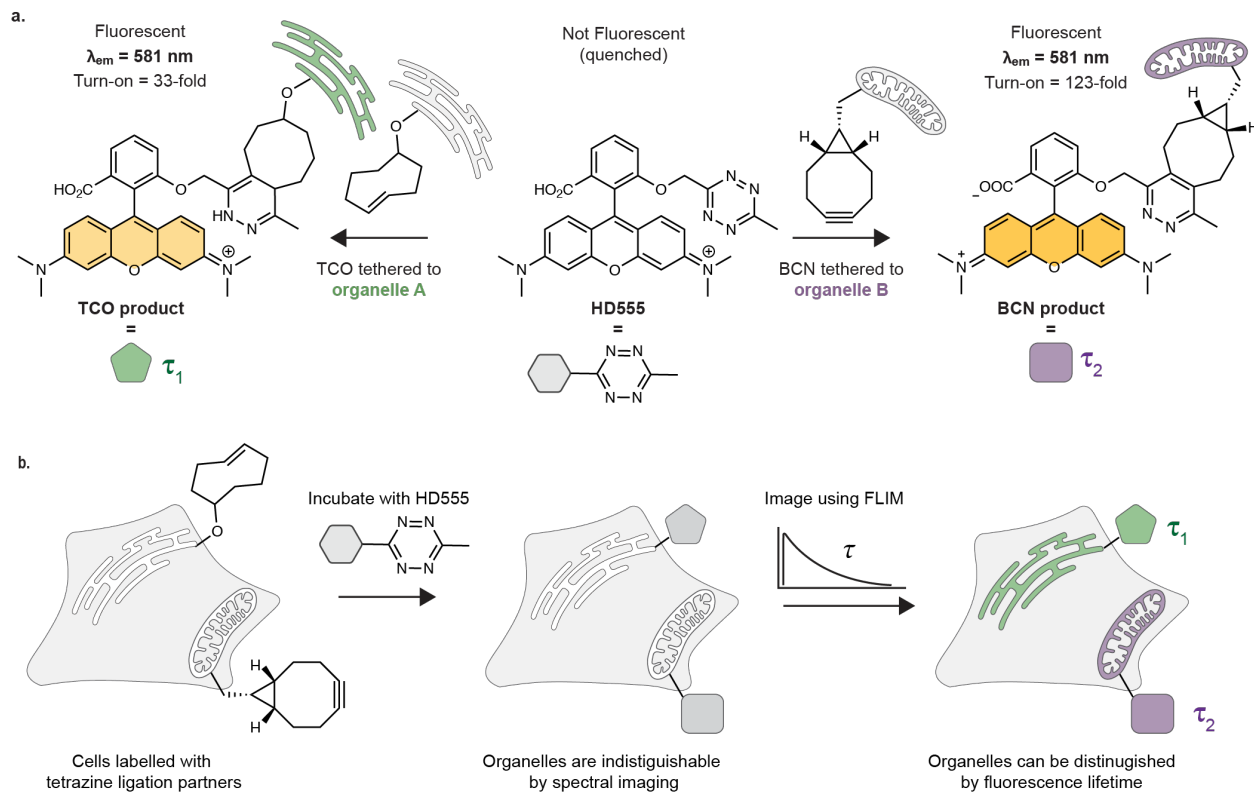


Fig. 1 | Using bioorthogonal reactions to modulate fluorescence lifetime. a, HD555, a tetrazine-quenched, fluorogenic rhodamine derivative, reacts with TCO or BCN to generate products that emit maximally at the same wavelength (581 nm) but with different intensities. The change in intensity upon reaction, referred to as the “turn-on efficiency”, is higher for reactions with BCN than with TCO, and would result in a difference in fluorescence lifetimes between the two products (see **Supplemental Notes** for further details). **b,** Schematic of live-cell imaging using bioorthogonal chemistry to induce changes in fluorescence lifetime. TCO and BCN are targeted to individual organelles using a small-molecule probes. Cells would then be incubated with HD555, leading to distinct products forming at each organelle. The reaction products would dictate the lifetime of HD555, thereby allowing one to separate the fluorescence signals from each organelle using FLIM.

Proof of principle: live-cell studies with self-labelling enzymes

We next asked if these structure-dependent differences in fluorescent lifetimes would be reproduced in cells. We made use of HeLa cells expressing Sec61 β -HaloTag and treated with a HaloTag substrate carrying either a terminal *trans*-cyclooctene (HTL-TCO) or a terminal bicyclo[6.1.0]nonyne (HTL-BCN) (**Fig. 2a, Supplementary Fig. 2**) to localize the two tetrazine reaction partners to the cytoplasmic face of the endoplasmic reticulum (ER). The cells were treated with HD555, incubated for 30 min, and visualized by fluorescence intensity or average photon arrival time (or fast FLIM) (**Fig. 2b**).

When visualized by fluorescent intensity, cells expressing Sec61B-HaloTag and reacted with HTL-TCO and then HD555 appeared identical to those reacted with HTL-BCN and then HD555 (**Fig. 2b, grayscale**). Organelle morphology for the ER showed no discernable differences. However, average photon arrival time told a different story: the ER in cells treated with HTL-TCO followed by HD555 had a lower average photon arrival time, than cells treated with HTL-BCN followed by HD555 (**Fig. 2b, Color-coded average photon arrival time**). When the decay curves from each population were fit to an exponential decay, The HTL-TCO population was characterized by a lower average intensity-weighted fluorescence lifetime (τ) (2.54 ± 0.05 ns) than the HTL-BCN population (2.75 ± 0.04 ns). The lifetime data was then transposed to a phasor plot⁵⁸. Phasor analysis provides a graphical, no-fit, method to visualize and analyze lifetime data. Phasor analysis transforms the time delays of lifetime data from each pixel in an image into a single point on a 2D polar-coordinate plot whose x- and y-axes represent the cosine and sine components, respectively of the phasor transformations⁵⁷. When the lifetime data for the HTL-TCO and HTL-BCN populations are plotted on the same phasor plot, (**Fig. 2b, middle plot**), one can easily determine that there are two lifetime populations. Furthermore, the phasor population for HTL-TCO-treated cells had a lower lifetime than the HTL-BCN treated cells, consistent with our hypothesis and previous data. Phasor analysis and separation was used to assign the images to BCN or TCO associated populations (**Supplementary Fig. 2**). Remarkably, the only difference between the two populations of cells was the bioorthogonal handle—a difference that suggests that the lifetime differences would exist in other cellular contexts.

Three additional experiments were performed to establish that the fluorescence lifetime of an HD555-derived fluorophore correlates with the structure of the reaction product. First, we altered the subcellular location of the HD555-derived fluorophore to the mitochondrial outer membrane instead of the ER using cells expressing TOMM20-HaloTag, modified with either HTL-TCO or HTL-BCN (**Supplementary Fig. 3**). Next, we altered the identity of the self-labeling protein used to localize HD555 to a given organelle membrane using cells expressing SNAPTag-Sec61B and modified with either BG-TCO or BG-BCN (**Supplementary Fig. 4**). Finally, we altered both the subcellular location of the HD555-derived fluorophore as well as the self-labeling tag using cells expressing Cox8A-SNAPTag, again modified with either BG-TCO or BG-BCN (**Supplementary fig. 5**). For each protein, when the cell population were treated with HD555 and visualized by average photon arrival time, populations containing the TCO reactive partner were characterized by a lower average arrival time than those containing the BCN reactive partner (**Supplementary Fig. 2c, 3c, 4c, 5c**). Fitting the decay curves for each population

further validated these results—the average intensity weighted lifetimes (**Fig. 2c**, **Supplementary Fig. 9 & 10; Supplementary Table 1**) and average amplitude weighted lifetimes (**Supplementary Fig. 9 & 10; Supplementary Table 1**) of HD555 were always higher for BCN treated cells than TCO treated cells. Single species phasor plots for BCN-treated cells always displayed clusters with higher lifetimes than TCO-treated cells (**Supplementary Fig. 11**), and, when analyzed, the cursor values from BCN populations were consistently higher than TCO populations (**Fig. 2d**).

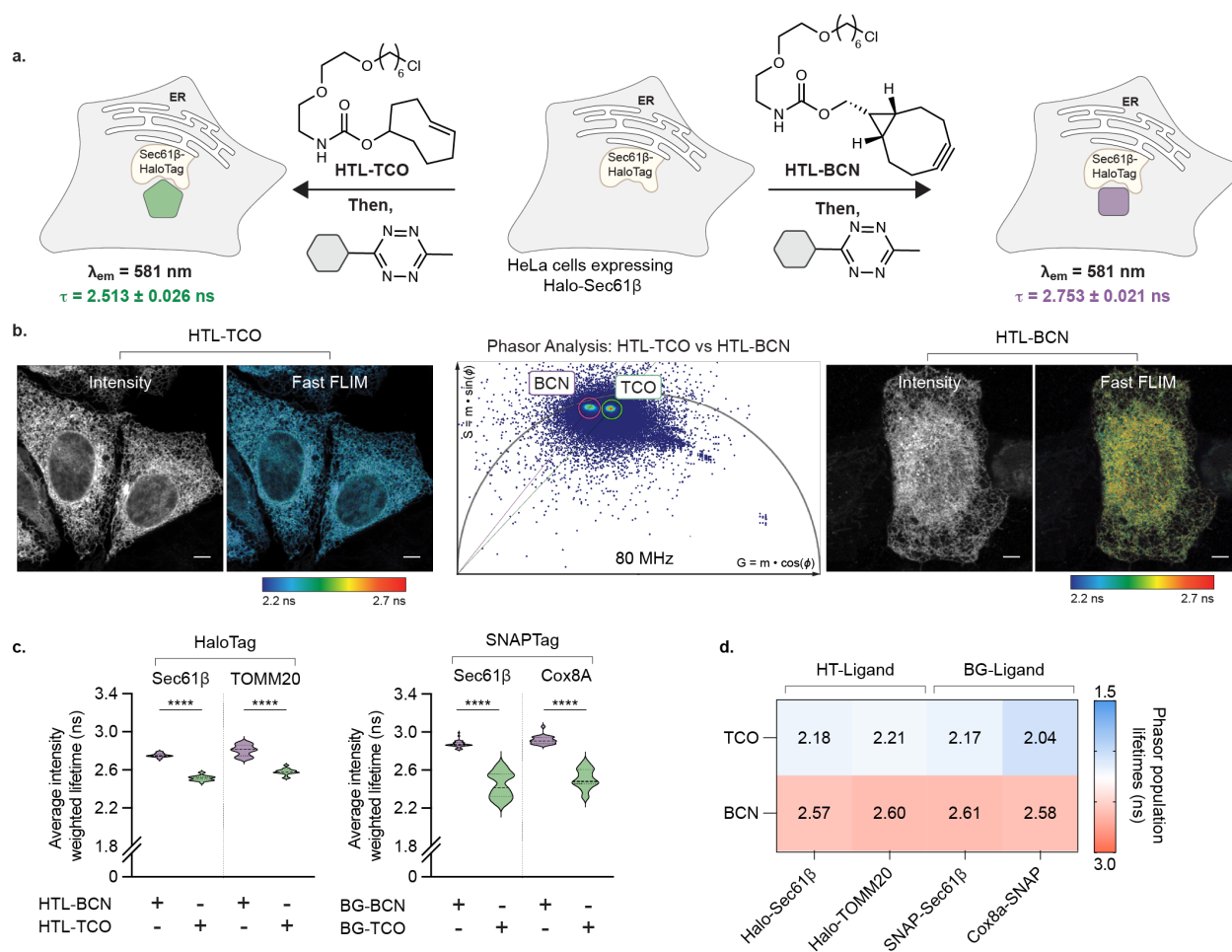


Fig. 2 | Bioorthogonal reactions predictably modulate fluorescence lifetime. **a**, Scheme of live cell experiment to determine if turn-on efficiency translates into differentiable fluorescence lifetimes (τ). HeLa cells expressing HaloTag-Sec61 β are treated first with HTL-TCO or HTL-BCN (10 μ M) followed by HD555 (1 μ M) and subsequently **(b)** imaged by detecting either fluorescence intensity or fluorescence lifetime. Composite phasor plot (**b**, middle plot) shows two lifetime populations, where cells treated with HTL-BCN have a higher lifetime than cells treated with HTL-TCO. Analogous experiments were performed with cells expressing TOMM20-HaloTag (**Supplementary Fig. 3**), Sec61B-SNAPTag (**Supplementary Fig. 4**) or Cox8A-SNAPTag (**Supplementary Fig. 5**). **c**, Plots showing the average intensity-weighted fluorescent lifetimes of eight cell populations, demonstrating consistent difference between BCN and TCO populations (for N , see **supplementary table 1**). **** $P < 0.0001$ for all pairwise comparisons between BCN and TCO populations: SNAP-Sec61 β ($\Delta = 0.4322 \text{ ns}$), Cox8A-SNAP ($\Delta = 0.4075 \text{ ns}$), Halo-Sec61 β ($\Delta = 0.2397 \text{ ns}$),

and Halo-TOMM20 ($\Delta = 0.2255$ ns). Adjusted P values calculated using Dunnett's T3 multiple comparisons test. Statistical significance was determined via Welch's and Brown-Forsythe ANOVA with post hoc Dunnett's T3 test accounting for heteroscedasticity. **d**, Cursor values extracted from phasor plots for the eight populations demonstrate that turn-on correlates with lifetime. Scale bars: **5 μ m**.

Thus, the product of reaction of HD555 with TCO exhibits a lower fluorescence lifetime than the product of reaction with BCN, regardless of whether this reaction occurs on the surface of the ER or the mitochondria, whether it is tethered to this organelle via SNAPTag or HaloTag, and where within the mitochondrial it is localized; TOMM20 is localized to the outer mitochondrial membrane whereas Cox8A is localized to the inner mitochondrial membrane. The shorter fluorescence lifetime of the TCO product is fully in line with the lower turn-on efficiency of HD555 upon reaction with a TCO reaction partner; given the same chromophore, a lower turn-on implies a lower fluorescence lifetime (**Supplementary Note 2**) for the TCO product compared to the BCN product. The differences in lifetime observed here are easily distinguished using phasor analysis^{20,57,58},. In all cases, the product resulting from the reaction of HD555 and TCO generated a distinct, separable lifetime population than the population resulting from reaction of HD555 with BCN (**Supplementary Fig. 9, 10, 11**), suggesting that the lifetime differences could support two-channel imaging.

To test this idea, cells expressing both Sec61 β -SNAPTag and TOMM20-HaloTag were treated with a mixture of HTL-TCO and BG-BCN (10 μ M) and then with HD555 (1 μ M) (**Fig. 3a**). When visualized by total fluorescence intensity of HD555, the ER and the mitochondria are indistinguishable (**Fig. 3b, grayscale**), however, average photon arrival time (fast FLIM, **Fig. 3b, false-colored**) established some contrast between two different morphologies. While the contrast is promising and allows us to visually distinguish the two organelles, image analysis and quantification require separate channels. We aimed to separate the organelles using phasor analysis. We used the single-species populations as ground truth and applied cursor values to the phasor plot from the two-species image. Upon separation, the ER and mitochondria are easily visualized and differentiated (**Fig. 3b**). The same was true when the cells were instead treated with a mixture of HTL-BCN and BG-TCO and then with HD555 (**Extended Data Fig. 2**).

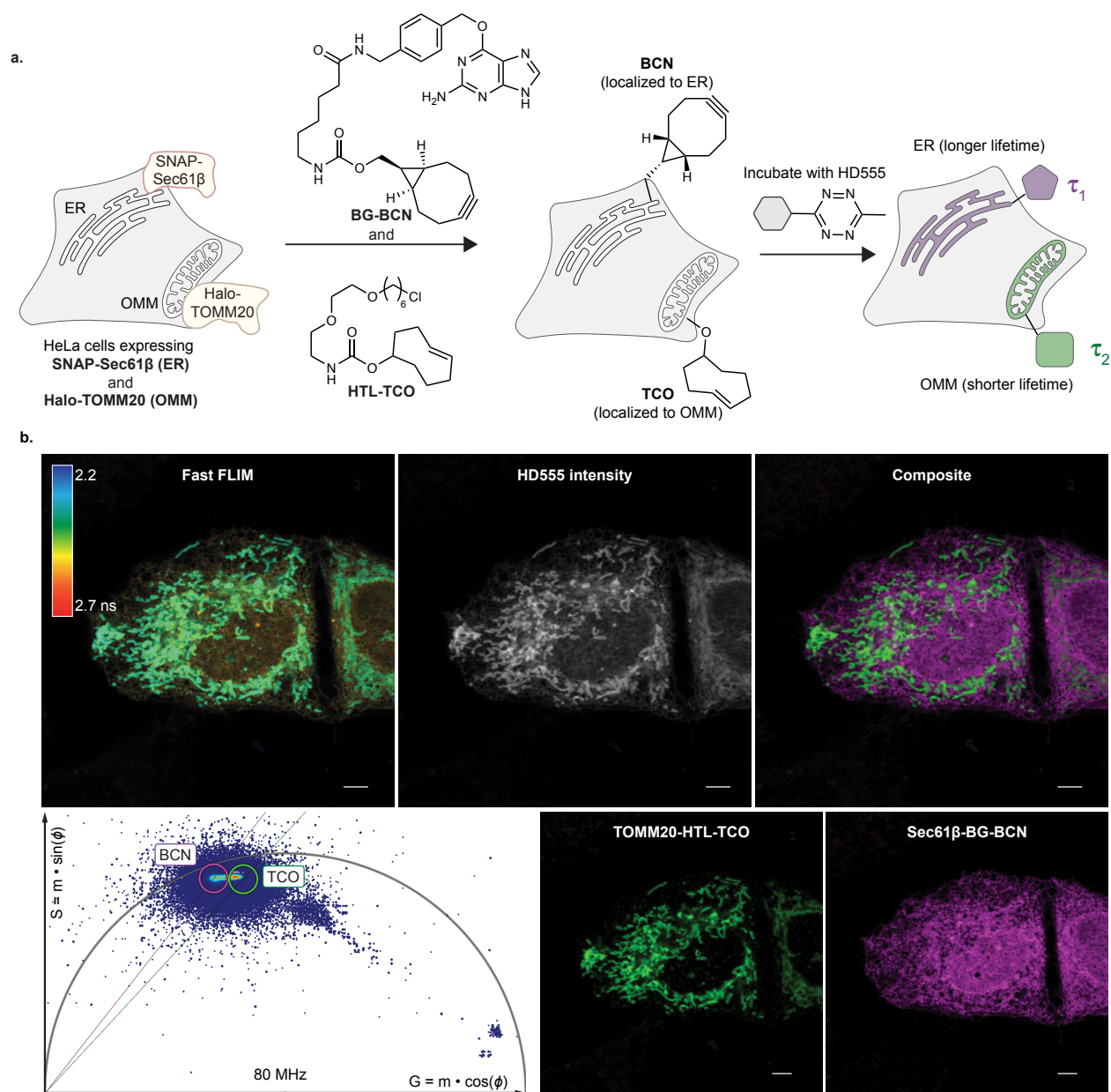


Fig. 3 | Two-channel imaging of ER and mitochondria in a cell using bioorthogonal handles to modulate fluorescence lifetime of HD555. **a.** Scheme of live cell experiment to image the ER and mitochondria, simultaneously, using a single dye. HeLa cells expressing Halo-TOMM20 and Halo-Sec61β are treated first with HTL-TCO and BG-BCN, followed by HD555. Given our single-species studies, we predict that the ER—targeted using BCN—will have a longer lifetime than the mitochondria—targeted using TCO. **b.** Total fluorescence intensity resulted in an image with indistinguishable organelles, however, fast FLIM images (**spectrum LUT**) showed two targets with contrasting lifetimes. Phasor analysis was used to separate the lifetimes based on the single-species phasor populations, allowing us to separate the organelles based on the bioorthogonal handle used to label the organelle. Subsequent experiments where the handles were flipped (**Extended Data Fig. 2**) confirmed that the bioorthogonal partner controlled the lifetime of HD555. Scale bars: 5 μm .

Small-molecule probes for transfection-free FLIM multiplexing with a single dye.

With these results in hand, we asked whether lifetime differences could be used to differentiate organelles in cells in which organelle membranes are targeted with organelle-selective lipid-like small molecules, not organelle-selective membrane proteins⁵⁹. Lipid-like small molecules have been used to selectively visualize the Golgi, ER, mitochondrial matrix, mitochondrial inner membrane, and the lysosome using super-resolution methods, often with greatly extended times^{39,60–66}. Others can selectively label the nucleus⁴³ and the cytoskeleton⁴². Labeling cells with small molecules offers expanded versatility, as doing so requires neither genetic manipulation nor transfection, and thus can be used to visualize primary cells or those that are difficult to transfect or culture. If different organelle-selective lipid-like small molecules carried different tetrazine reaction partners, and the products displayed differentiable lifetimes, then the organelle structure and dynamics could be visualized without transfection or other genetic engineering using multi-channel fluorescence lifetime imaging.

To evaluate whether reaction partner-guided changes in fluorescence lifetime would translate to organelle imaging by cell-permeant small molecules, we synthesized TCO and BCN conjugates of hexyl acridine orange (HAO)⁶⁶—a lipid-like small molecule that localizes to the mitochondrial inner membrane—(**Extended Data Fig. 3a**), Hoechst 33258 (which localizes within the nucleus)⁴³ (**Extended Data Fig. 3b**), and Docetaxel (which binds to microtubules)⁴² (**Extended Data Fig. 3c**). While the small molecule probes are based off bonafide organelle targeting moieties, colocalization studies would bolster the claim regarding their organelle specificity.

Ligating HD555 to small-molecule probes presents a new challenge. Unlike the microenvironment of self-labelling enzymes—which shields the fluorophore from the organelle—small-molecule probes expose the fluorophore to the microenvironment of the organelle. First, we wanted to ensure that the lifetime differences between BCN and TCO were reproducible with these probes. HeLa cells were incubated with either HAO-BCN or HAO-TCO (100 nM), followed by HD555 (200 nM) (**Fig. 4a, Supplementary Fig. 6**) and each population was imaged using FLIM. As observed previously, when visualized by intensity (**Fig. 4b, grayscale**) both populations looked identical. However, fast FLIM images showed a stark contrast in average photon arrival times between the two populations (**Fig. 4b, false-colored**), and the composite phasor plot showed large difference between HAO-BCN and HAO-TCO (**Fig. 4b, middle panel**).

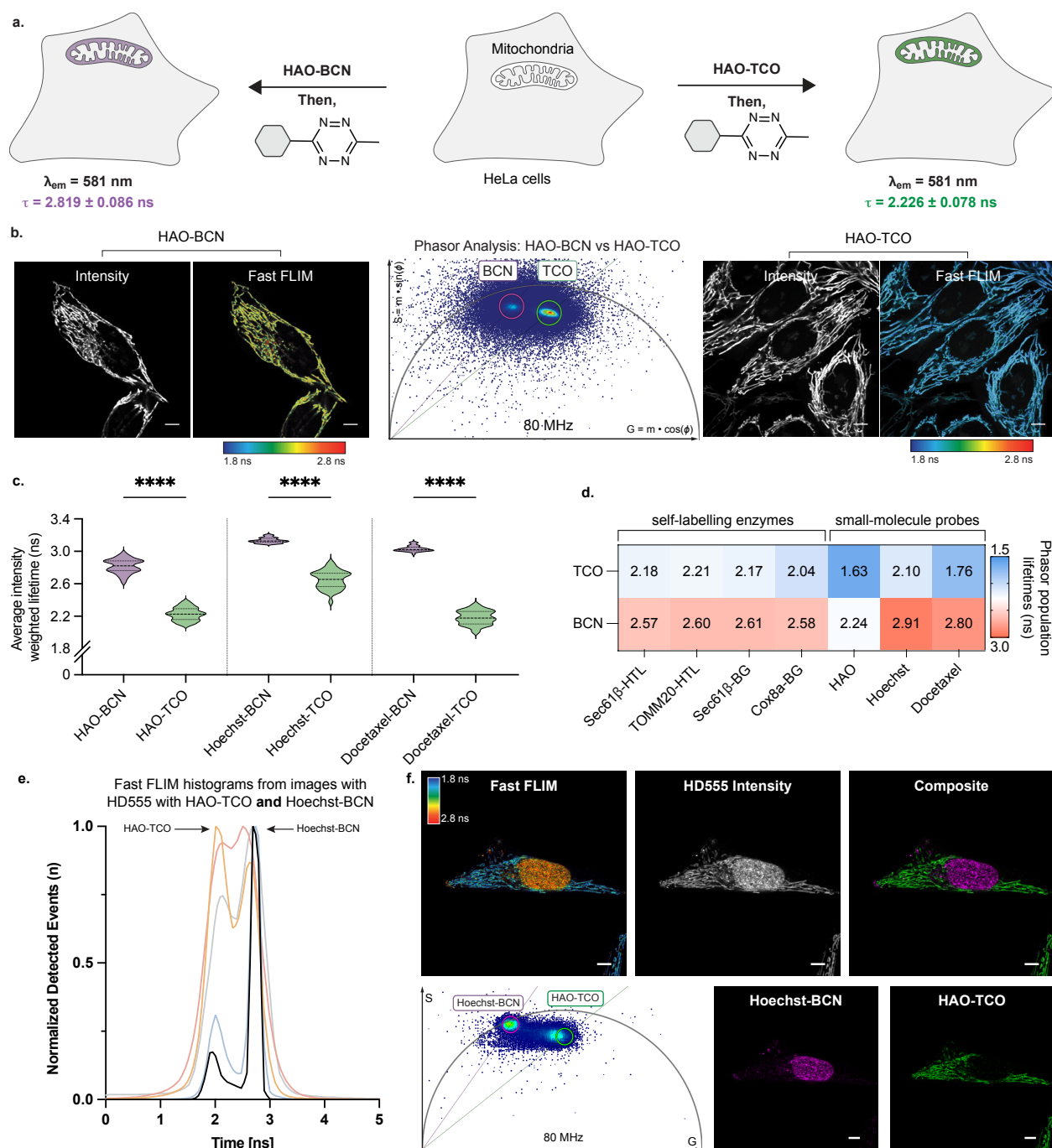


Fig. 4 | Using small-molecule probes for transfection-free FLIM multiplexing with a single dye. Small-molecules can be used to target organelles—we synthesized BCN and TCO derivatives of HAO (mitochondria-targeting), Hoechst (nucleus-targeting), and Docetaxel (tubulin-binding) (**Extended Data Fig. 3**) to demonstrate modulation of lifetimes (τ) using bioorthogonal partners. **a**, Scheme of live cell experiment to determine if turn-on efficiency translates into differentiable fluorescence lifetimes using HAO-BCN or HAO-TCO to target mitochondria. HeLa cells were incubated with HAO-BCN or HAO-TCO (100 nM) followed by HD555 (200 nM), and then **(b)** imaged by detecting either fluorescence intensity or fluorescence lifetime. Phasor analysis of multiple images (**b, middle plot**) shows two lifetime populations, where cells treated with HAO-BCN have a higher lifetime than cells treated with HAO-TCO. Analogous

experiments were performed with cells labelled with Hoechst-BCN or Hoechst-TCO (**Supplementary Fig. 7**), and Docetaxel-BCN or Docetaxel-TCO (**Supplementary Fig. 8**). **c**, Plots showing the average intensity-weighted fluorescent lifetimes of the six cell populations, ****P < 0.0001 for all pairwise comparisons between BCN and TCO small molecules-derivatives: HAO ($\Delta = 0.5930$ ns), Hoechst ($\Delta = 0.4853$ ns), and Docetaxel ($\Delta = 0.8504$ ns). Adjusted P values calculated using Dunnett's T3 multiple comparisons test. Statistical significance was determined via Welch's and Brown-Forsythe ANOVA with post hoc Dunnett's T3 test accounting for heteroscedasticity. **d**, Lifetimes extracted from phasor plots across populations demonstrate that turn-on correlates with lifetime, and that small-molecule probes demonstrate the larger differences in lifetime, compared to self-labelling enzymes. To demonstrate FLIM multiplexing with a single fluorophore and no transfection, HeLa cells were incubated with HAO-TCO and Hoechst-BCN, followed by HD555 (**Extended Data Fig. 4**). **e**, Fast FLIM histograms from the images depicted two lifetime populations, which could be fit to gaussian or Lorentzian distributions (**Supplemental Fig. 11**). **f**, Total fluorescence intensity resulted in an image with indistinguishable organelles, however, fast FLIM images (**spectrum LUT**) showed two targets with contrasting lifetimes. Phasor analysis was used to separate the lifetimes based on the single-species phasor populations, allowing us to separate the organelles based on the bioorthogonal handle used to label the organelle. Scale bars: **5 μ m**.

We repeated these measurements with Hoechst-BCN and Hoescht-TCO (**Supplementary Fig. 7**), and Docetaxel-BCN and Docetaxel-TCO (**Supplementary Fig. 8**). In all cases, the product resulting from reaction of HD555 with TCO had a lower average photon arrival time than that of the analogous BCN reaction product (HAO: **Supplementary Fig. 6c**; Hoechst: **Supplementary Fig. 7c**; Docetaxel: **Supplementary Fig. 8c**). Fitting the decays from each single-species population reflected the differences in BCN and TCO analogues: average intensity-weighted lifetimes for BCN were always higher than TCO analogues (**Fig. 4c**), as was the case with amplitude weighted lifetimes (**Supplementary Fig. 9b**, **Supplementary Fig. 10a**). Finally, phasor plots from each single-species population showed one cluster of lifetimes (**Supplementary Fig. 12**) for each analogue, and TCO analogues had consistently lower lifetimes clusters than BCN analogues.

Although the magnitude and absolute value of the difference varies, this observation holds true regardless of whether reaction occurred within the inner mitochondrial membrane, the outer mitochondrial membrane, the ER, microtubules, or within the nucleus (**Supplementary Fig. 9**, **Supplementary Fig. 10**). The difference in intensity-weighted lifetime between the TCO and BCN products when linked to a self-labeling tag ranged from 2.5 ns to 2.7 ns for HaloTag and 2.5 to 2.9 ns for SNAPTag (**Supplementary Table 1**), whereas when linked to a small-molecule probe, the difference ranged from 2.2 to 2.8 ns for HAO, and 2.7 to 3.2 ns for Hoechst and 2.2 to 3.3 ns for Docetaxel. Given that phasor analysis seems appropriate for multiplexed imaging, we analyzed all the lifetime clusters across every single-species experiment (**Fig. 4d**). Remarkably, the lifetime of HD555 can be modulated by **~1.3 ns**, across different bioorthogonal pairing and cellular contexts. The greatest differences between BCN and TCO can be seen between the small-molecule parings. These data suggest that bioorthogonal chemistry can be used to fine-tune the lifetime of a fluorophore, even in cases where the cellular environment results in a large lifetime shift. The data also suggest that multiplexing would be feasible and robust.

We wanted to demonstrate lifetime multiplexing using bioorthogonal chemistry—the possibility of imaging of two cellular targets simultaneously, without transfection, using the same fluorophore, and separating their signals using FLIM. To do this, we incubated the cells with Hoechst-BCN and HAO-TCO, followed by HD555 (**Extended Data Fig. 4a**). When visualized by fluorescence intensity (**Fig. 4f**, **Extended Data Fig. 4c**), one could distinguish nucleus from mitochondria based on organelle morphology, however, the fluorescence signals were identical. Visualizing the image using average photon arrival time resulted in striking contrast between the two organelles (**Fig. 4f**, **Extended Data Fig. 4c**). Additionally, the Fast FLIM histograms of the images reflected this contrast—two distinct and separable lifetime distributions are present in each image (**Fig. 4e**). We were able to fit the curves to either gaussian or Lorentzian distributions, demonstrating that the signals are easily separable (**Extended Data Fig. 4b**, **Supplementary Fig. 13**). The phasor plot for the two-species images reported two distinct lifetime populations (**Fig. 4f**) for the same cell. Applying cursor values from the single-species lifetimes for HD555 with Hoechst-BCN and HAO-TCO allowed us to separate the signal from the mitochondria and the nucleus (**Fig. 4f**). Additional images and replicates (**Extended Data Fig. 4c**, **Supplementary fig. 13**) demonstrated that the method is robust and reproducible. To our knowledge, this is the first example of FLIM multiplexing facilitated by bioorthogonal chemistry.

Given the range of fluorescence lifetimes of HD555 ligated to these different small molecule probes, we set out to image three cellular targets by bioorthogonally-induced FLIM multiplexing. First, we wanted to determine the minimal resolvable difference in fluorescence lifetime by phasor analysis. To do this, we incubated cells with HAO-BCN and Hoechst-BCN followed by HD555 (**Extended Data Fig. 4d**). When visualized by average photon arrival time, we observed a contrast between the two organelles, albeit less of a contrast than with HAO-TCO and Hoechst-BCN (**Extended Data Fig. 4f**). The lower contrast is also represented in the fast FLIM histogram (**Extended Data Fig. 4e**) seeing as there is one broad peak, as opposed to two distinct peaks. However, phasor analysis of the two-species image displayed two lifetime populations. (**Extended Data Fig. 4f**). Each lifetime population in the multiplexed image, corresponded specifically to the lifetime clusters observed in the single-species images. When separated into individual channels, the organelles were distinct and specific to the targeting probe. Additional images and replicates demonstrated that these probes were separable and the method was robust (**Supplementary Fig. 14**).

Occupying the higher lifetime ranges with the BCN-based probes, we wanted to see whether it was possible to add Docetaxel-TCO to the cells, and simultaneously resolved three cellular targets, using a single fluorophore. We incubated cells with Hoechst-BCN (nucleus), HAO-BCN (mitochondria), and Docetaxel-TCO (microtubules), followed by HD555 (**Fig. 5a**), and proceeded to image them via FLIM. Fluorescence intensity from HD555 resulted in an image where the organelles could not be distinguished from each other (**Fig. 5c**). However, when visualized by average photon arrival time (**Fig. 5b**), we could observe contrast between the three cellular targets. Finally, phasor separation (**Fig. 5d**) resulted in three channels, one for each of the three cellular targets (**Fig. 5c**,

Supplementary Fig. 15). To our knowledge, this is the first instance of using bioorthogonal chemistry to modulate fluorescence lifetimes of a single fluorophore, and image three targets in a cell, simultaneously.

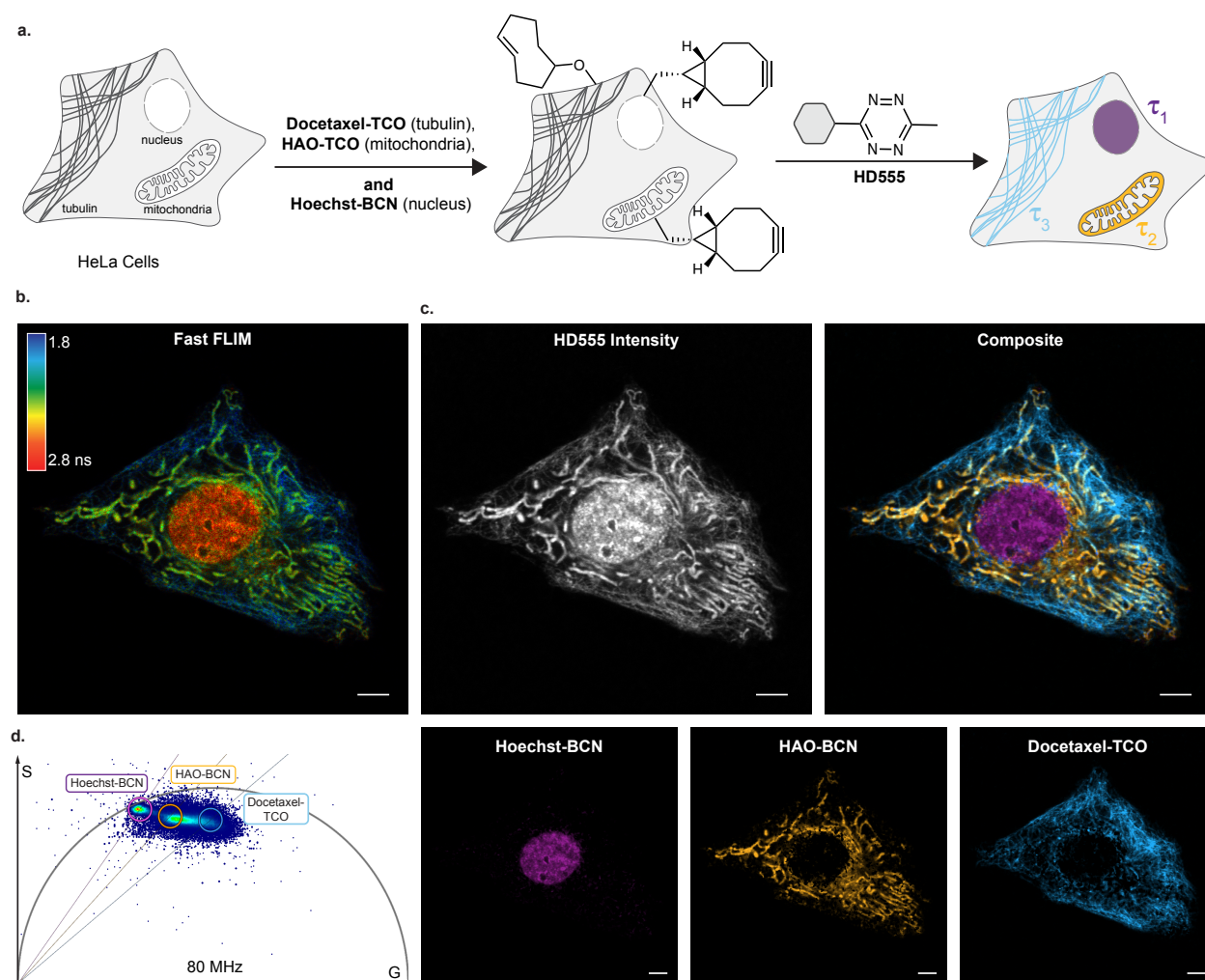


Fig. 5 | Three-channel FLIM multiplexing of nucleus, mitochondria, and microtubules in a live-cell using a single dye, and only chemical probes. **a**, Scheme of live cell experiment to image the nucleus, mitochondria, and microtubules, simultaneously, using a single dye with no genetic manipulation. HeLa cells were incubated with Hoechst-BCN, HAO-BCN, and Docetaxel-TCO followed by HD555. Given our single-species studies, we predict that the nucleus—targeted using Hoechst-BCN—will have the longest lifetime, followed by mitochondria—targeted using HAO-BCN—and finally, microtubules—targeted by Docetaxel-TCO—will have the shortest lifetime. **b**, Total fluorescence intensity resulted in an image with indistinguishable organelles, however, fast FLIM images (**spectrum LUT**) showed distinct contrasting lifetimes. Phasor analysis was used to separate the lifetimes based on the single-species phasor populations, allowing us to separate the organelles based on the bioorthogonal handle used to label the organelle. Replicates (**Supplementary Fig. 13**) confirmed that the bioorthogonal partner controlled the lifetime of HD555. Scale bars: **5 μ m**.

Discussion

Innovations have propelled the capabilities of fluorescence microscopy, from high content imaging to super-resolution imaging, and FLIM. However, chemical tools and our ability to label cellular targets remains a hurdle. Our work describes a tool, or a platform, that can expand the capabilities of FLIM multiplexing to image multiple organelles.

We demonstrate that bioorthogonal chemistry can be used to modulate fluorescence lifetime of HD555, a tetrazine-triggered fluorogenic dye. Using small-molecule probes, we were able to localize bioorthogonal reaction partners to specific organelles. Furthermore, we demonstrated that HD555 could react with these partners in situ, and the identity of the bioorthogonal partner dictated the lifetime of HD555. The differences in fluorescence lifetime arise from the structures of the products after bioorthogonal conjugation with either TCO or BCN. In seven different cellular contexts, the lifetime of the product between TCO and HD555 was always shorter than the product with BCN. This trend in lifetime correlates with the trend in fluorescence turn-on, suggesting that behavior of these probes are predictable and can be tuned.

Existing FLIM multiplexing methods that rely on multiple structurally distinct dyes and specifically targeting them to organelles, or genetically encoded self-labelling enzymes. Our strategy requires no transfection and leverages bioorthogonal targeting. These data expand the utility of FLIM multiplexing to systems where genetic manipulation is infeasible, such as primary cells or organoids. Not only do a plethora of iEDDA partners exist^{53,54,67}, but also a large swath of tetrazine-triggered fluorophores^{68–79}. The scope to expand on this platform is massive, and could provide a solution to multiplexed imaging. Our work establishes a framework for transfection-free, chemically programmable lifetime imaging—a framework with incredible scope for applications and tool development.

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References

1. Betzig, E. *et al.* Imaging Intracellular Fluorescent Proteins at Nanometer Resolution. *Science* **313**, 1642–1645 (2006).
2. Moerner, W. E. (William E.). Nobel Lecture: Single-molecule spectroscopy, imaging, and photocontrol: Foundations for super-resolution microscopy. *Reviews of Modern Physics* **87**, 1183–1212 (2015).
3. Möckl, L., Lamb, D. C. & Bräuchle, C. Super-resolved Fluorescence Microscopy: Nobel Prize in Chemistry 2014 for Eric Betzig, Stefan Hell, and William E. Moerner. *Angewandte Chemie International Edition* **53**, 13972–13977 (2014).
4. Betzig, E. Nobel Lecture: Single molecules, cells, and super-resolution optics. *Reviews of Modern Physics* **87**, 1153–1168 (2015).
5. Hell, S. W. Nobel Lecture: Nanoscopy with freely propagating light. *Reviews of Modern Physics* **87**, 1169–1181 (2015).
6. Willig, K. I., Rizzoli, S. O., Westphal, V., Jahn, R. & Hell, S. W. STED microscopy reveals that synaptotagmin remains clustered after synaptic vesicle exocytosis. *Nature* **440**, 935–939 (2006).
7. Hein, B., Willig, K. I. & Hell, S. W. Stimulated emission depletion (STED) nanoscopy of a fluorescent protein-labeled organelle inside a living cell. *Proceedings of the National Academy of Sciences* **105**, 14271–14276 (2008).
8. Nagerl, U. V., Willig, K. I., Hein, B., Hell, S. W. & Bonhoeffer, T. Live-cell imaging of dendritic spines by STED microscopy. *Proceedings of the National Academy of Sciences* **105**, 18982–18987 (2008).
9. Westphal, V. *et al.* Video-Rate Far-Field Optical Nanoscopy Dissects Synaptic Vesicle Movement. *Science* **320**, 246–249 (2008).
10. Voleti, V. *et al.* Real-time volumetric microscopy of in vivo dynamics and large-scale samples with SCAPE 2.0. *Nature Methods* **16**, 1054–1062 (2019).
11. Li, D. *et al.* Extended-resolution structured illumination imaging of endocytic and cytoskeletal dynamics. *Science* **349**, aab3500 (2015).
12. Legant, W. R. *et al.* High-density three-dimensional localization microscopy across large volumes. *Nature Methods* **13**, 359–365 (2016).
13. Valm, A. M. *et al.* Applying systems-level spectral imaging and analysis to reveal the organelle interactome. *Nature* **546**, 162–167 (2017).
14. Chen, B.-C. *et al.* Lattice light-sheet microscopy: Imaging molecules to embryos at high spatiotemporal resolution. *Science* **346**, 1257998 (2014).
15. Berezin, M. Y. & Achilefu, S. Fluorescence Lifetime Measurements and Biological Imaging. *Chemical Reviews* **110**, 2641–2684 (2010).
16. Tantama, M., Hung, Y. P. & Yellen, G. Imaging Intracellular pH in Live Cells with a Genetically Encoded Red Fluorescent Protein Sensor. *Journal of the American Chemical Society* **133**, 10034–10037 (2011).

17. Okabe, K. *et al.* Intracellular temperature mapping with a fluorescent polymeric thermometer and fluorescence lifetime imaging microscopy. *Nature Communications* **3**, 705 (2012).
18. Colom, A. *et al.* A fluorescent membrane tension probe. *Nature Chemistry* **10**, 1118–1125 (2018).
19. Datta, R., Heaster, T. M., Sharick, J. T., Gillette, A. A. & Skala, M. C. Fluorescence lifetime imaging microscopy: fundamentals and advances in instrumentation, analysis, and applications. *Journal of Biomedical Optics* **25**, 071203–071203 (2020).
20. Malacrida, L., Ranjit, S., Jameson, D. M. & Gratton, E. The Phasor Plot: A Universal Circle to Advance Fluorescence Lifetime Analysis and Interpretation. *Annual Review of Biophysics* **50**, 575–593 (2021).
21. Frei, M. S., Koch, B., Hiblot, J. & Johnsson, K. Live-Cell Fluorescence Lifetime Multiplexing Using Synthetic Fluorescent Probes. *ACS Chem. Biol.* **17**, 1321–1327 (2022).
22. Frei, M. S. *et al.* Engineered HaloTag variants for fluorescence lifetime multiplexing. *Nat Methods* **19**, 65–70 (2022)..
23. Linders, P. T. A., Ioannidis, M., Beest, M. ter & Bogaart, G. van den. Fluorescence Lifetime Imaging of pH along the Secretory Pathway. *ACS Chemical Biology* **17**, 240–251 (2022).
24. Pisfil, M. G. *et al.* Stimulated emission depletion microscopy with a single depletion laser using five fluorochromes and fluorescence lifetime phasor separation. *Scientific Reports* **12**, 14027 (2022).
25. Guo, Y. *et al.* Visualizing Intracellular Organelle and Cytoskeletal Interactions at Nanoscale Resolution on Millisecond Timescales. *Cell* **175**, 1430-1442.e17 (2018).
26. Grimm, J. B. & Lavis, L. D. Synthesis of Rhodamines from Fluoresceins Using Pd-Catalyzed C–N Cross-Coupling. *Organic Letters* **13**, 6354–6357 (2011).
27. Wysocki, L. M. *et al.* Facile and General Synthesis of Photoactivatable Xanthene Dyes. *Angewandte Chemie International Edition* **50**, 11206–11209 (2011).
28. Grimm, J. B. *et al.* Carbofluoresceins and Carborhodamines as Scaffolds for High-Contrast Fluorogenic Probes. *ACS Chemical Biology* **8**, 1303–1310 (2013).
29. Grimm, J. B. *et al.* A general method to improve fluorophores for live-cell and single-molecule microscopy. *Nature Methods* **12**, 244–250 (2015).
30. Grimm, J. B. *et al.* Synthesis of a Far-Red Photoactivatable Silicon-Containing Rhodamine for Super-Resolution Microscopy. *Angewandte Chemie International Edition* **55**, 1723–1727 (2015).
31. Grimm, J. B. *et al.* Synthesis of a Far-Red Photoactivatable Silicon-Containing Rhodamine for Super-Resolution Microscopy. *Angewandte Chemie* **128**, 1755–1759 (2015).

32. Grimm, J. B. *et al.* Bright photoactivatable fluorophores for single-molecule imaging. *Nature Methods* **13**, nmeth.4034 (2016).
33. Grimm, J. B., Brown, T. A., Tkachuk, A. N. & Lavis, L. D. General Synthetic Method for Si-Fluoresceins and Si-Rhodamines. *ACS Central Science* **3**, 975–985 (2017).
34. Grimm, J. B. *et al.* A general method to fine-tune fluorophores for live-cell and in vivo imaging. *Nature Methods* **14**, e04236 14 (2017).
35. Spahn, C., Grimm, J. B., Lavis, L. D., Lampe, M. & Heilemann, M. Whole-Cell, 3D, and Multicolor STED Imaging with Exchangeable Fluorophores. *Nano Letters* **19**, 500–505 (2018).
36. Miura, T. *et al.* Rational Design Principle for Modulating Fluorescence Properties of Fluorescein-Based Probes by Photoinduced Electron Transfer. *Journal of the American Chemical Society* **125**, 8666–8671 (2003).
37. Uno, S. *et al.* A spontaneously blinking fluorophore based on intramolecular spirocyclization for live-cell super-resolution imaging. *Nature Chemistry* **6**, 681–689 (2014).
38. Lukinavičius, G. *et al.* Fluorogenic Probes for Multicolor Imaging in Living Cells. *Journal of the American Chemical Society* **138**, 9365–9368 (2016).
39. Takakura, H. *et al.* Long time-lapse nanoscopy with spontaneously blinking membrane probes. *Nature Biotechnology* **35**, 773–780 (2017).
40. Uno, S.-N., Kamiya, M., Morozumi, A. & Urano, Y. A green-light-emitting, spontaneously blinking fluorophore based on intramolecular spirocyclization for dual-colour super-resolution imaging. *Chemical communications (Cambridge, England)* **54**, 102–105 (2017).
41. Lukinavičius, G. *et al.* A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins. *Nature Chemistry* **5**, 132–139 (2013).
42. Lukinavičius, G. *et al.* Fluorogenic probes for live-cell imaging of the cytoskeleton. *Nat Methods* **11**, 731–733 (2014).
43. Lukinavičius, G. *et al.* SiR–Hoechst is a far-red DNA stain for live-cell nanoscopy. *Nature Communications* **6**, 8497 (2015).
44. Yang, Z. *et al.* A Nile Red/BODIPY-based bimodal probe sensitive to changes in the micropolarity and microviscosity of the endoplasmic reticulum. *Chem. Commun.* **50**, 11672–11675 (2014).
45. Lin, H., Herman, P. & Lakowicz, J. R. Fluorescence lifetime-resolved pH imaging of living cells. *Cytometry Pt A* **52A**, 77–89 (2003).
46. Agronskaia, A. V., Tertoolen, L. & Gerritsen, H. C. Fast fluorescence lifetime imaging of calcium in living cells. *J. Biomed. Opt.* **9**, 1230 (2004).
47. Gest, A. M. M., Grenier, V. & Miller, E. W. Optical Estimation of Membrane Potential Values Using Fluorescence Lifetime Imaging Microscopy and Hybrid Chemical-Genetic Voltage Indicators. *Bioelectricity* **6**, 34–41 (2024).

48. Lazzari-Dean, J. R., Gest, A. M. & Miller, E. W. Optical estimation of absolute membrane potential using fluorescence lifetime imaging. *eLife* **8**, e44522 (2019).
49. Sarfraz, N. *et al.* Visualizing orthogonal RNAs simultaneously in live mammalian cells by fluorescence lifetime imaging microscopy (FLIM). *Nature Communications* **14**, 867 (2023).
50. Werther, P. *et al.* Bio-orthogonal Red and Far-Red Fluorogenic Probes for Wash-Free Live-Cell and Super-resolution Microscopy. *ACS Cent. Sci.* **7**, 1561–1571 (2021).
51. Hild, F., Werther, P., Yserentant, K., Wombacher, R. & Hertel, D.-P. A dark intermediate in the fluorogenic reaction between tetrazine fluorophores and trans-cyclooctene. *Biophysical Reports* **2**, 100084 (2022).
52. Yu, Z. & Lin, Q. Design of Spiro[2.3]hex-1-ene, a Genetically Encodable Double-Strained Alkene for Superfast Photoclick Chemistry. *J. Am. Chem. Soc.* **136**, 4153–4156 (2014).
53. Murrey, H. E. *et al.* Systematic Evaluation of Bioorthogonal Reactions in Live Cells with Clickable HaloTag Ligands: Implications for Intracellular Imaging. *Journal of the American Chemical Society* **137**, 11461–11475 (2015).
54. Oliveira, B. L., Guo, Z. & Bernardes, G. J. L. Inverse electron demand Diels–Alder reactions in chemical biology. *Chemical Society Reviews* **46**, 4895–4950 (2017).
55. Ramil, C. P. *et al.* Spirohexene-Tetrazine Ligation Enables Bioorthogonal Labeling of Class B G Protein-Coupled Receptors in Live Cells. *Journal of the American Chemical Society* **139**, 13376–13386 (2017).
56. Li, Y. & Fu, H. Bioorthogonal Ligations and Cleavages in Chemical Biology. *ChemistryOpen* **9**, 835–853 (2020).
57. Torrado, B., Pannunzio, B., Malacrida, L. & Digman, M. A. Fluorescence lifetime imaging microscopy. *Nat Rev Methods Primers* **4**, 80 (2024).
58. Digman, M. A., Caiolfa, V. R., Zamai, M. & Gratton, E. The Phasor Approach to Fluorescence Lifetime Imaging Analysis. *Biophysical Journal* **94**, L14–L16 (2008).
59. Dadina, N., Tyson, J., Zheng, S., Lesiak, L. & Schepartz, A. Imaging organelle membranes in live cells at the nanoscale with lipid-based fluorescent probes. *Current Opinion in Chemical Biology* **65**, 154–162 (2021).
60. Erdmann, R. S. *et al.* Super-Resolution Imaging of the Golgi in Live Cells with a Bioorthogonal Ceramide Probe. *Angewandte Chemie-International Edition* **53**, 10242–10246 (2014).
61. Gupta, A., Rivera-Molina, F., Xi, Z., Toomre, D. & Schepartz, A. Endosome motility defects revealed at super-resolution in live cells using HIDE probes. *Nature Chemical Biology* **16**, 408–+ (2020).
62. Tyson, J. *et al.* Extremely Bright, Near-IR Emitting Spontaneously Blinking Fluorophores Enable Ratiometric Multicolor Nanoscopy in Live Cells. *ACS Cent. Sci.* acscentsci.1c00670 (2021) doi:10.1021/acscentsci.1c00670.

63. Thompson, A. D., Bewersdorf, J., Toomre, D. & Schepartz, A. HIDE Probes: A New Toolkit for Visualizing Organelle Dynamics, Longer and at Super-Resolution. *Biochemistry* **56**, 5194–5201 (2017).
64. Thompson, A. D. *et al.* Long-Term Live-Cell STED Nanoscopy of Primary and Cultured Cells with the Plasma Membrane HIDE Probe Dil-SiR. *Angewandte Chemie International Edition* **56**, 10408–10412 (2017).
65. Chu, L. *et al.* Two-color nanoscopy of organelles for extended times with HIDE probes. *Nat Commun* **11**, 4271 (2020)..
66. Zheng, S. *et al.* Long-term super-resolution inner mitochondrial membrane imaging with a lipid probe. *Nat Chem Biol* **20**, 83–92 (2024).
67. Blackman, M. L., Royzen, M. & Fox, J. M. Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron-Demand Diels–Alder Reactivity. *Journal of the American Chemical Society* **130**, 13518–13519 (2008).
68. Klier, P. *et al.* Bioorthogonal, fluorogenic targeting of voltage-sensitive fluorophores for visualizing membrane potential dynamics in cellular organelles. doi:10.26434/chemrxiv-2022-db0kk.
69. Shieh, P., Hangauer, M. J. & Bertozzi, C. R. Fluorogenic Azidofluoresceins for Biological Imaging. *Journal of the American Chemical Society* **134**, 17428–17431 (2012).
70. Yang, J., Šečkutė, J., Cole, C. M. & Devaraj, N. K. Live-Cell Imaging of Cyclopropene Tags with Fluorogenic Tetrazine Cycloadditions. *Angewandte Chemie International Edition* **51**, 7476–7479 (2012).
71. Carlson, J. C. T., Meimetis, L. G., Hilderbrand, S. A. & Weissleder, R. BODIPY–Tetrazine Derivatives as Superbright Bioorthogonal Turn-on Probes. *Angewandte Chemie International Edition* **52**, 6917–6920 (2013).
72. Wieczorek, A., Buckup, T. & Wombacher, R. Rigid tetrazine fluorophore conjugates with fluorogenic properties in the inverse electron demand Diels–Alder reaction. *Organic & Biomolecular Chemistry* **12**, 4177–4185 (2014).
73. Shieh, P. *et al.* CalFluors: A Universal Motif for Fluorogenic Azide Probes across the Visible Spectrum. *Journal of the American Chemical Society* **137**, 7145–7151 (2015).
74. Wieczorek, A., Werther, P., Euchner, J. & Wombacher, R. Green- to far-red-emitting fluorogenic tetrazine probes – synthetic access and no-wash protein imaging inside living cells. *Chemical Science* **8**, 1506–1510 (2016).
75. Kozma, E., Girona, G. E., Paci, G., Lemke, E. A. & Kele, P. Bioorthogonal double-fluorogenic siliconrhodamine probes for intracellular super-resolution microscopy. *Chemical Communications* **53**, 6696–6699 (2017).
76. Beliu, G. *et al.* Bioorthogonal labeling with tetrazine-dyes for super-resolution microscopy. *Communications biology* **2**, 261 (2019).

77. Siegl, S. J., Galeta, J., Dzijak, R., Dračinský, M. & Vrabel, M. Bioorthogonal Fluorescence Turn-On Labeling Based on Bicyclononyne–Tetrazine Cycloaddition Reactions that Form Pyridazine Products. *ChemPlusChem* **84**, 493–497 (2019).
78. Zheng, Q. *et al.* Rational Design of Fluorogenic and Spontaneously Blinking Labels for Super-Resolution Imaging. *ACS central science* **5**, 1602–1613 (2019).
79. Mao, W. *et al.* A General Strategy to Design Highly Fluorogenic Far-Red and Near-Infrared Tetrazine Bioorthogonal Probes. *Angewandte Chemie International Edition* **60**, 2393–2397 (2021).

Methods

Materials and general considerations.

Materials for chemical synthesis and their sources are included in the supplementary notes. Culturing medium DMEM (12430054, 21063029 and A1443001), Opti-MEM with no phenol red (11058021) and FBS and were all purchased from Thermo. Plasmid encoding Halo-TOMM20 was a gift from K. McGowan (HHMI Janelia, Halo-TOMM20-N-10, Addgene plasmid 123284). Plasmid encoding COX8A-SNAP was a gift from A. Egana (New England Biolabs, pSNAPf-COX8A, Addgene plasmid 101129). Plasmid encoding Halo-Sec61-C-18 was a gift from Kevin McGowan (Addgene plasmid # 123285; RRID:Addgene_123285). Plasmid encoding SNAP-Sec61B was a gift from Gia Voeltz (Addgene plasmid # 141152 ; RRID:Addgene_141152).

General cell culture notes

HeLa cells (University of California, Berkeley, Cell Culture Facility) were cultured in DMEM (Thermo Fisher) supplemented with 10% FBS, penicillin (100 U ml^{-1}) and streptomycin ($100 \mu\text{g ml}^{-1}$). Cells were cultured at 37°C in a humidified CO_2/air (5%/95%) incubator. All cells were bona fide cell lines obtained from the University of California, Berkeley, Cell Culture Facility and were periodically tested for *Mycoplasma* by using DNA methods. Cells for imaging were seeded in four-well glass-bottom μ slides (Ibidi®, 80427, number #1.5H, 2.5 cm^2) at the indicated densities.

Microscope specifications

All imaging experiments were performed on a Leica Stellaris 8 microscope (Leica Microsystems) equipped with a Leica DMI8 CS scanhead, an HC Plan-Apo $\times 63/1.4\text{-NA}$ water immersion objective, and a pulsed white-light laser (440–790 nm; 440 nm: $>1.1 \text{ mW}$; 488 nm: $>1.6 \text{ mW}$; 560 nm: $>2.0 \text{ mW}$; 630 nm: $>2.6 \text{ mW}$; 790 nm: $>3.5 \text{ mW}$, 78 MHz). Fluorescence Lifetime Imaging was performed using HyD X detectors in photon counting mode. Live-cell imaging conditions were maintained using a blacked out cage enclosure from Okolab. The temperature was maintained by heating the enclosure and was monitored by using Oko-Touch. The pH was maintained by supplying humidified 5% CO_2 to the sample chamber.

Synthesis and characterization.

Experimental procedures for the synthesis and characterization of HTL, HTL, BG, HAO, Hoechst, and Docetaxel derivatives (and intermediates), and their respective characterization data are included in the supplementary notes.

In vitro determination of lifetimes of HD555 with bioorthogonal partners.

100 μ M solutions of the bioorthogonal handles (BCN, TCO, and SpH) in DPBS were prepared by diluting a 10 mM DMSO stock. A 10 μ M solution of HD555 in DPBS was prepared by diluting a 2mM DMSO stock solution. In an 8-well microscopy dish, 150 μ L of the reaction partner was combined with 150 μ L of HD555, bringing the total concentration of the reaction partner to 50 μ M and HD555 to 5 μ M. The mixture was incubated at 37°C for 2 hrs before being imaged on the microscope. Images were acquired using the settings detailed below. Multiple frames were acquired per sample till >500 photons per pixel were acquired. Curves were fit to tri-exponential decay models ($\chi^2 < 1.0$) and the average weighted lifetimes determined from the fitted curves.

Transfection and labelling of HeLa cells with Halo-TOMM20 for single-species imaging.

Forty-eight hours before imaging, HeLa cells were plated (1.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were transfected with plasmids encoding Halo-TOMM20 (500 ng/well) using FuGENE HD (1.5 μ L/well) transfection reagent (Promega), according to the manufacturer's protocol, incubated for 6 hours, and washed three times with DMEM ph(-) supplemented with 10% FBS. The following day, the cells were labeled with 700 μ L of either 10 μ M of HTL-BCN or 10 μ M HTL-TCO in Opti-MEM ph(-) for 30 minutes, washed three times with 700 μ L of DMEM ph(-), incubated with 700 μ L of 1 μ M HD555 in Opti-MEM ph(-) for 0.5 h, washed three times with DMEM ph(-) supplemented with 10% FBS, and imaged.

Transfection and labelling of HeLa cells with Halo-Sec61 β for single-species imaging.

Forty-eight hours before imaging, HeLa cells were plated (1.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were transfected with plasmids encoding Halo-Sec61 β (500 ng/well) using FuGENE HD (1.5 μ L/well) transfection reagent (Promega), according to the manufacturer's protocol, incubated for 6 hours, and washed three times with DMEM ph(-) supplemented with 10% FBS. The following day, the cells were labeled with 700 μ L of either 10 μ M of HTL-BCN or 10 μ M HTL-TCO in Opti-MEM ph(-) for 30 minutes, washed three times with 700 μ L of DMEM

ph(–), incubated with 700 μ L of 1 μ M HD555 in Opti-MEM ph(–) for 0.5 h, washed three times with DMEM ph(–) supplemented with 10% FBS, and imaged.

Transfection and labelling of HeLa cells with SNAP-Sec61 β for single-species imaging.

Forty-eight hours before imaging, HeLa cells were plated (1.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were transfected with plasmids encoding SNAP-Sec61 β (500 ng/well) using FuGENE HD (1.5 μ L/well) transfection reagent (Promega), according to the manufacturer's protocol, incubated for 6 hours, and washed three times with DMEM ph(–) supplemented with 10% FBS. The following day, the cells were labeled with 700 μ L of either 10 μ M of BG-BCN or 10 μ M BG-TCO in Opti-MEM ph(–) for 30 minutes, washed three times with 700 μ L of DMEM ph(–), incubated with 700 μ L of 1 μ M HD555 in Opti-MEM ph(–) for 0.5 h, washed three times with DMEM ph(–) supplemented with 10% FBS, and imaged.

Transfection and labelling of HeLa cells with Cox8a-SNAP for single-species imaging.

Forty-eight hours before imaging, HeLa cells were plated (1.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were transfected with plasmids encoding Cox8A-SNAP (500 ng/well) using FuGENE HD (1.5 μ L/well) transfection reagent (Promega), according to the manufacturer's protocol, incubated for 6 hours, and washed three times with DMEM ph(–) supplemented with 10% FBS. The following day, the cells were labeled with 700 μ L of either 10 μ M of BG-BCN or 10 μ M BG-TCO in Opti-MEM ph(–) for 30 minutes, washed three times with 700 μ L of DMEM ph(–), incubated with 700 μ L of 1 μ M HD555 in Opti-MEM ph(–) for 0.5 h, washed three times with DMEM ph(–) supplemented with 10% FBS, and imaged.

Labelling of HeLa cells for single-species imaging using HAO-BCN.

Twenty-four hours before imaging, HeLa cells were plated (2.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were labeled with 700 μ L of 100 nM HAO-BCN in Opti-MEM ph(–) for 1 hr, washed three times with 700 μ L of DMEM ph(–), incubated with 700 μ L of 200 nM HD555 in Opti-MEM ph(–) for 30 min, washed three times with DMEM ph(–) supplemented with 10% FBS. Cells were rested for 1 hr before imaging.

Labelling of HeLa cells for single-species imaging using HAO-TCO.

Twenty-four hours before imaging, HeLa cells were plated (2.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were labeled with 700 μ L of 100 nM HAO-TCO in Opti-MEM ph(–) for 1 h, washed three times with 700 μ L of DMEM ph(–) supplemented with 10% FBS, allowed cells to rest for 1 h, incubated with 700 μ L of 200 nM HD555 in Opti-MEM ph(–) for 0.5 h, washed three times with DMEM ph(–) supplemented with 10% FBS, and imaged.

Labelling of HeLa cells for single-species imaging using Hoechst-BCN or Hoechst-TCO.

Twenty-four hours before imaging, HeLa cells were plated (2.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were labeled with 700 μ L of either 200 nM Hoechst-BCN or 200 nM Hoechst-TCO in Opti-MEM ph(–) for 0.5 h, washed three times with 700 μ L of DMEM ph(–), incubated with 700 μ L of 200 nM HD555 in Opti-MEM ph(–) for 0.5 h, washed three times with DMEM ph(–) supplemented with 10% FBS, and imaged.

Labelling of HeLa cells for single-species imaging using Docetaxel-BCN or Docetaxel-TCO.

Twenty-four hours before imaging, HeLa cells were plated (2.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were labeled with 700 μ L of either 100 nM Docetaxel-BCN or 100 nM Docetaxel-TCO in Opti-MEM ph(–) for 1 hr, washed three times with 700 μ L of DMEM ph(–), incubated with 700 μ L of 500 nM HD555 in Opti-MEM ph(–) for 0.5 h, washed three times with DMEM ph(–) supplemented with 10% FBS, and imaged.

Transfection and Labelling of HeLa cells for two-species imaging using Halo-TOMM20+HTL-BCN and SNAP-Sec61 β +BG-TCO.

Forty-eight hours before imaging, HeLa cells were plated (1.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were transfected with two separate plasmids encoding Halo-TOMM20 (375 ng/well) and SNAP-Sec61 β (125 ng/well) using FuGENE HD (1.5 μ L/well) transfection reagent (Promega), according to manufacturer's protocol, incubated for 6 hrs, and washed three times with DMEM ph(–) supplemented with 10% FBS. The following day, the cells were labeled with 700 μ L of

4 μM HTL-BCN and 1 μM BG-TCO in Opti-MEM ph(–) for 30 min, washed three times with 700 μL of DMEM ph(–), incubated with 700 μL of 4 μM HD555 in Opti-MEM ph(–) for 30 min, washed three times with DMEM ph(–) supplemented with 10% FBS, and imaged

Transfection and Labelling of HeLa cells for two-species imaging using Halo-TOMM20+HTL-TCO and SNAP-Sec61 β +BG-BCN.

Forty-eight hours before imaging, HeLa cells were plated (1.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were transfected with two separate plasmids encoding Halo-TOMM20 (250 ng/well) and SNAP-Sec61 β (500 ng/well) using FuGENE HD (1.5 μL /well) transfection reagent (Promega), according to manufacturer's protocol. The plasmids were added simultaneously to the well, incubated for 6 hrs, and washed three times with DMEM ph(–) supplemented with 10% FBS. The following day, the cells were labeled with 700 μL of 1 μM HTL-TCO and 4 μM BG-BCN in Opti-MEM ph(–) for 30 min, washed three times with 700 μL of DMEM ph(–), incubated with 700 μL of 4 μM HD555 in Opti-MEM ph(–) for 30 min, washed three times with DMEM ph(–) supplemented with 10% FBS, and imaged.

Labelling of HeLa cells for two-species imaging using HAO-TCO and Hoechst-BCN.

Twenty-four hours before imaging, HeLa cells were plated (2.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were labeled with 700 μL of 100 nM HAO-TCO in Opti-MEM ph(–) for 1 h, washed three times with 700 μL of DMEM ph(–) supplemented with 10% FBS, then allowed to rest for 0.5 h. Cells were subsequently incubated with 700 μL of 200 nM Hoechst-BCN in Opti-MEM ph(–) for 0.5 h, washed three times with 700 μL of DMEM ph(–), then incubated with 700 μL of 400 nM HD555 in Opti-MEM ph(–) for 0.5 h, washed three times with DMEM ph(–) supplemented with 10% FBS, and imaged.

Labelling of HeLa cells for two-species imaging using HAO-BCN and Hoechst-BCN.

Twenty-four hours before imaging, HeLa cells were plated (2.5×10^4 cells per well) in a four-well chamber and incubated overnight. After 16 h, the cells were labeled with 700 μL of 100 nM HAO-BCN in Opti-MEM ph(–) for 1 h, washed three times with 700 μL of DMEM ph(–), incubated with 700 μL of 400 nM HD555 in Opti-MEM ph(–) for 0.5 h, then washed three times with DMEM ph(–). Cells were subsequently incubated with 700 μL of 200 nM Hoechst-BCN in Opti-MEM ph(–) for 0.5 h, washed three times with 700 μL of DMEM ph(–), incubated with 700 μL of 400 nM HD555 in Opti-MEM ph(–) for 0.5 h, then washed three times with DMEM ph(–) supplemented with 10% FBS, and imaged.

Labelling of HeLa cells for three-species imaging using Hoechst-BCN, HAO-BCN and Docetaxel-TCO.

Twenty-four hours before imaging, HeLa cells were plated (4.0×10^4 cells per well) in a two-well chamber and incubated overnight. After 16 h, the cells were labeled with 700 μ L of 100 nM HAO-BCN in Opti-MEM ph(–) for 1 h, washed three times with 700 μ L of DMEM ph(–), incubated with 700 μ L of 1 μ M HD555 in Opti-MEM ph(–) for 0.5 h, then washed three times with DMEM ph(–). Cells were then labeled with 700 μ L of 200 nM Docetaxel-TCO in Opti-MEM ph(–) for 0.5 h and washed three times with 700 μ L of DMEM ph(–). These cells were subsequently incubated with a 700 μ L mixture containing both 200 nM Docetaxel-TCO and 100 nM Hoechst-BCN for 0.5 h, washed three times with 700 μ L of DMEM ph(–), incubated with 700 μ L of 500 nM HD555 in Opti-MEM ph(–) for 0.5 h, then washed three times with DMEM ph(–) supplemented with 10% FBS, and imaged.

General fluorescence lifetime image acquisition settings.

All experiments were performed on a Leica Stellaris 8 (as detailed above) equipped with FALCON for fluorescence lifetime imaging microscopy (FLIM). All images were acquired using the same beam path and scanhead settings. Given the fact that only one fluorophore was imaged across all experiments, settings remained consistent. The HC Plan-Apo $\times 63/1.4$ -NA water immersion objective was used for all experiments, and the correction collar was adjusted to correct for differences in coverslip thickness. **Beam path setting:** The excitation wavelength was set to 561 nm and the laser power (1-5%) was adjusted to collect between 0.3 and 0.5 photons per laser pulse. The HyD X detector window was set between 575 nm – 750 nm and the detector was set to photon counting mode. A 561 nm filter was engaged to filter out stray excitation photons. **Scanhead settings:** The pixel size was kept constant over all images at 60.06 x 60.06 nm. The field of view was also kept constant at 61.5 x 61.5 μ m. The pixel dwell time was set to 3.8375 μ s (scanning frequency of 200 lines per second). Line accumulation (between 8-12) was adjusted to ensure that >150 photons per pixel (>200 for multispecies) were collected per image. **IRF:** The instrument response function (IRF) for each data set (i.e. image) was automatically calculated by the LAS X FLIM-FCS Module.

Fluorescence lifetime determination of single-species images.

All data were processed and worked up using the LAS X and LAS X FLIM-FCS Module (Leica Microsystems). Initially, images were screened to ensure that photons per pixel was >150. Then a brightness (photon count) threshold was set to exclude background photons from media. Next, a region of interest (ROI) was drawn around the cell/organelle

to ensure that lifetime data was only coming from pixels belonging to the respective organelles. The decay histogram pertaining to an ROI was fit using the n-exponential reconvolution fitting model (Leica Microsystems—detailed equations included in the **supplemental information**). Intensity weighted and amplitude weighted lifetimes were determined by fitting the curves to bi- or tri-exponential decay models. The number of exponents was determined by the best fit ($\chi^2 < 1.8$) with the least degrees of freedom. To determine the average intensity-weighted lifetime of a population of cells, the intensity weighted lifetime of >20 ROIs was averaged. To determine the average amplitude-weighted lifetime of a population of cells, the amplitude-weighted lifetime of >20 ROIs was averaged.

To determine phasor populations, single-species images were processed in the LAS X FLIM-FCS modules. First, images were threshold to reduce background photons. Median filtering (filter strength = 9) was applied, and the cursor (radius = 15) was placed over the cluster that encapsulated the pixels from the ROI in the image.

Curve fitting for fast FLIM histograms for HAO-TCO and Hoechst-BCN images

Histograms of average photon arrival times (Fast FLIM histograms) were exported from LAS X software and plotted using GraphPad Prism. Data points representing lifetime distributions were then fit to Gaussian or Lorentzian functions ($R^2 > 0.95$).

Phasor separation of multi-species images

FLIM data from multispecies images was processed and plotted on a phasor plot using the LAS X FLIM-FCS software package. First, images were thresholded to remove background photons, and a median filter was applied (strength = 9). Cursor position were determined using the lifetimes and positions from the homogenous, single species images. Once the cursors were positioned for each channel, the pixels populations were separated into their individual channels.

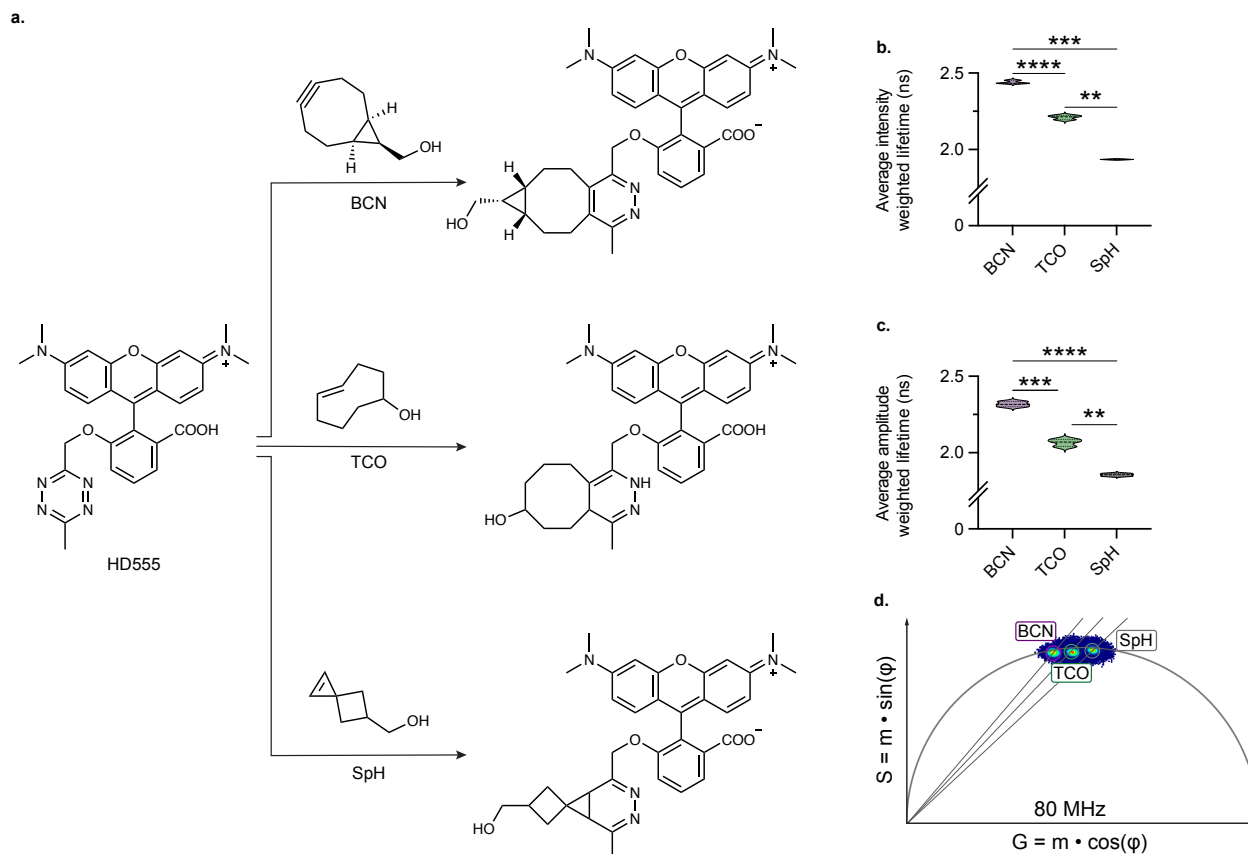
Software and image processing.

Images were acquired using Leica LAS X and curves were fitted using the LAS X FLIM-FCS Module. Fast FLIM Images were analyzed and exported using the LAS X software package. Statistical analysis and plotting were performed using GraphPad Prism 10.4.1. All images were processed with ImageJ/Fiji^{80,81}.

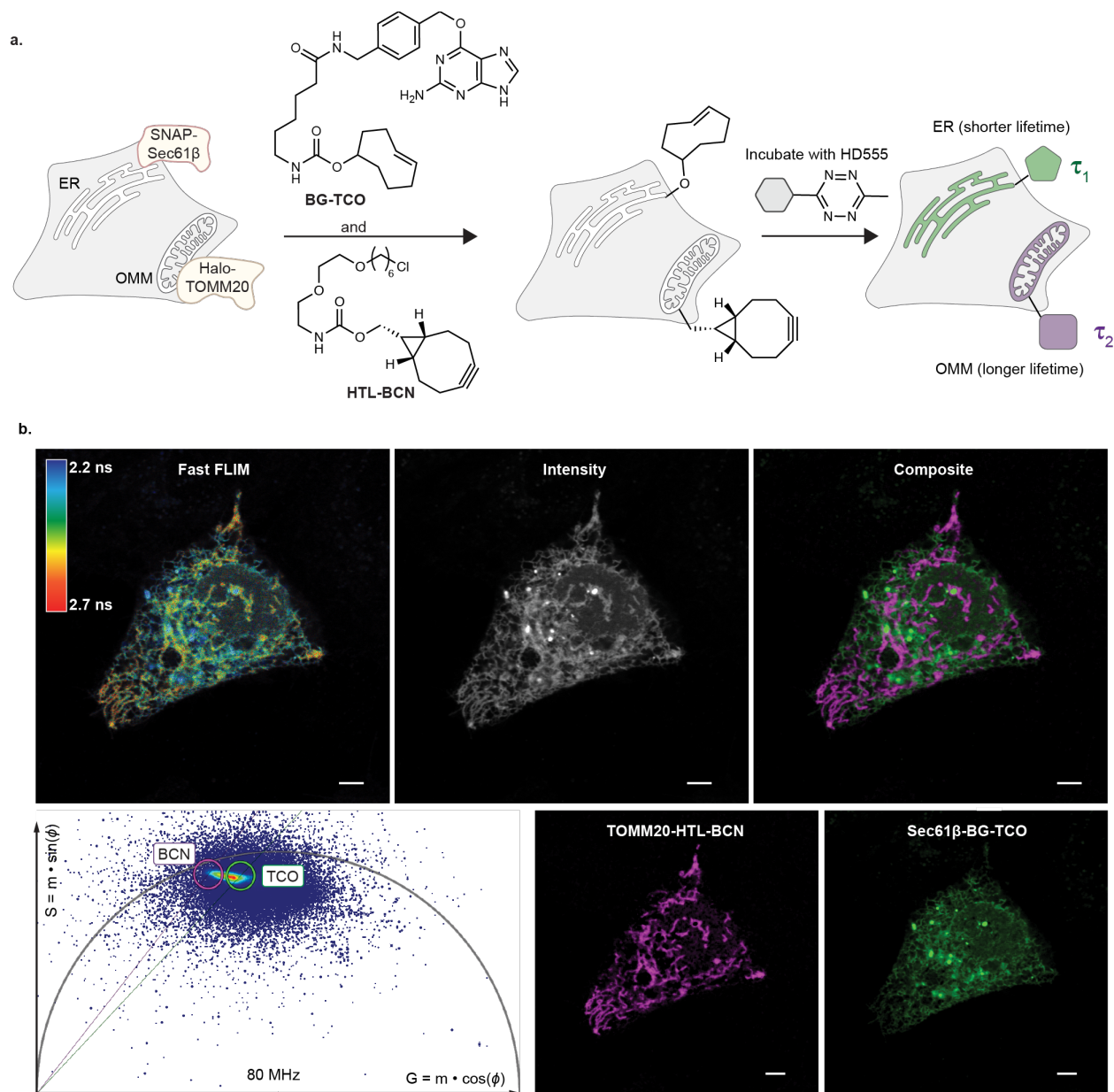
Methods References

80. Rueden, C. T. *et al.* ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics* **18**, (2017).
81. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676–682 (2012).

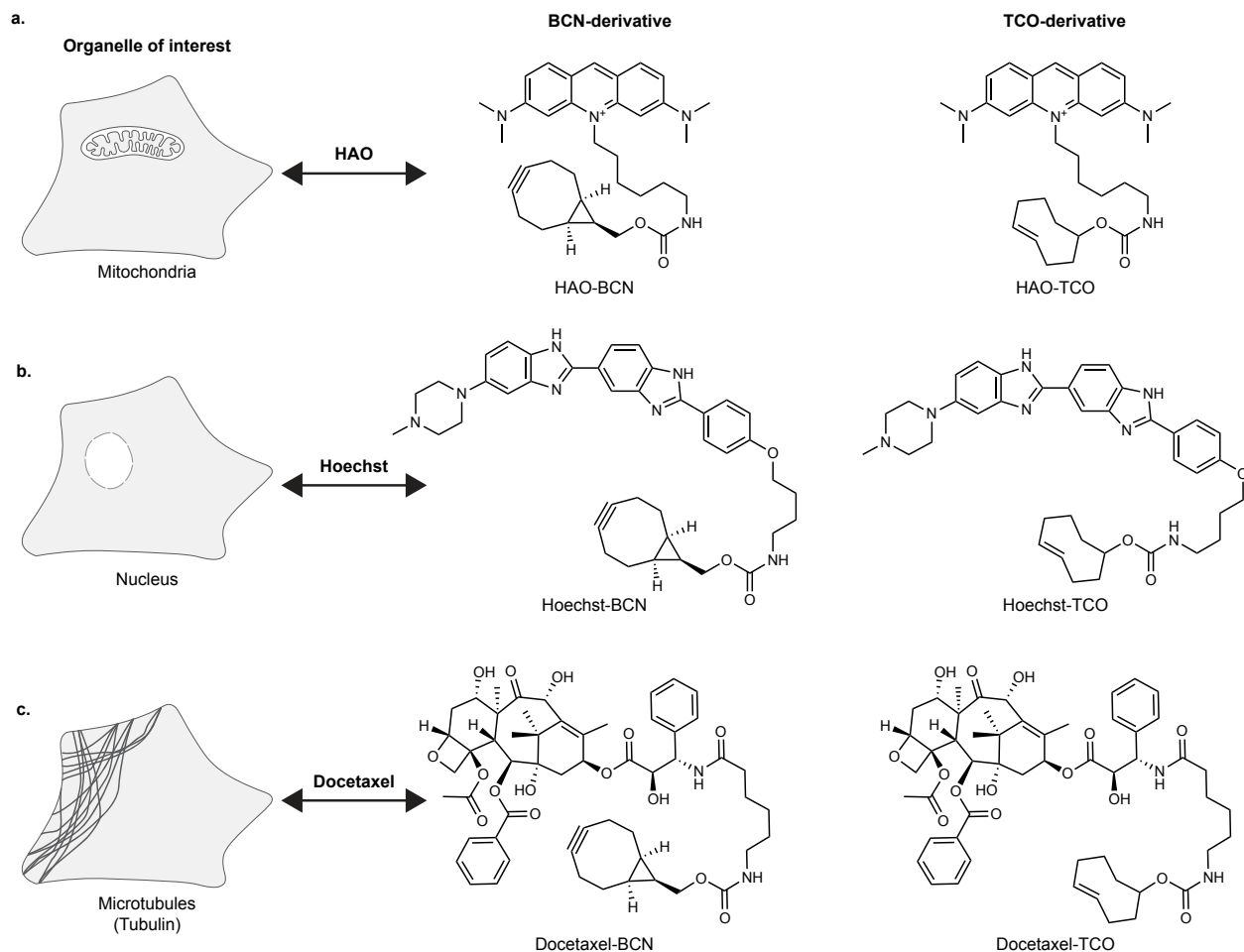
Extended Data Figures



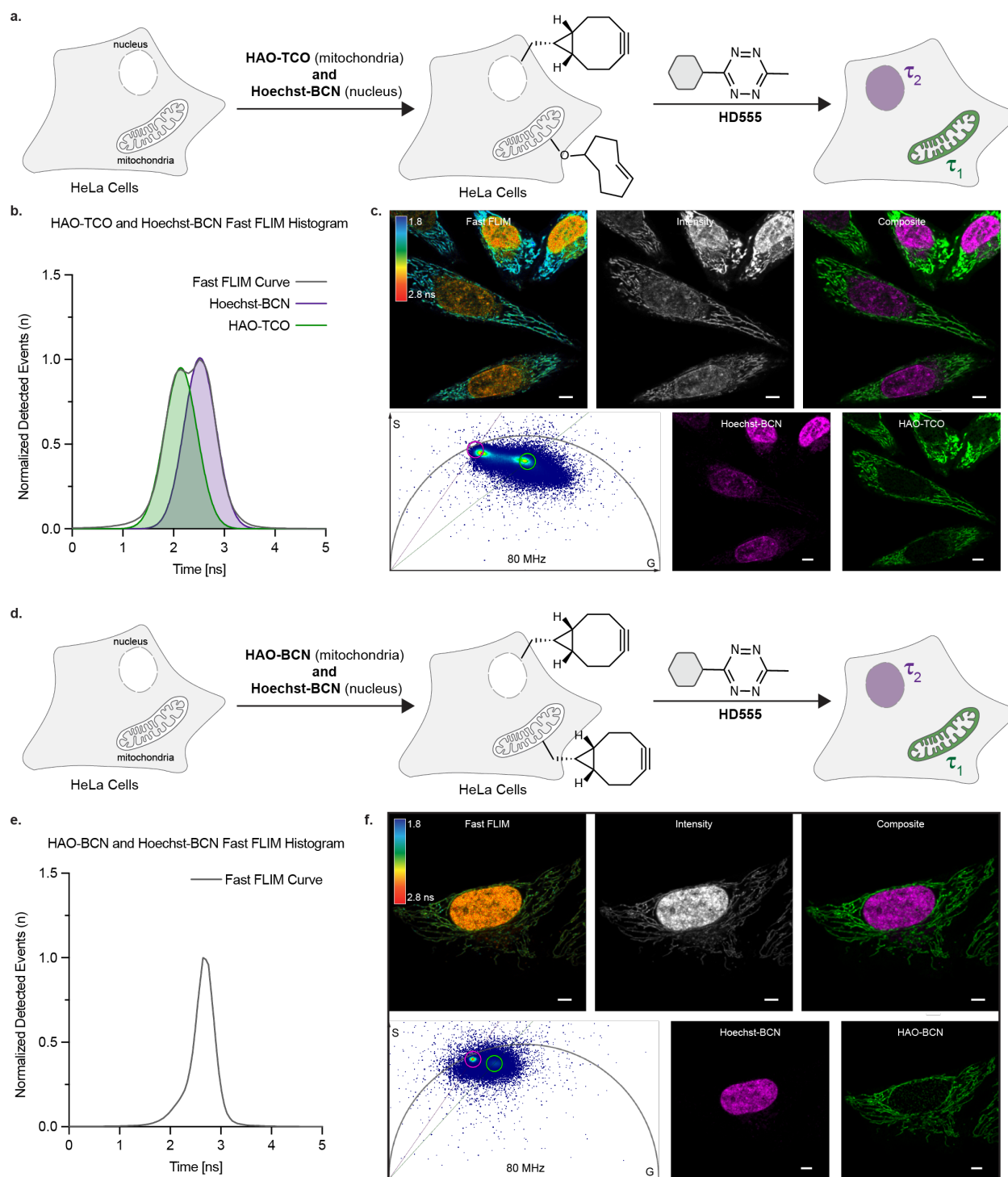
Extended Data Fig. 1 | Reaction products of HD555 with inverse electron demand Diels-Alder tetrazine ligation partners. The tetrazine moiety on HD555 can react with different dienophiles to form different ligation products, each with different electronic properties, and different fluorescence lifetimes. **a**, HD555 was reacted with BCN, TCO, and SpH for 2 hours, at 37 °C (see **online methods**), leading to three chemically distinct reaction products. Lifetimes for each of the three products was then determined using FLIM. **b**, Average intensity-weighted lifetimes for **BCN** (2.440 ± 0.011 ns), **TCO** (2.211 ± 0.015 ns), and **SpH** (1.935 ± 0.002 ns), and **c**, average amplitude-weighted lifetimes for **BCN** (2.318 ± 0.017 ns), **TCO** (2.067 ± 0.024 ns), and **SpH** (1.865 ± 0.009 ns) were measured using FLIM. **d**, Phasor analysis of lifetime data from in vitro studies shows differences in fluorescence lifetime between the three bioorthogonal handles. Each product forms a distinct population on the phasor plot. For **b**, BCN vs. TCO ($\Delta = 0.2292$, **** $P < 0.0001$), BCN vs. SpH ($\Delta = 0.5050$, *** $P = 0.0003$), and TCO vs. SpH ($\Delta = 0.2758$, ** $P = 0.0021$) all showed statistically significant differences. Adjusted P values calculated using Dunnett's T3 multiple comparisons test. Statistical significance was determined via Welch's and Brown-Forsythe ANOVA with post hoc Dunnett's T3 test accounting for heteroscedasticity. For **c**, BCN vs. TCO ($\Delta = 0.2510$, *** $P = 0.0003$), BCN vs. SpH ($\Delta = 0.4617$, **** $P < 0.0001$), and TCO vs. SpH ($\Delta = 0.2107$, ** $P = 0.0018$) all showed statistically significant differences. Adjusted P values calculated using Dunnett's T3 multiple comparisons test. Statistical significance was determined via Welch's and Brown-Forsythe ANOVA with post hoc Dunnett's T3 test accounting for heteroscedasticity.



Extended Data Fig. 2 | Two-channel imaging of ER and mitochondria in a cell using bioorthogonal handles to modulate fluorescence lifetime of HD555. a, Scheme of live cell experiment to image the ER and mitochondria, simultaneously, using a single dye. HeLa cells expressing Halo-TOMM20 and Halo-Sec61 β are treated first with BG-TCO and HTL-BCN, followed by HD555. Given our single-species studies, we predict that mitochondria—targeted using BCN—will have a longer lifetime than the ER—targeted using TCO. **b,** Total fluorescence intensity resulted in an image with indistinguishable organelles, however, fast FLIM images (**spectrum LUT**) showed two targets with contrasting lifetimes. Phasor analysis was used to separate the lifetimes based on the single-species phasor populations, allowing us to separate the organelles based on the bioorthogonal handle used to label the organelle. Complementing the data in **Fig. 3**, we confirm that the bioorthogonal partner controls the lifetime of HD555. Scale bars: **5 μ m**.



Extended Data Fig. 3 | Small-molecule targeting strategies for subcellular compartments using BCN and TCO derivatives. Small-molecule probes were synthesized to enable specific localization of bioorthogonal ligation partners, BCN and TCO. **a**, Mitochondria-targeting molecules HAO-BCN and HAO-TCO. **b**, Hoechst, used for nuclear localization, is functionalized to give Hoechst-BCN and Hoechst-TCO. **c**, Tubulin-targeting Docetaxel is conjugated to BCN and TCO to yield Docetaxel-BCN and Docetaxel-TCO to selectively label microtubules.



Extended Data Fig. 4 | Using small-molecule probes to image two organelles, for transfection-free FLIM multiplexing with a single dye. **a**, Scheme of live cell experiment to image the nucleus, and mitochondria, simultaneously, using a single dye with no genetic manipulation. HeLa cells were incubated with Hoechst-BCN, and HAO-TCO, followed by HD555. Given our single-species studies, we predict that the nucleus—targeted using Hoechst-BCN—will have the longest lifetime, and the mitochondria—targeted

using HAO-TCO—will have the shortest lifetime. **b**, Fast FLIM histograms from the images depicted two lifetime populations, which could be fit to gaussian or Lorentzian distributions. **c**, Total fluorescence intensity resulted in an image with indistinguishable organelles, however, fast FLIM images (**spectrum LUT**) showed two targets with contrasting lifetimes. Phasor analysis was used to separate the lifetimes based on the single-species phasor populations, allowing us to separate the organelles based on the bioorthogonal handle used to label the organelle. To determine the minimal resolvable difference by phasor analysis, **(d)** HeLa cells were incubated with Hoechst-BCN, and HAO-BCN, followed by HD555. Given our single-species studies, we predicted that the nucleus—targeted using Hoechst-BCN—will have the longest lifetime, and the mitochondria—targeted using HAO-BCN—will have the shortest lifetime. **e**, Fast FLIM histograms show one broad peak, with less distinguishable populations, further supporting the argument that bioorthogonal partners are beneficial in tuning the fluorescence lifetimes of HD555. **f**, Total fluorescence intensity resulted in an image with indistinguishable organelles, however, fast FLIM images (**spectrum LUT**) showed two targets with contrasting lifetimes. Phasor analysis was used to separate the lifetimes based on the single-species phasor populations, allowing us to separate the organelles based on the probe and bioorthogonal handle used to label the organelle. Scale bars: **5 μ m**.

Supplemental Information

Supplemental Note 1: Decay curve fitting and determination of Intensity and amplitude weighted lifetimes

Fluorescence lifetime decay histograms were fit according to the following equations.

$$y(t) = \{IRF(t + Shift_{IRF}) + Bkgr_{IRF}\} \otimes \left\{ \sum_{i=0}^{n-1} A[i] e^{-\left(\frac{t}{\tau[i]}\right)} + Bkgr \right\}$$

$$I_{Sum} = \sum_{k=0}^{n-1} I[k]$$

$$A_{Sum} = \sum_{k=0}^{n-1} A[k]$$

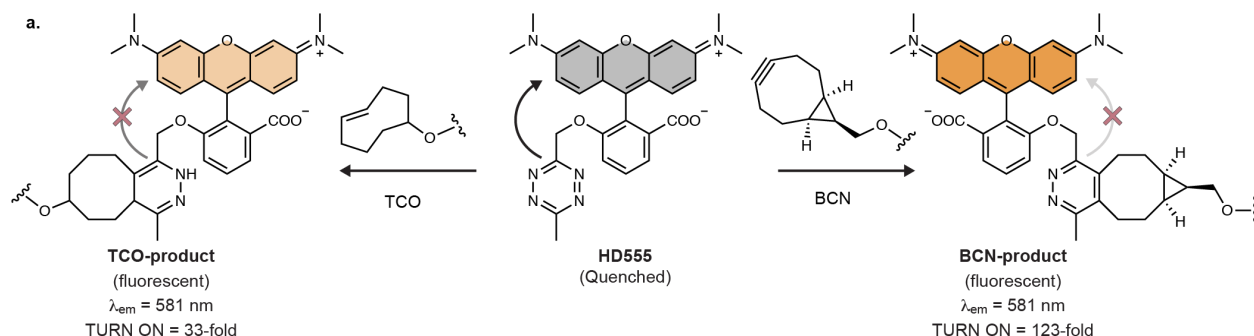
$$\tau_{Av Int} = \frac{\sum_{k=0}^{n-1} I[k] \tau[k]}{I_{Sum}}$$

$$\tau_{Av Amp} = \frac{\sum_{k=0}^{n-1} A[k] \tau[k]}{A_{Sum}}$$

Where:

n	Number of exponential components
A	Amplitudes – Exponential pre-factors
τ	Exponential Decay Times (e.g. lifetimes)
$Bkgr$	Tail Offset - Correction for background (after pulsing, dark counts, environmental light)
$Shift_{IRF}$	IRF Shift - Correction for IRF displacement (e.g. wavelength dependent zero time of the detector)
$Bkgr_{IRF}$	IRF Background - Correction for IRF background
I	Intensities associated with each exponential component, normalized to the time resolution of the measured decay curve to be displayed in photon counts
I_{Sum}	Sum of Fluorescence Intensity for all components
A_{Sum}	Sum of Fluorescence Intensity for all components at time zero
$\tau_{Av Int}$	Mean Photon Arrival Time - Intensity weighted average lifetime
$\tau_{Av Amp}$	Mean Decay Time - Amplitude weighted average lifetime

Supplementary Note 2: Correlation between fluorescence turn-on and lifetime.



Supplementary Fig. 1| Reaction of HD555 with BCN and TCO leading to differences in fluorescence turn-on. HD555 is a rhodamine-based fluorophore with a tetrazine on the pendant aryl ring (**middle**). The tetrazine quenches the fluorescence of the xanthene dye head. Upon reacting with **bicyclo[6.1.0]non-4-yne (BCN, right arrow)**, the tetrazine quenching is disrupted and a 123-fold fluorescence enhancement is observed. When HD555 is reacted with **trans-cyclooctene (TCO, left arrow)** a different ligation product is formed with a lower turn-on.

It is known that the fluorescence quantum yield (Φ) can be represented as a fraction of rate constants

$$\Phi = \frac{k_{fl}}{k_{fl} + k_{nr}}$$

Where k_{fl} is the rate constant for fluorescence decay and k_{nr} is the sum of all non-fluorescent decay. The observable fluorescence lifetime is then

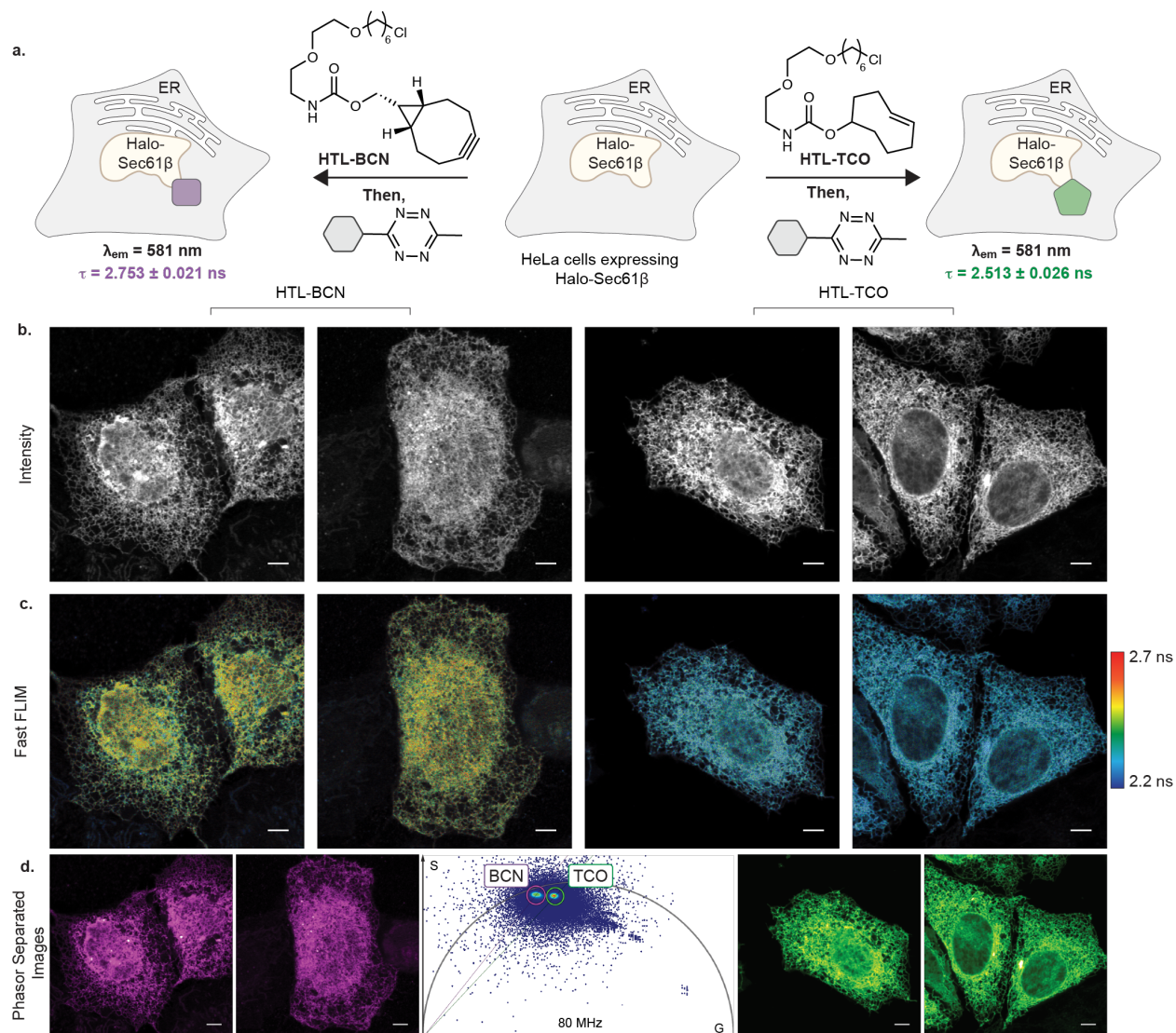
$$\tau = \frac{1}{k_{fl} + k_{nr}}$$

It is therefore reasonable to assume that the quantum yield is proportional to the observable fluorescence lifetime. Given that fluorescence turn-on of HD555 changes based on the reaction product, the bioorthogonal product affects the quantum yield of the xanthene core. Therefore, a difference in fluorescence turn-on for different dienophiles should reflect a difference in fluorescence lifetime. Furthermore, any trend for fluorescence turn-ons across dienophiles (for a given fluorophore) should parallel any trend in observed fluorescence lifetimes.

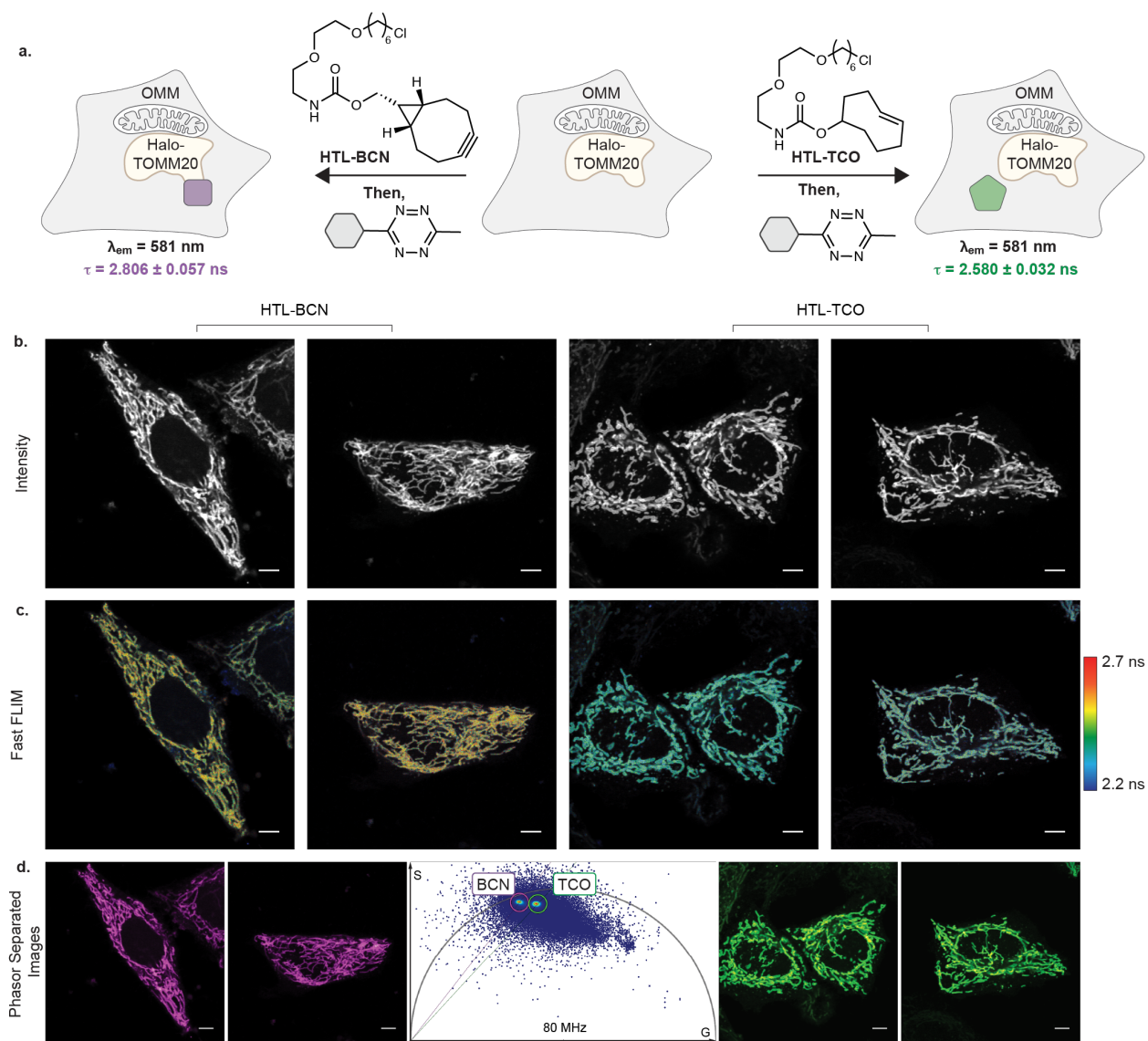
Supplementary Table 1. Lifetime data for HD555 with bioorthogonal reaction partners in multiple contexts.

		Average intensity weighted lifetime (ns)	Average amplitude weighted lifetime (ns)	Phasor plot lifetime (ns)	<i>n</i>
<i>In Vitro</i>					
	BCN	2.440 ± 0.011	2.318 ± 0.017	2.384	3
	TCO	2.211 ± 0.015	2.067 ± 0.024	2.138	3
	SpH	1.935 ± 0.002	1.856 ± 0.009	1.903	3
<i>Self-labelling enzymes</i>					
Halo- TOMM20	HTL-BCN	2.806 ± 0.057	2.438 ± 0.198	2.603	26
	HTL-TCO	2.580 ± 0.032	2.004 ± 0.107	2.221	30
Halo- Sec61β	HTL-BCN	2.753 ± 0.021	2.371 ± 0.112	2.566	27
	HTL-TCO	2.513 ± 0.026	1.989 ± 0.101	2.180	25
Cox8a- SNAP	BG-BCN	2.917 ± 0.044	2.452 ± 0.135	2.583	23
	BG-TCO	2.509 ± 0.103	1.656 ± 0.261	2.040	21
SNAP- Sec61β	BG-BCN	2.874 ± 0.035	2.448 ± 0.105	2.605	34
	BG-TCO	2.441 ± 0.134	1.763 ± 0.225	2.174	26
<i>Small-molecule probes</i>					
	HAO-BCN	2.819 ± 0.086	1.941 ± 0.118	2.238	37
	HAO-TCO	2.226 ± 0.078	1.243 ± 0.086	1.627	51
	Hoechst-BCN	3.134 ± 0.032	2.961 ± 0.031	2.905	32
	Hoechst-TCO	2.649 ± 0.112	1.502 ± 0.241	2.095	34
	Docetaxel-BCN	3.033 ± 0.035	2.812 ± 0.038	2.799	38
	Docetaxel-TCO	2.182 ± 0.087	1.190 ± 0.129	1.763	31

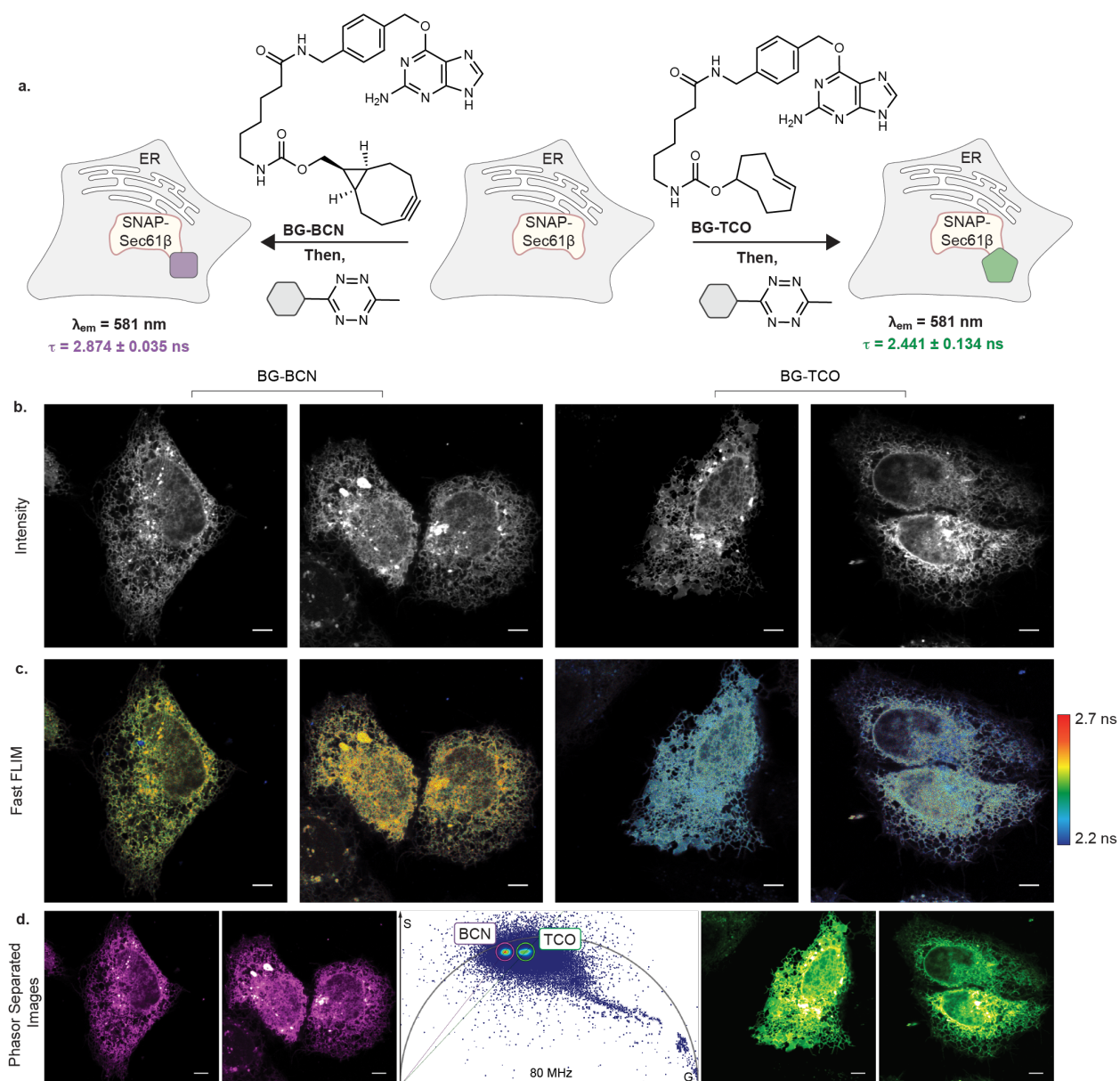
Supplemental Figures: Determining fluorescence lifetimes of HD555 with bioorthogonal partners localized to organelles using self-labelling enzymes.



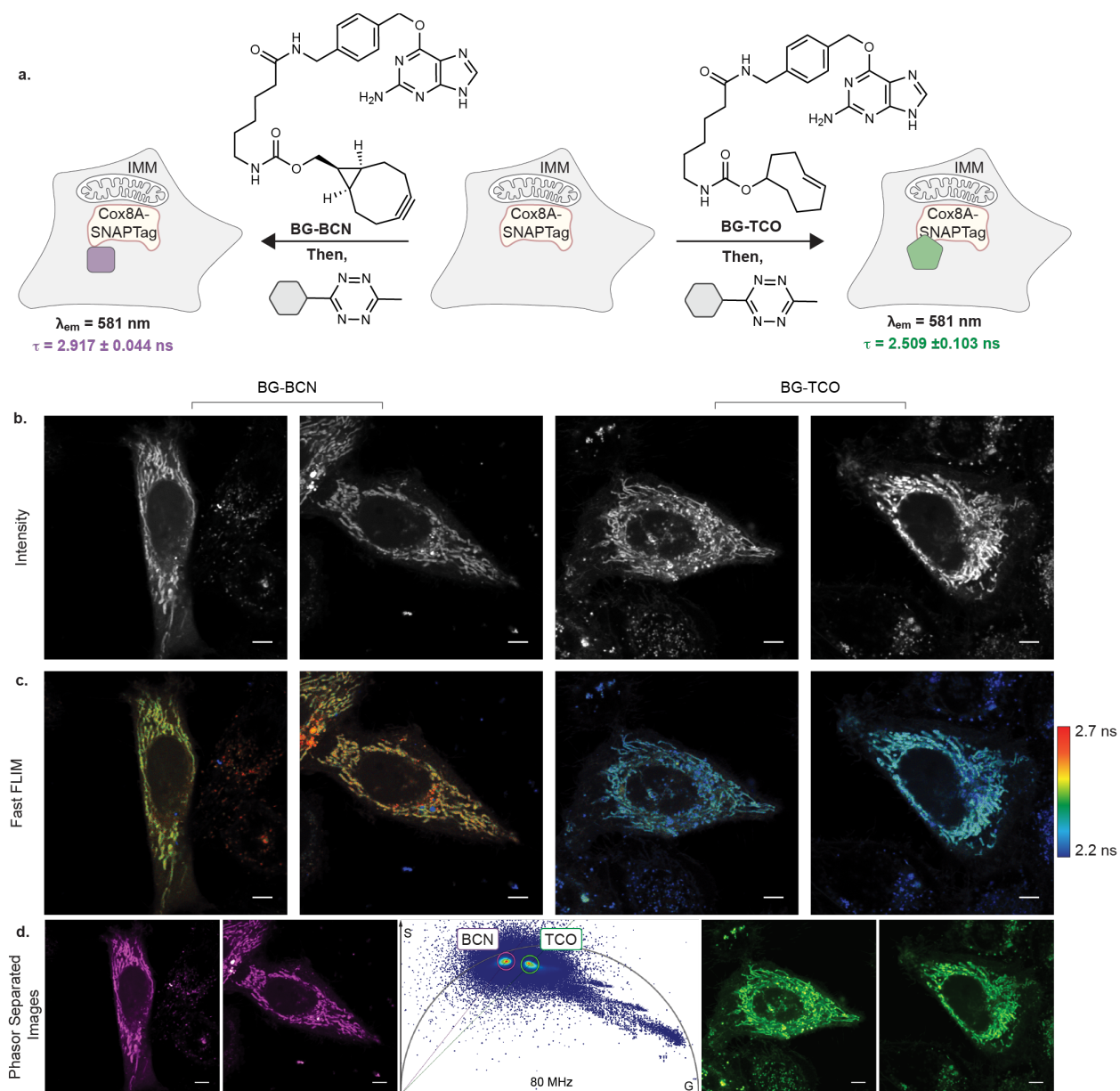
Supplementary Fig. 2 | Single-species FLIM of HD555 with BCN or TCO localized using Halo-Sec61β.
a, Scheme of live cell experiment to determine if turn-on efficiency translates into differentiable fluorescence lifetimes (τ). HeLa cells expressing **HaloTag-Sec61β** (endoplasmic reticulum) are treated first with **HTL-TCO** or **HTL-BCN** (10 μM , 30 min) followed by **HD555** (1 μM , 30 min) and subsequently visualized by fluorescence intensity (**b**) or average photon arrival time—fast FLIM (**c**). **d**, Composite phasor plot (**middle plot**) was then carried out on the data sets, and the phasor plot was used to assign the images to the **BCN** channel (**magenta, left**) or the **TCO** channel (**green, right**). **Scale bars, 5 μm .**



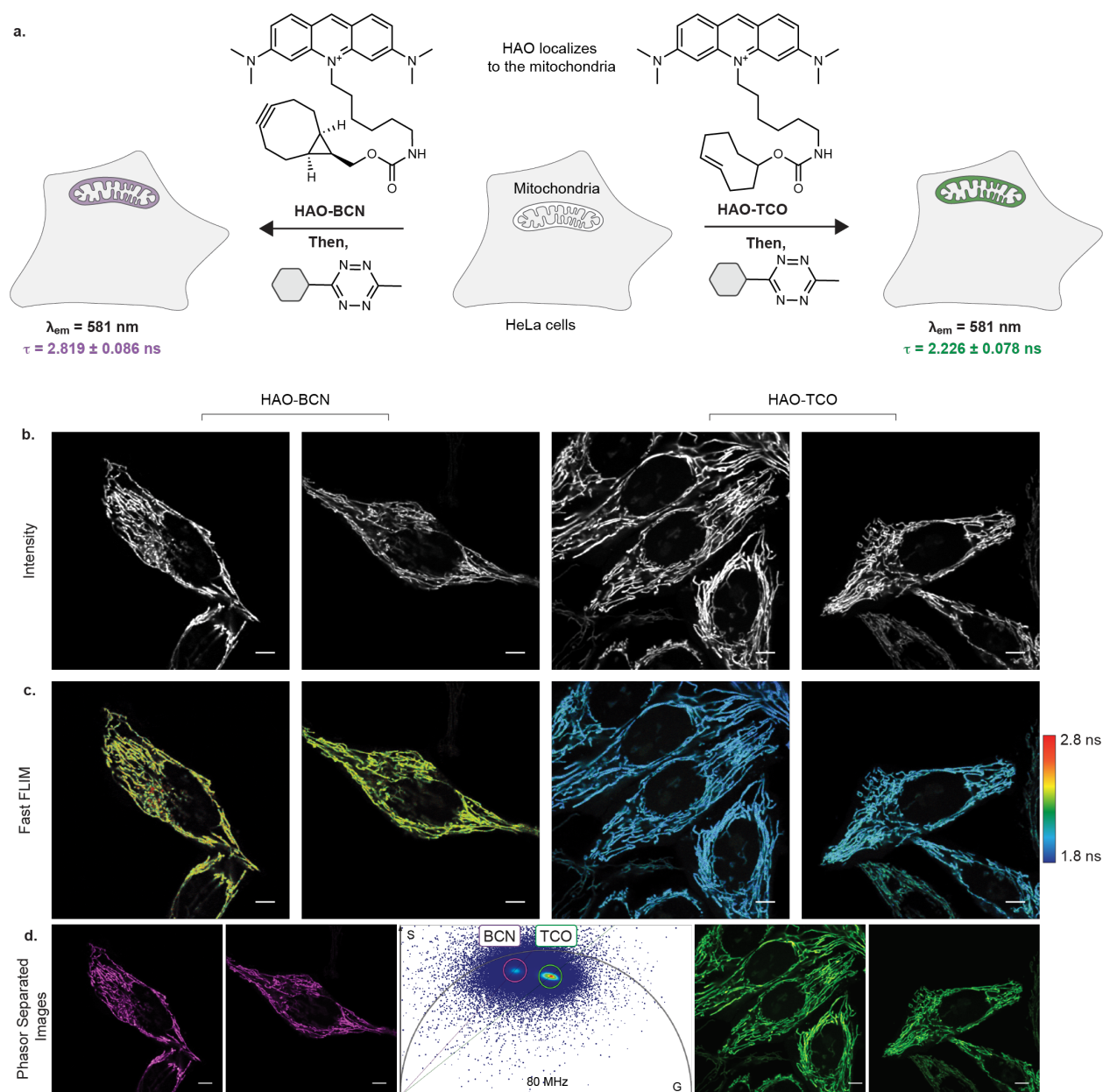
Supplementary Fig. 3 | Single-species FLIM of HD555 with BCN or TCO localized using Halo-TOMM20. **a**, Scheme of live cell experiment to determine if turn-on efficiency translates into differentiable fluorescence lifetimes (τ). HeLa cells expressing **HaloTag-TOMM20** (outer mitochondrial membrane) are treated first with **HTL-TCO** or **HTL-BCN** (10 μ M, 30 min) followed by **HD555** (1 μ M, 30 min) and subsequently visualized by fluorescence intensity (**b**) or average photon arrival time—fast FLIM (**c**). **d**, Phasor analysis (**middle plot**) was then carried out on the data sets, and the phasor plot was used to assign the images to the **BCN** channel (**magenta, left**) or the **TCO** channel (**green, right**). **Scale bars, 5 μ m**.



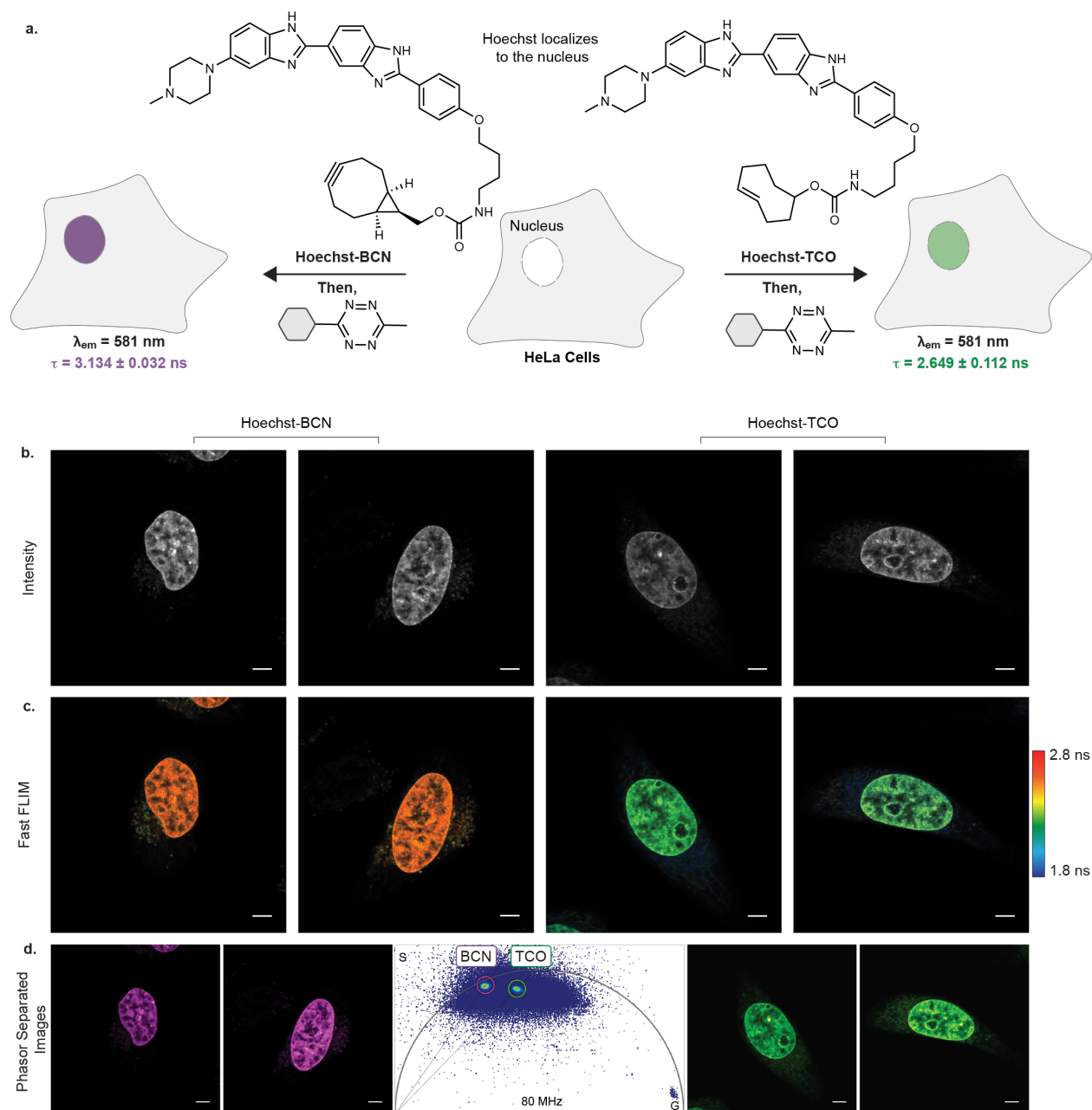
Supplementary Fig. 4 | Single-species FLIM of HD555 with BCN or TCO localized using SNAP-Sec61β. **a**, Scheme of live cell experiment to determine if turn-on efficiency translates into differentiable fluorescence lifetimes (τ). HeLa cells expressing **SNAPTag-Sec61β** (endoplasmic reticulum) are treated first with **BG-TCO** or **BG-BCN** (10 μM , 30 min) followed by **HD555** (1 μM , 30 min) and subsequently visualized by fluorescence intensity (**b**) or average photon arrival time—fast FLIM (**c**). **d**, Phasor analysis (**middle plot**) was then carried out on the data sets, and the phasor plot was used to assign the images to the **BCN** channel (**magenta, left**) or the **TCO** channel (**green, right**). Scale bars, 5 μm .



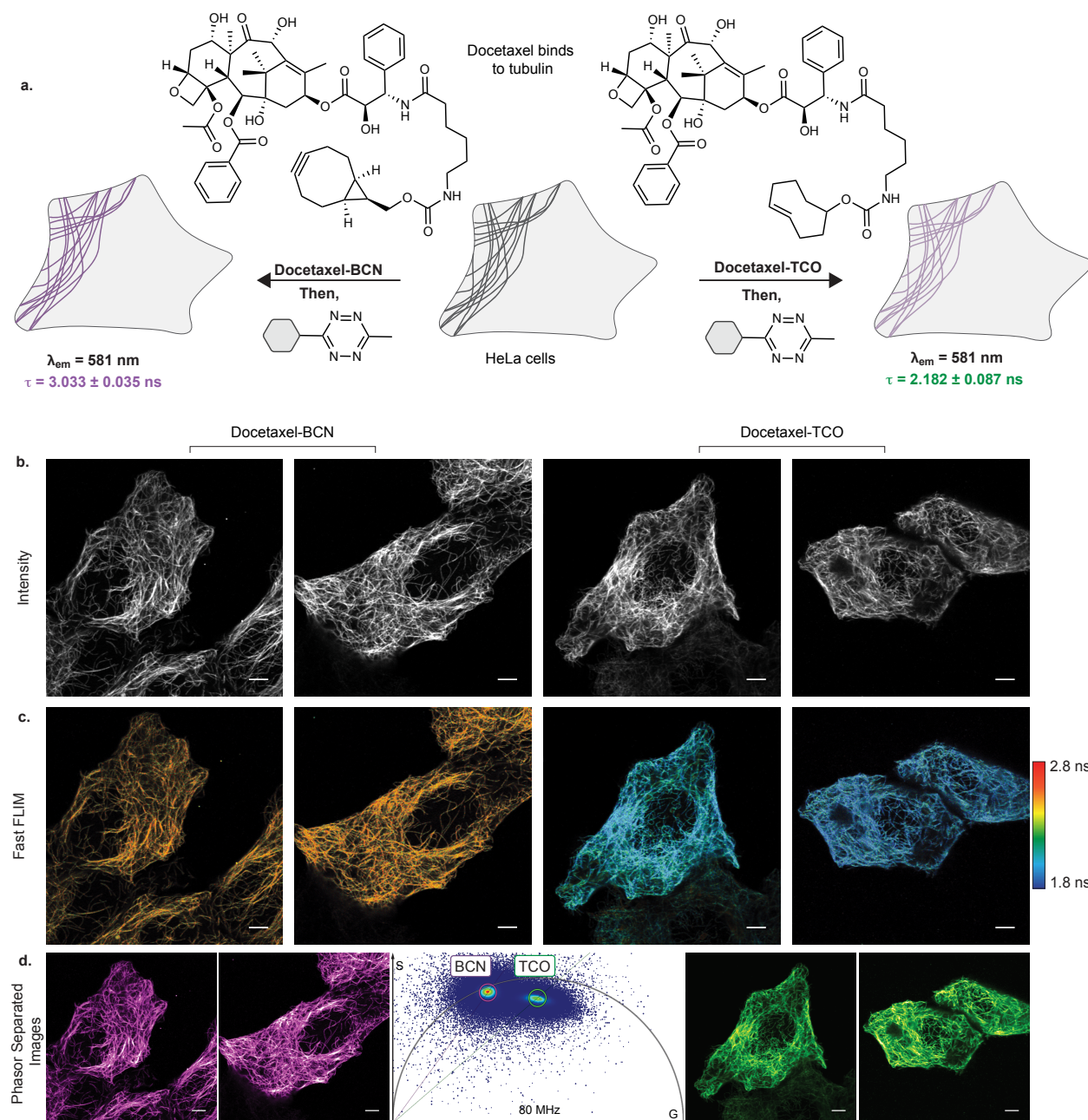
Supplementary Fig. 5 | Single-species FLIM of HD555 with BCN or TCO localized using Cox8a-SNAP.
a. Scheme of live cell experiment to determine if turn-on efficiency translates into differentiable fluorescence lifetimes (τ). HeLa cells expressing **Cox8a-SNAP** (inner mitochondrial membrane) are treated first with **BG-TCO** or **BG-BCN** (10 μM , 30 min) followed by **HD555** (1 μM , 30 min) and subsequently visualized by fluorescence intensity (**b**) or average photon arrival time—fast FLIM (**c**). **d.** Phasor analysis (**middle plot**) was then carried out on the data sets, and the phasor plot was used to assign the images to the **BCN** channel (**magenta, left**) or the **TCO** channel (**green, right**). **Scale bars, 5 μm .**



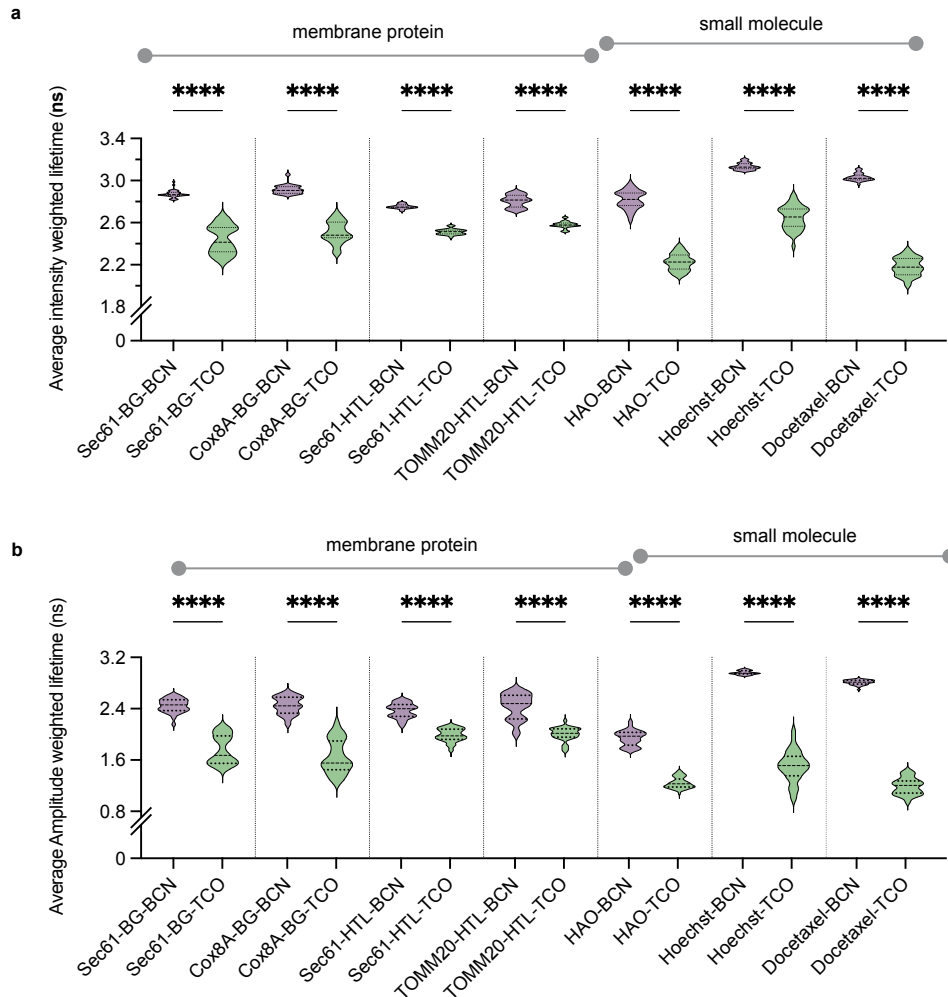
Supplementary Fig. 6 | Single-species FLIM of HD555 with HAO-BCN or HAO-TCO localized to mitochondria. **a**, Scheme of live cell experiment to determine if turn-on efficiency translates into differentiable fluorescence lifetimes (τ) with small-molecule probes. HeLa cells are treated first with **HAO-TCO** or **HAO-BCN** (100 nM, 1 hr) followed by **HD555** (200 nM, 30 min) and subsequently visualized by fluorescence intensity (**b**) or average photon arrival time—fast FLIM (**c**). **d**, Phasor analysis (**middle plot**) was then carried out on the data sets, and the phasor plot was used to assign the images to the **BCN** channel (**magenta, left**) or the **TCO** channel (**green, right**). **Scale bars, 5 μm .**



Supplementary Fig. 7 | Single-species FLIM of HD555 with Hoechst-BCN or Hoechst-TCO localized to nuclei. **a**, Scheme of live cell experiment to determine if turn-on efficiency translates into differentiable fluorescence lifetimes (τ) with small-molecule probes. HeLa cells are treated first with **Hoechst-TCO** or **Hoechst-BCN** (200 nM, 30 min) followed by **HD555** (200 nM, 30 min) and subsequently visualized by fluorescence intensity (**b**) or average photon arrival time—fast FLIM (**c**). **d**, Phasor analysis (**middle plot**) was then carried out on the data sets, and the phasor plot was used to assign the images to the **BCN** channel (**magenta, left**) or the **TCO** channel (**green, right**). **Scale bars, 5 μm .**

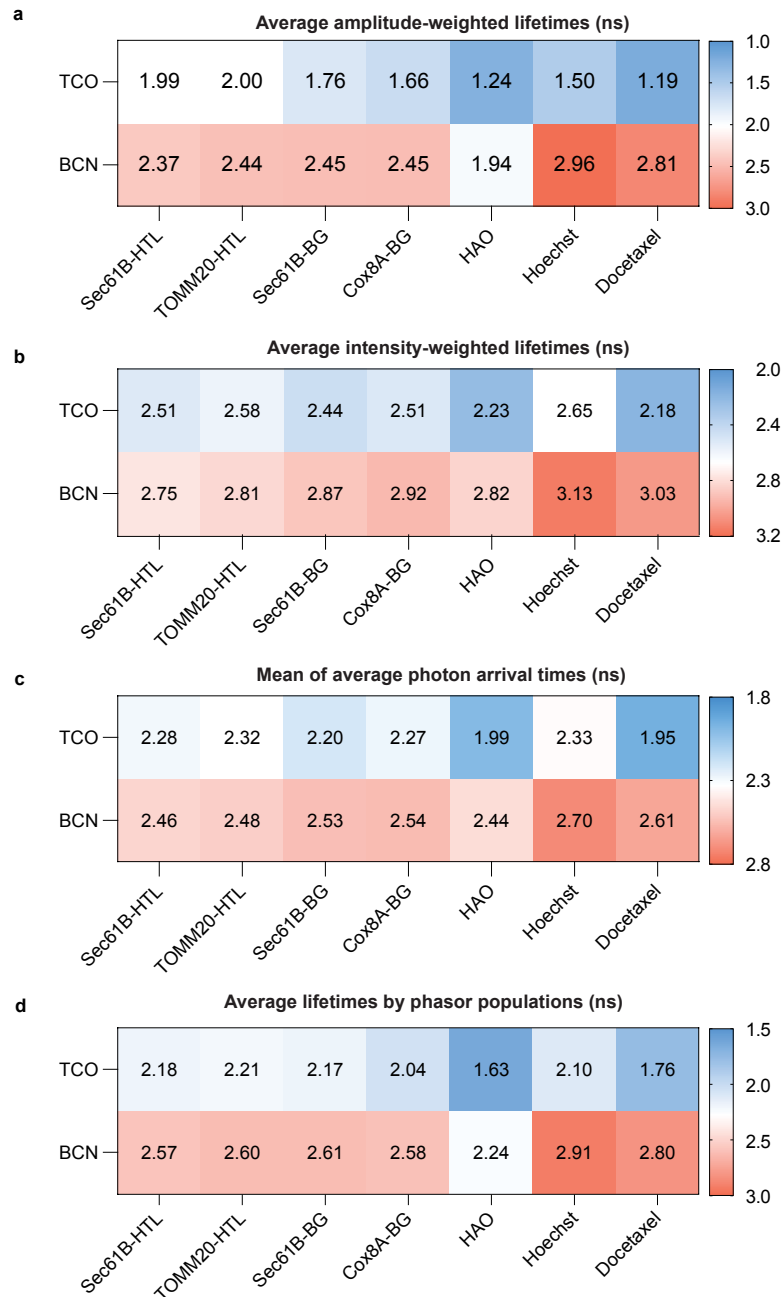


Supplementary Fig. 8 | Single-species FLIM of HD555 with Docetaxel-BCN or Docetaxel-TCO bound to tubulin. **a**, Scheme of live-cell experiment to determine if turn-on efficiency translates into differentiable fluorescence lifetimes (τ) with small-molecule probes. HeLa cells are treated first with **Docetaxel-BCN** or **Docetaxel-TCO** (100 nM, 1 hr) followed by **HD555** (200 nM, 30 min) and subsequently visualized by fluorescence intensity (**b**) or average photon arrival time—fast FLIM (**c**). **d**, Phasor analysis (**middle plot**) was then carried out on the data sets, and the phasor plot was used to assign the images to the **BCN** channel (**magenta, left**) or the **TCO** channel (**green, right**). **Scale bars, 5 μm .**

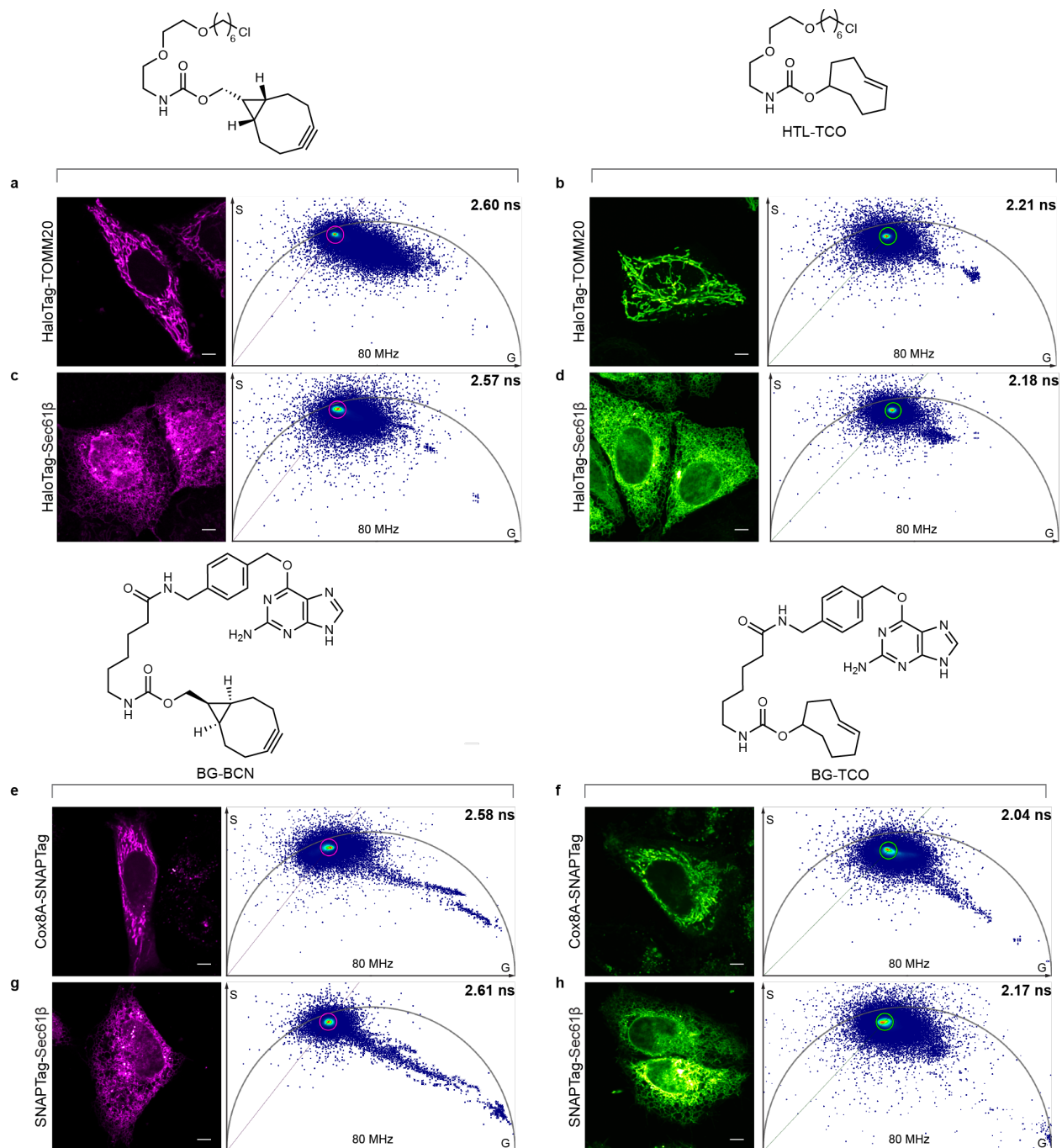


Supplementary Fig. 9 | Comparative analysis lifetimes of HD555 with bioorthogonal handles BCN and TCO across membrane proteins and small molecules. **A**, Average intensity-weighted lifetime (ns) and, **b**, average amplitude-weighted lifetime (ns) are shown for various HD555 with BCN and TCO in various cellular contexts. For **a**, **** $P < 0.0001$ for all tested comparisons using Dunnett's T3 multiple comparisons test: Sec61-BG-BCN (mean = 2.874) vs. Sec61-BG-TCO (mean = 2.441), $\Delta = 0.4322$, 95% CI [0.3546–0.5098], DF = 27.66 | Cox8A-BG-BCN (mean = 2.917) vs. Cox8A-BG-TCO (mean = 2.509), $\Delta = 0.4075$, 95% CI [0.3402–0.4749], DF = 30.23 | Sec61-HTL-BCN (mean = 2.753) vs. Sec61-HTL-TCO (mean = 2.513), $\Delta = 0.2397$, 95% CI [0.2212–0.2582], DF = 45.55 | TOMM20-HTL-BCN (mean = 2.806) vs. TOMM20-HTL-TCO (mean = 2.580), $\Delta = 0.2255$, 95% CI [0.1902–0.2608], DF = 40.20 | HAO-BCN (mean = 2.819) vs. HAO-TCO (mean = 2.226), $\Delta = 0.5930$, 95% CI [0.5425–0.6435], DF = 68.45 | Hoechst-BCN (mean = 3.134) vs. Hoechst-TCO (mean = 2.649), $\Delta = 0.4853$, 95% CI [0.4290–0.5417], DF = 38.62 | Docetaxel-BCN (mean = 3.033) vs. Docetaxel-TCO (mean = 2.182), $\Delta = 0.8504$, 95% CI [0.7910–0.9099], DF = 22.88. For **b**, **** $P < 0.0001$ for all tested comparisons using Dunnett's T3 multiple comparisons test: Sec61-BG-BCN (mean = 2.448) vs. Sec61-BG-TCO (mean = 1.763), $\Delta = 0.6851$, 95% CI [0.5493–0.8208], DF = 33.33 | Cox8A-BG-BCN (mean = 2.452) vs. Cox8A-BG-TCO (mean = 1.656), $\Delta = 0.7958$, 95% CI [0.6200–0.9716], DF = 33.67 | Sec61-HTL-BCN (mean = 2.371) vs. Sec61-HTL-TCO (mean = 1.989), $\Delta = 0.3812$, 95% CI [0.2990–0.4634], DF = 49.96 | TOMM20-HTL-BCN (mean = 2.438) vs. TOMM20-HTL-TCO (mean = 2.004), $\Delta = 0.4340$, 95% CI [0.3109–0.5570], DF = 37.14 | HAO-BCN (mean = 1.941) vs. HAO-

TCO (mean = 1.243), Δ = 0.6983, 95% CI [0.6353–0.7614], DF = 62.41 | Hoechst-BCN (mean = 2.961) vs. Hoechst-TCO (mean = 1.502), Δ = 1.459, 95% CI [1.340–1.577], DF = 34.12 | Docetaxel-BCN (mean = 2.812) vs. Docetaxel-TCO (mean = 1.190), Δ = 1.623, 95% CI [1.555–1.691], DF = 34.20. All comparisons were statistically significant with adjusted P values < 0.0001.

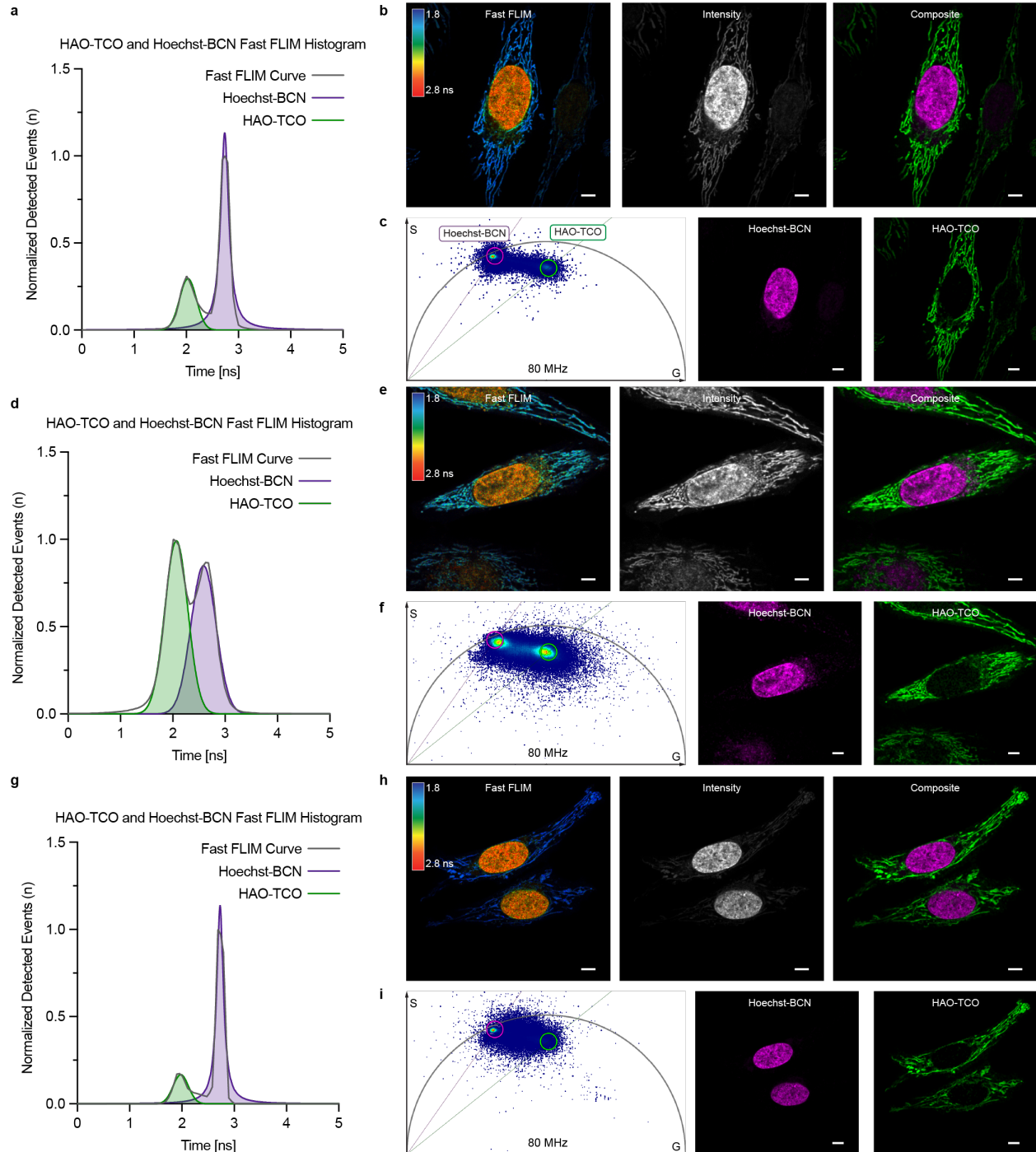


Supplementary Fig. 10 | Comparative analysis lifetimes of HD555 with bioorthogonal handles BCN and TCO across membrane proteins and small molecules. a, Average amplitude-weighted lifetime (ns) and, **b,** average intensity-weighted lifetime (ns), **c,** mean average photon arrival time, **d,** lifetimes by phasor analysis are shown for various HD555 with BCN and TCO in various cellular contexts.



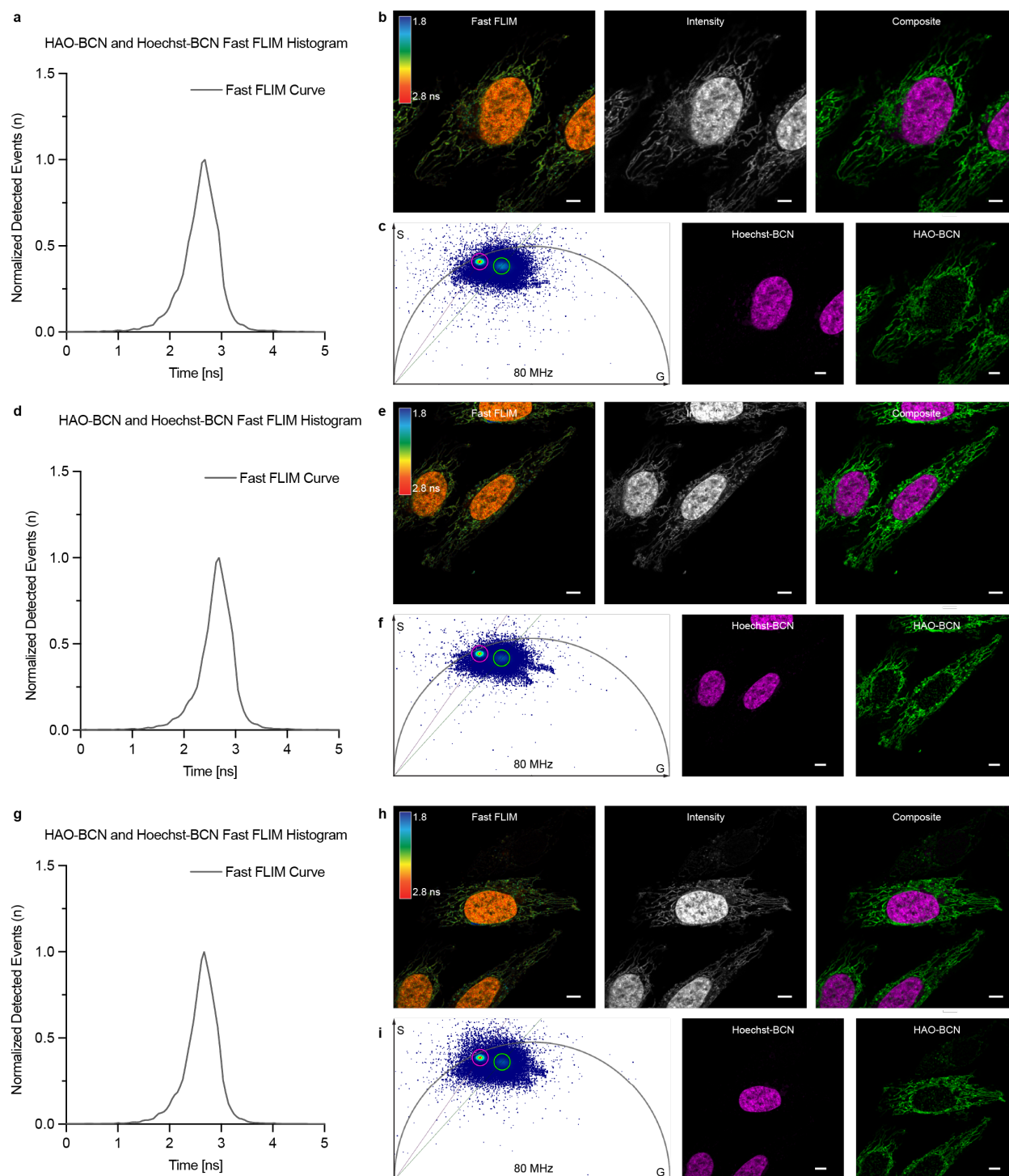
Supplementary Fig. 11 | Phasor plots for single-species lifetimes of HD555 with self-labelling enzymes. Phasor plots from imaging HD555 when localized using Halo-TOMM20 and reacted with HTL-BCN (a) or HTL-TCO (b). We notice single population in multiple cellular contexts: with Halo-Sec61β and HTL-BCN (c), vs. HTL-TCO (d); Cox8A-SNAP and BG-BCN (e), vs. BG-TCO (f); SNAPTag-Sec61β and BG-BCN (h), vs. BG-TCO (h). Lifetime from the phasor cursor is overlaid on the plot.

Supplemental Figures: Multiplexed FLIM with HD555 and small-molecule probes.



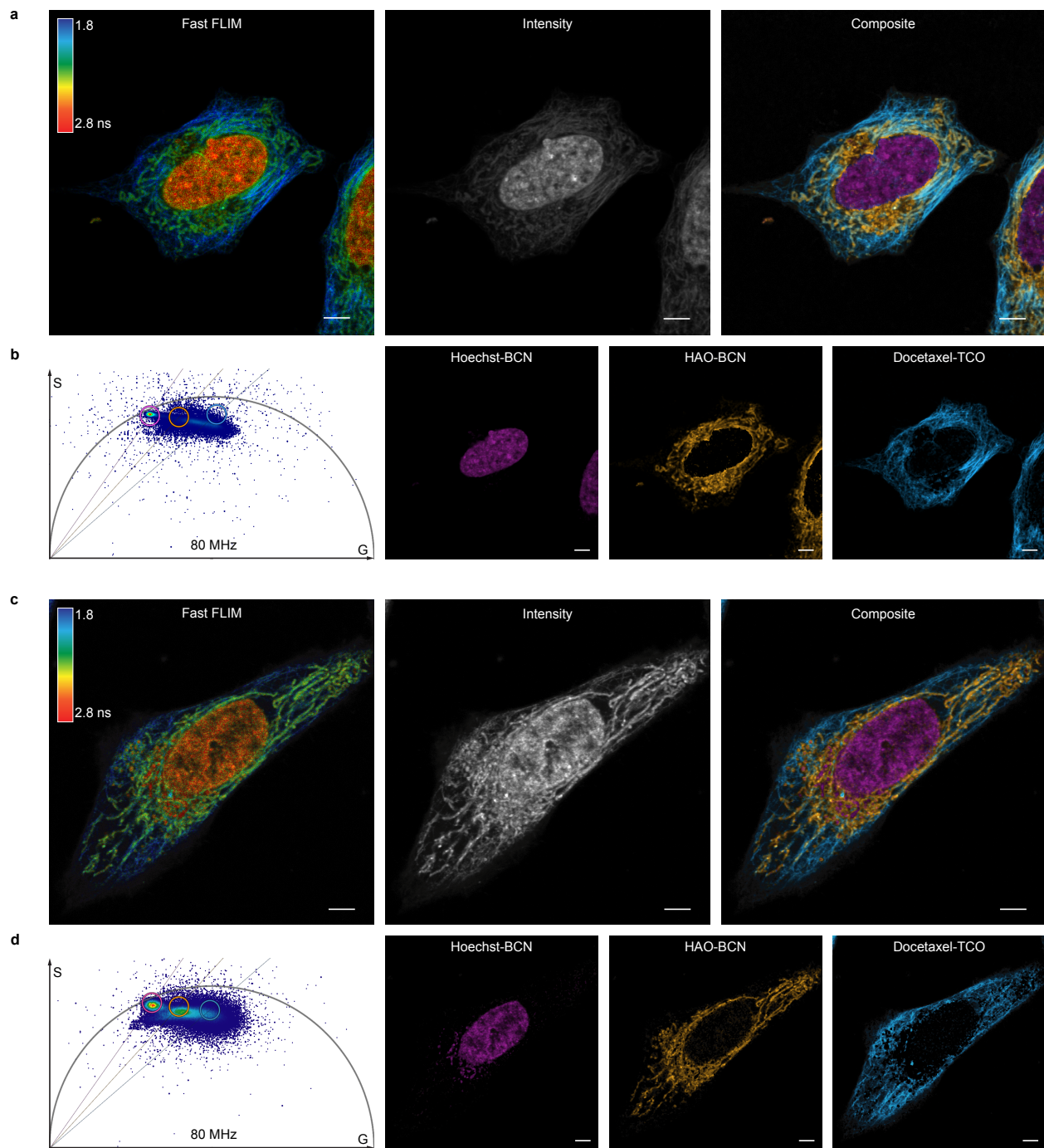
Supplementary Fig. 13 | Multi-species FLIM of HD555 with Hoechst-BCN and HAO-TCO. HeLa cells were treated with HAO-TCO (100 nM) and Hoechst-BCN (200 nM), then with HD555 (400 nM) (see **online methods** for more details). When visualized on the basis of total fluorescence intensity (**b,e,h—grayscale images**) of HD555, the organelles are recognizable, but are not distinguishable. Using fast FLIM (**b,e,h—heatmap image**), we observe a difference in average photon arrival time between the nucleus (**Hoechst-BCN**) and the mitochondria (**HAO-TCO**). When the fast FLIM histograms (**a, d, g**) were plotted, two distinct

peaks were observed. Each peak could be fitted to either a gaussian or Lorentzian function to obtain two distinct lifetime distribution populations. The FLIM phasor analysis was used to separate the HAO-TCO associated HD555 signal from the Hoechst-BCN associated HD555 signal (**c, f, i**), resulting in an image with two distinct and separable channels belonging to each organelle. $R^2 > 0.95$ for Gaussian and Lorentzian curve fits. **Scale bars, 5 μm .**



Supplementary Fig. 14 | Multi-species FLIM of HD555 with Hoechst-BCN and HAO-BCN. HeLa cells were treated with HAO-BCN (100 nM) and Hoechst-BCN (200 nM), then with HD555 (400 nM) (see **online methods** for more details). When visualized on the basis of total fluorescence intensity (**b,e,h—grayscale images**) of HD555, the organelles are recognizable, but are not distinguishable. Using fast FLIM (**b,e,h—heatmap image**), we observe a difference in average photon arrival time between the nucleus (**Hoechst-BCN**) and the mitochondria (**HAO-BCN**), however the contrast is less pronounced than in **Sup. Fig. 8**.

When the fast FLIM histograms (**a, d, g**) were plotted, The distribution of lifetimes was broad and indistinguishable. However, FLIM phasor analysis could be used to separate the HAO-BCN associated HD555 signal from the Hoechst-BCN associated HD555 signal, resulting in an image with two distinct and separable channels belonging to each organelle. **Scale bars, 5 μ m.**



Supplementary Fig. 15 | Imaging the nucleus, mitochondria, and microtubules simultaneously, with one dye and no transfection. Multi-species FLIM of HD555 with Hoechst-BCN, HAO-BCN, and Docetaxel-TCO—separating three lifetime species by modulating fluorescence lifetime of HD555 with bioorthogonal partners. HeLa cells were treated with Docetaxel-TCO (200 nM), HAO-BCN (100 nM), and Hoechst-BCN (100 nM), then with HD555 (1 μ M) (see **online methods** for more details). When visualized on the basis of total fluorescence intensity (**a, c—grayscale images**) of HD555, the organelles are indistinguishable. Using fast FLIM (**a, c—heatmap image**), we observe a difference in average photon arrival time between the three

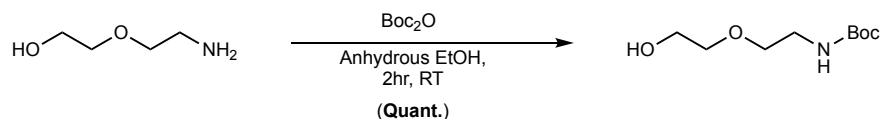
organelles. FLIM phasor analysis (**b**, **d**) could be used to separate the signals from each organelle probe.
Scale bars, 5 μm .

Supplemental Note: Synthesis methods for small-molecule probes and ligands

General Considerations

All reactions were performed under inert conditions (under nitrogen, and anhydrous). Reaction vessels were flame dried or oven dried, then cooled under vacuum and refilled with nitrogen. Chemicals used for synthesis were purchased from commercial manufacturers (Combi-block Inc., Sigma-Aldrich®, BroadPharm®, ThermoScientific Inc., TCI America™) and used as is, or prepared according to literature procedures. Flash chromatography was performed on a Teledyne ISCO CombiFlash® NextGen 300+ using RediSep® Silica (40–60 µm) or Alumina (40–60 µm) prepackaged columns. Preparative HPLC was performed on a Waters 150 HPLC using a 95-10% Water/Acetonitrile gradient with 0.1% TFA additive. The HPLC was equipped with a reverse-phase C18 column, a 200-800 nm UV/vis detector, an automatic fraction collector, and an autosampler with a 7 mL injection loop. ¹H NMR and ¹³C NMR spectra were recorded using a Bruker NEO-500, NEO-501, AV-600, or AV-700 (frequencies listed in spectra). NMR spectra were analyzed using MestreLab MesReNova 15.0.1. Chemical shift values (δ) are reported in parts per million (ppm) relative to the solvent residual signals (reported individually for each compound). Reaction monitoring by LC/MS was carried out on a low-resolution Waters SQD2 using a 90-10% Water/Acetonitrile gradient with a 0.1% formic acid additive. The LCMS was equipped with a reverse-phase C18 column, a 200-800 nm UV/vis detector, a 300-1000 nm fluorescence detector, and an autosampler. High-resolution mass spectra (HRMS) were recorded on an Agilent QTOF LCMS with ESI connected to an Agilent UHPLC. HPLC purifications were performed on a reverse-phase column (YMC-Triart C18, 150 mm × 10 mm S-5 µm, 12 nm) using an Agilent HPLC system.

Synthesis of HaloTag Ligands (HTL)



1 – tert-butyl (2-(2-hydroxyethoxy)ethyl)carbamate.

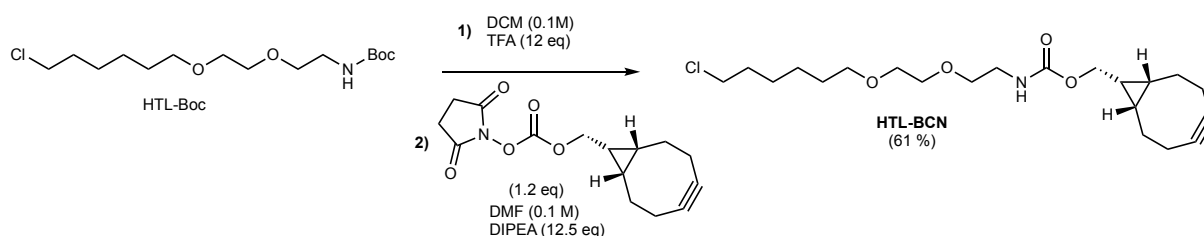
The procedure was adopted from previously published methods⁸². To a stirred solution of 2-(2-aminoethoxy)ethanol (1 g, 9.511 mmol, 1.0 equiv.) in anhydrous ethanol (0.5 M, 19.0 mL) under a nitrogen atmosphere, cooled to 0 °C in an ice bath, was added di-tert-butyl dicarbonate (2.076 g, 9.511 mmol, 1.0 equiv.) dropwise. After stirring at 0 °C for 20 min, the ice bath was removed, and the reaction mixture was allowed to warm to room temperature under nitrogen and stirred for an additional 2 h. After completion, the solvent was evaporated under reduced pressure. The residue was diluted with water, transferred into a separatory funnel, and extracted with dichloromethane (4 × 20 mL). The combined organic layers were washed with brine, dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and evaporated under vacuum. Residual solvent was removed under a stream of nitrogen overnight, providing the product as a colorless oil (1.95 g). The crude product was sufficiently pure and used directly in subsequent steps without further purification. Characterization agreed with previously reported spectra. ^1H NMR (500 MHz, CDCl_3) δ 4.89 (s, 1H), 3.81 – 3.68 (m, 2H), 3.56 (dt, J = 12.7, 4.9 Hz, 4H), 3.33 (t, J = 5.2 Hz, 2H), 1.45 (s, 9H). ^{13}C NMR (126 MHz, CDCl_3) δ 156.07, 79.46, 72.14, 70.32, 61.82, 40.42, 28.41.



HTL-Boc – tert-butyl (2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamate.

The procedure was adopted from previously published methods⁸². Under an inert atmosphere, 1 (0.500 g, 2.436 mmol) was added to a microwave vial. Anhydrous THF (0.69 M, 3.53 mL) and DMF (1.390 M, 1.75 mL) were added, and the solution stirred under nitrogen at 0 °C until equilibrated. Sodium hydride (60% dispersion in oil, 0.117 g, 2.923 mmol, 1.2 equiv.) was quickly added under nitrogen (!!!generates hydrogen gas!!!). The vial was capped, sealed, and stirred for an additional 30 min. 1-chloro-6-iodohexane (0.841 g, 3.410 mmol, 1.4 equiv.) was added via syringe, and the reaction mixture was allowed to warm to room temperature overnight. The reaction was quenched with saturated aqueous NH_4Cl , diluted with dichloromethane, and transferred to a separatory

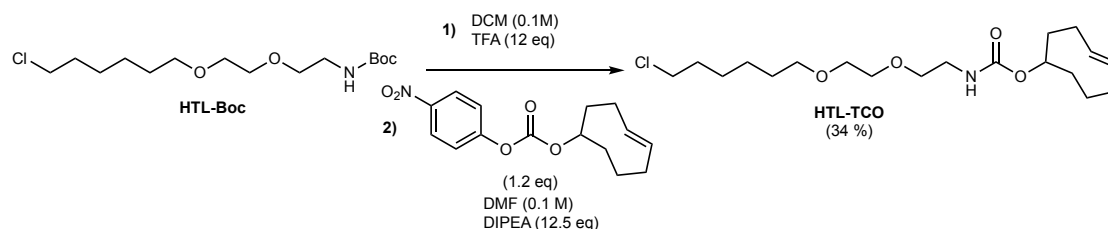
funnel. Crude was extracted with DCM—combined organic layers, were washed with brine, dried (anhydr. Na_2SO_4), filtered, and evaporated. Purification by flash chromatography (Silica, hexanes/ethyl acetate gradient: 0–50% EtOAc) yielded **HTL-Boc** (0.303 g, 37%) as a clear oil (R_f = 0.25, 70:30 hexanes/EtOAc, stains black with PMA). Characterization agreed with previously reported spectra. ^1H NMR (500 MHz, CDCl_3) δ 4.99 (s, 1H), 3.60 (dd, J = 5.7, 3.0 Hz, 2H), 3.58 – 3.50 (m, 6H), 3.46 (t, J = 6.6 Hz, 2H), 3.34 – 3.28 (m, 2H), 1.77 (p, J = 6.9 Hz, 2H), 1.61 (p, J = 6.8 Hz, 2H), 1.46 (d, J = 8.5 Hz, 2H), 1.44 (s, 9H), 1.42 – 1.32 (m, 2H). ^{13}C NMR (126 MHz, CDCl_3) δ 156.12, 79.32, 71.42, 70.42, 70.35, 70.17, 45.18, 40.49, 32.67, 31.07, 29.57, 28.56, 26.83, 25.56.



HTL-BCN – ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-((6chlorohexyl)oxy) ethoxy)ethyl)carbamate.

HTL-Boc (50 mg, 0.154 mmol) was dissolved in DCM (1.54 mL) and TFA (0.118 mL , 1.544 mmol, 10 equiv.). The reaction was stirred until completion (monitored by TLC and LCMS). Reaction mixture was concentrated and dried under vacuum. Deprotected amine was used without any further purification. In a separate vial, BCN-NHS ester (54 mg, 0.185 mmol, 1.2 equiv.) was dissolved in DMF (0.1 M, 1.54 mL), and DIPEA (0.249 g, 1.930 mmol, 12.5 equiv.) was added. The deprotected chloroalkane intermediate was dissolved separately in DMF (1.54 mL), and the BCN-NHS ester solution was quickly added. The reaction was stirred at room temperature and monitored by TLC (basic alumina, 75:25 hexanes/ethyl acetate; stains: KMnO_4 , I_2) and LCMS until completion. Upon completion, the reaction was concentrated and purified by column chromatography (Basic alumina, 0–50% ethyl acetate in hexanes). **HTL-BCN** was isolated as a colorless oil (37 mg, 61%). **Note:** The product eluted early but showed low mobility on TLC.

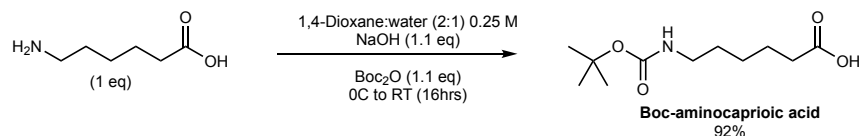
^1H NMR (400 MHz, CDCl_3) δ 5.18 (s, 1H), 4.13 (d, J = 8.1 Hz, 2H), 3.63 – 3.53 (m, 4H), 3.54 (s, 2H), 3.55 – 3.44 (m, 3H), 3.44 (d, J = 6.7 Hz, 2H), 3.36 (q, J = 5.4 Hz, 2H), 2.24 (dq, J = 15.4, 9.7, 3.6 Hz, 5H), 1.76 (p, J = 7.0 Hz, 2H), 1.60 (p, J = 6.9 Hz, 3H), 1.50 – 1.27 (m, 5H), 0.99 – 0.86 (m, 2H). ^{13}C NMR (126 MHz, CDCl_3) δ 156.12, 79.32, 71.42, 70.42, 70.35, 70.17, 45.18, 40.49, 32.67, 31.07, 29.57, 28.56, 26.83, 25.56. HRMS (ES+) calcd for $(\text{C}_{21}\text{H}_{34}\text{ClNO}_4)$ $[\text{M}+\text{K}^+]$ 438.18079, found 438.37069.



HTL-TCO – (E)-cyclooct-4-en-1-yl (2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)carbamate.

HTL-Boc (50 mg, 0.154 mmol) was dissolved in DCM (1.54 mL) and TFA (0.118 mL, 1.544 mmol, 10 equiv.). The reaction was stirred until completion (monitored by TLC and LCMS). Reaction mixture was concentrated and dried under vacuum. Deprotected amine was used without any further purification. In a separate vial, TCO-PNB ester (54 mg, 0.185 mmol, 1.2 equiv.) was dissolved in DMF (0.1 M, 1.54 mL), and DIPEA (0.249 g, 1.930 mmol, 12.5 equiv.) was added. The deprotected chloroalkane intermediate was dissolved separately in DMF (1.54 mL), and the TCO-PNB ester solution was quickly added. The reaction was stirred at room temperature and monitored by TLC (Silica, 50:50 hexanes/ethyl acetate; stains: KMnO_4 , I_2) and LCMS until completion. Upon completion, the reaction was concentrated and purified by column chromatography (basic alumina, 0–50% ethyl acetate in hexanes). **HTL-TCO** was isolated as a yellowish oil (17.9 mg, 34%). ^1H NMR (500 MHz, CDCl_3) δ 5.61 – 5.44 (m, 2H), 5.07 (t, J = 6.0 Hz, 1H), 4.32 (dd, J = 11.0, 5.9 Hz, 1H), 3.62 – 3.56 (m, 2H), 3.58 – 3.49 (m, 6H), 3.44 (t, J = 6.6 Hz, 2H), 3.37 – 3.30 (m, 2H), 2.39 – 2.27 (m, 3H), 2.04 – 1.98 (m, 1H), 2.00 – 1.85 (m, 3H), 1.81 – 1.71 (m, 3H), 1.74 – 1.67 (m, 1H), 1.66 – 1.40 (m, 5H), 1.43 – 1.31 (m, 2H), 1.26 (s, 1H). ^{13}C NMR (126 MHz, CDCl_3) δ 156.12, 79.32, 71.42, 70.42, 70.35, 70.17, 45.18, 40.49, 32.67, 31.07, 29.57, 28.56, 26.83, 25.56. HRMS (ES+) calcd for ($\text{C}_{19}\text{H}_{34}\text{ClNO}_4$) $[\text{M}+\text{Na}]^+$ 398.20686, found 398.20199

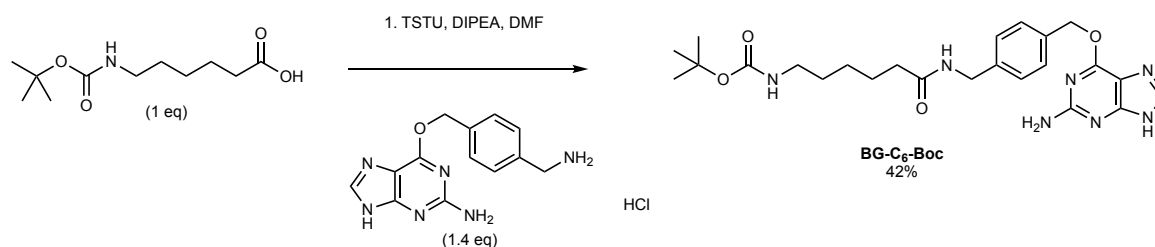
Synthesis of Benzyl Guanine Ligands (BG)



Boc-aminocaproic acid – 6-((tert-butoxycarbonyl)amino)hexanoic acid.

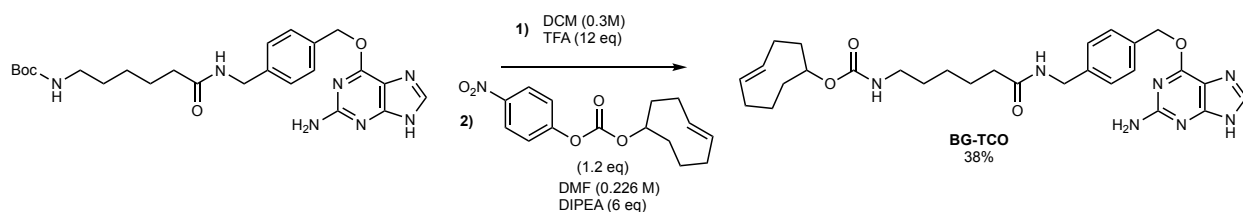
NaOH (1.1 eq, 189 mg, 4.738 mmol) was dissolved in dioxane/ H_2O (90.0 mL, 2:1). 6-aminocaproic acid (1 eq, 565 mg, 4.307 mmol) was then added to the reaction vessel, and the flask was cooled to 0°C. Ditetert-butyl dicarbonate (1.1 eq, 1.04 g, 4.738 mmol) was added to the flask in portions. The reaction mixture was stirred for 30 min, then was warmed up to ambient temperature overnight. The mixture was concentrated, and the

crude dissolved in H₂O (50 mL) and washed with ethyl acetate (3 × 50.0 mL). The aqueous layer was then acidified to pH 2 with aqueous 0.1 M HCl. The aqueous phase was extracted with ethyl acetate (3 × 50 mL). Organic layers were combined and dried with Na₂SO₄, filtered and concentrated under reduced pressure to yield the title compound (922 mg, 92%) as a colourless oil (crystalizes in white solid upon drying and cooling). Spectra agree with previously published data⁸³. ¹H NMR (500 MHz, MeOD) δ 3.03 (t, *J* = 6.6 Hz, 2H), 2.33 – 2.26 (m, 2H), 1.61 (h, *J* = 6.4 Hz, 2H), 1.51 – 1.45 (m, 3H), 1.43 (s, 8H), 1.35 (h, *J* = 7.0 Hz, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 156.12, 79.32, 71.42, 70.42, 70.35, 70.17, 45.18, 40.49, 32.67, 31.07, 29.57, 28.56, 26.83, 25.56.



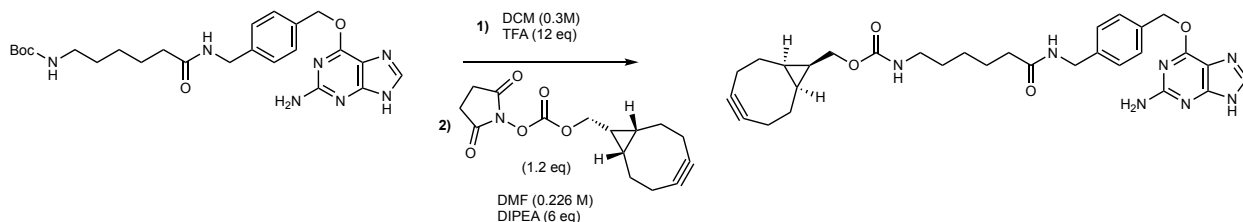
BG-C6-Boc – tert-butyl (6-((4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)amino)-6-oxohexyl)carbamate.

Boc-aminocaproic acid (0.250 g, 1.082 mmol, 1.0 equiv.) was dissolved in DMF (2.4 mL) and DIPEA (0.885 mL, 5.083 mmol, 4.7 equiv.) was added. The mixture was stirred and equilibrated. TSTU (0.391 g, 1.298 mmol, 1.2 equiv.) added to the vial and the mixture was stirred for 10 min under nitrogen. Conversion to the NHS-ester was monitored by LCMS. After 1 hour, we observed complete conversion to NHS-ester. 6-((4-(aminomethyl)benzyl)oxy)-9H-purin-2-amine hydrochloride (0.409 g, 1.514 mmol, 1.4 equiv.) was then dissolved separately in DMF (2.4 mL, 0.226 M for total reaction volume), added dropwise to the NHS-ester solution, and stirred at room temperature. Reaction completion was monitored by LCMS. After completion, the mixture was concentrated under vacuum, redissolved in 50:50 acetonitrile/water containing 0.1% formic acid, and purified via reverse-phase HPLC (gradient: 5–95% acetonitrile in water, 0.1% TFA). The fraction were combined and lyophilized affording the desired product (0.220 g, 42%) as a white solid. ¹H NMR (500 MHz, MeOD) δ 7.85 (s, 0H), 7.53 – 7.48 (m, 1H), 7.34 – 7.28 (m, 1H), 5.55 (s, 1H), 4.38 (s, 1H), 3.03 (t, *J* = 7.0 Hz, 1H), 2.25 (t, *J* = 7.5 Hz, 1H), 1.65 (p, *J* = 7.6 Hz, 1H), 1.54 – 1.45 (m, 1H), 1.44 (s, 4H), 1.40 – 1.30 (m, 1H). ¹³C NMR (126 MHz, MeOD) δ 174.61, 160.29, 157.16, 138.71, 135.52, 128.30, 127.28, 78.41, 67.25, 47.89, 42.42, 39.79, 35.57, 29.24, 27.39, 26.05, 25.33.



BG-TCO – (E)-cyclooct-4-en-1-yl (6-(((4-(((2-amino-9H-purin-6-yl)oxy)methyl)benzyl)amino)-6-oxohexyl)carbamate.

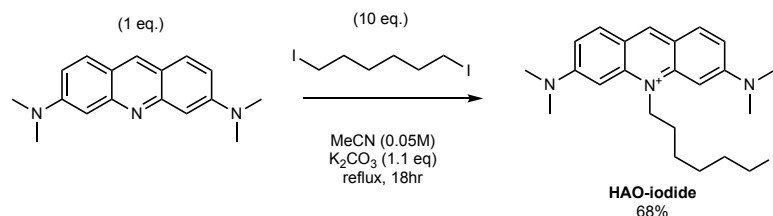
Prepared similarly to **HTL-BCN**—adapted procedure below: **BG-C₆-Boc** (35 mg, 0.072 mmol, 1 eq.) was dissolved in DCM (1.54 mL) and TFA (0.118 mL, 1.544 mmol). The reaction was stirred until completion (monitored by TLC and LCMS). Reaction mixture was concentrated and dried under vacuum. Deprotected amine was used without any further purification. In a separate vial, TCO-PNB ester (21 mg, 0.072 mmol, 1 equiv.) was dissolved in DMF (0.226 M, 0.320 mL), and DIPEA (0.076 mL, 0.434 mmol, 6 equiv.) was added. The deprotected chloroalkane intermediate was dissolved separately in DMF (0.320 mL), and the TCO-PNB ester solution was quickly added. The reaction was stirred at room temperature and monitored by LCMS until completion. After completion, the mixture was concentrated under vacuum, redissolved in 50:50 acetonitrile/water containing 0.1% formic acid, and purified via reverse-phase HPLC (gradient: 5–95% acetonitrile in water, 0.1% TFA). The fraction were combined and lyophilized affording the desired product (15 mg, 38%) as a white solid. ¹H NMR (500 MHz, MeOD) δ 8.34 (s, 1H), 7.54 – 7.49 (m, 2H), 7.35 – 7.30 (m, 2H), 5.64 (s, 2H), 5.57 (ddd, *J* = 15.1, 10.2, 4.6 Hz, 1H), 5.44 (ddd, *J* = 16.0, 11.0, 3.6 Hz, 1H), 4.37 (s, 2H), 4.36 – 4.24 (m, 1H), 3.04 (t, *J* = 6.9 Hz, 2H), 2.35 – 2.29 (m, 2H), 2.24 (t, *J* = 7.5 Hz, 2H), 1.92 (dddd, *J* = 23.3, 15.4, 8.2, 3.3 Hz, 3H), 1.76 – 1.51 (m, 4H), 1.47 (p, *J* = 7.2 Hz, 2H), 1.37 – 1.29 (m, 2H). ¹³C NMR (126 MHz, MeOD) δ 176.05, 162.54, 162.25, 161.10, 158.72, 158.04, 153.46, 143.78, 141.06, 136.07, 135.22, 133.72, 130.25, 128.79, 118.99, 116.67, 107.92, 81.54, 70.95, 49.45, 49.28, 43.71, 42.21, 41.47, 39.63, 36.92, 35.15, 33.48, 32.10, 30.68, 30.63, 27.38, 26.67, 24.21. HRMS (ES⁺) calcd for (C₂₈H₃₇N₇O₄) [M+H]⁺ 536.29798, found 536.28416.



BG-BCN – ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (6-((4-(((2-amino-9H-purin-6-yl)oxy)methyl) benzyl) amino)-6-oxohexyl) carbamate.

Prepared similarly to **HTL-BCN**—adapted procedure below: **BG-C₆-Boc** (35 mg, 0.072 mmol, 1 eq.) was dissolved in DCM (1.54 mL) and TFA (0.118 mL, 1.544 mmol). The reaction was stirred until completion (monitored by TLC and LCMS). Reaction mixture was concentrated and dried under vacuum. Deprotected amine was used without any further purification. In a separate vial, BCN-NHS ester (21 mg, 0.072 mmol, 1 equiv.) was dissolved in DMF (0.226 M, 0.320 mL), and DIPEA (0.076 mL, 0.434 mmol, 6 equiv.) was added. The deprotected chloroalkane intermediate was dissolved separately in DMF (0.320 mL), and the BCN-NHS ester solution was quickly added. The reaction was stirred at room temperature and monitored by LCMS until completion. After completion, the mixture was concentrated under vacuum, redissolved in 50:50 acetonitrile/water containing 0.1% formic acid, and purified via reverse-phase HPLC (gradient: 5–95% acetonitrile in water, 0.1% TFA). The fraction were combined and lyophilized affording the desired product (12 mg, 29%) as a white solid. ¹H NMR (500 MHz, MeOD) δ 8.32 (s, 1H), 7.54 – 7.48 (m, 2H), 7.36 – 7.30 (m, 2H), 5.64 (s, 2H), 4.37 (s, 2H), 4.13 (dd, *J* = 12.9, 8.2 Hz, 2H), 3.07 (t, *J* = 7.0 Hz, 3H), 2.68 – 2.44 (m, 1H), 2.38 – 2.09 (m, 6H), 2.05 – 1.75 (m, 2H), 1.71 – 1.54 (m, 5H), 1.50 (p, *J* = 7.1 Hz, 3H), 1.35 (dd, *J* = 11.8, 4.5 Hz, 3H), 1.36 – 1.27 (m, 1H), 1.27 – 1.17 (m, 1H), 1.03 – 0.65 (m, 2H). ¹³C NMR (126 MHz, MeOD) δ 174.64, 159.72, 152.38, 142.10, 139.65, 133.96, 128.82, 127.39, 98.08, 69.42, 62.15, 61.79, 48.23, 48.06, 47.88, 45.04, 42.32, 40.14, 39.72, 35.54, 29.23, 28.75, 28.16, 25.98, 25.28, 23.24, 20.51, 19.96, 19.46, 18.32, 17.87, 17.55. HRMS (ES⁺) calcd for (C₃₀H₃₇N₇O₄) [M+H]⁺ 560.29798, found 560.28841.

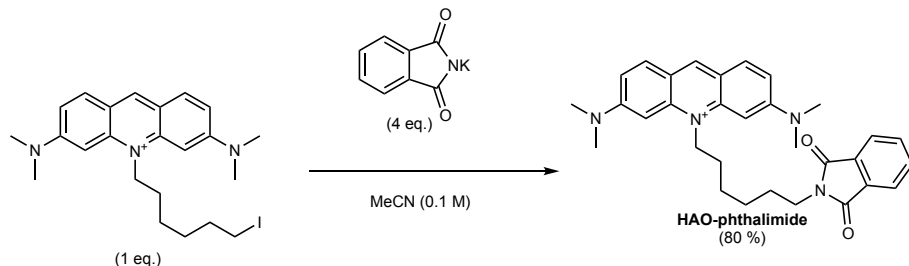
Synthesis of Hexyl Acridine Orange (HAO) derivatives



HAO-iodide – 3,6-bis(dimethylamino)-10-(6-iodohexyl)acridin-10-ium.

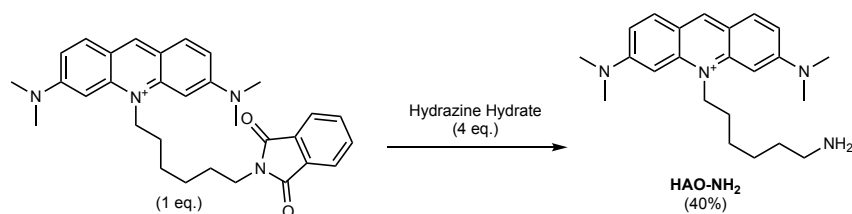
Acridine Orange free base was prepared according to the procedure published in Zheng et al⁶⁶. The free base (0.100 g, 0.377 mmol, 1.0 equiv.) and potassium carbonate (0.062 g, 0.452 mmol, 1.2 equiv.) were combined, and the vial was sealed and filled with nitrogen. Anhydrous acetonitrile (7.54 mL, 0.05 M) was added, and the mixture was stirred for 15 min to equilibrate. Diiodo-hexane (1.274 g, 0.621 mL, 3.768 mmol, 10.0 equiv.) was then added to the mixture dropwise. The reaction mixture was stirred at 85 °C for 18 h. To determine the reaction end point, LCMS monitoring confirmed complete consumption of AO. The reaction mixture was filtered and concentrated under vacuum.

The compound was purified by flash chromatography on neutral alumina (0–10% MeOH in DCM), with an isocratic hold upon elution of the product. **HAO-iodide** (124 mg, 68%) was isolated as a waxy orange solid. ^1H NMR (600 MHz, CDCl_3) δ 8.71 (s, 1H), 7.90 (d, J = 9.2 Hz, 2H), 7.03 (dd, J = 9.3, 2.1 Hz, 2H), 6.63 (d, J = 2.2 Hz, 2H), 4.90 (t, J = 8.1 Hz, 2H), 3.33 (s, 12H), 3.24 (t, J = 6.8 Hz, 2H), 1.98 (p, J = 7.6 Hz, 2H), 1.87 (h, J = 7.1 Hz, 2H), 1.75 (p, J = 7.6 Hz, 2H), 1.60 (d, J = 9.9 Hz, 6H). ^{13}C NMR (151 MHz, CDCl_3) δ 155.70, 143.09, 142.80, 133.40, 117.30, 114.23, 93.15, 48.46, 41.45, 33.13, 30.51, 26.26, 26.21, 7.78.



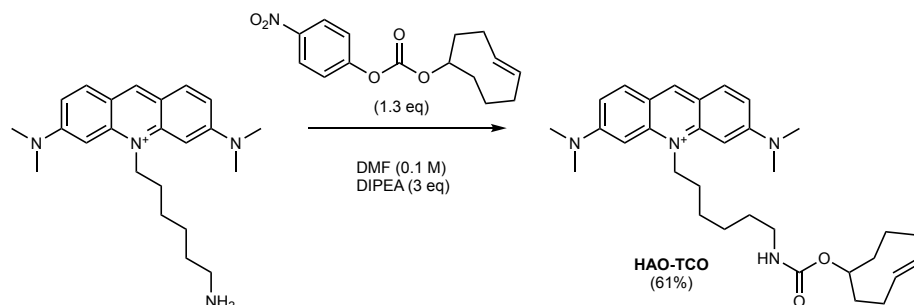
HAO-phthalimide – 3,6-bis(dimethylamino)-10-(6-(1,3-dioxoisindolin-2-yl)hexyl)acridin-10-ium.

HAO-iodide (0.382 g, 0.802 mmol, 1.0 equiv.) was added to microwave vial and dried overnight under vacuum with gentle heating. This additional step of drying is not necessary, but does result in a higher yield. Potassium phthalimide (0.594 g, 3.207 mmol, 4.0 equiv.) was added, the vial was sealed, filled with nitrogen. Anhydrous acetonitrile (8.02 mL, 0.1 M) was then added, and the reaction mixture was stirred at 60°C. LCMS monitoring showed reaction completion after 18 h. The reaction mixture was cooled and concentrated under vacuum. The crude was resuspended in DCM and the organic fraction was washed with water. The organic layer was then dried over anhydrous Na_2SO_4 , filtered, and concentrated under vacuum. The crude was purified using flash chromatography on neutral alumina (0–5% MeOH/DCM). **HAO-phthalimide** was isolated as an orange solid (0.40 g, 80% yield). ^1H NMR (500 MHz, CDCl_3) δ 8.70 (s, 1H), 7.87 (d, J = 9.3 Hz, 2H), 7.76 (dd, J = 5.4, 3.1 Hz, 2H), 7.68 (dd, J = 5.5, 3.0 Hz, 2H), 7.00 (dd, J = 9.3, 2.1 Hz, 2H), 6.49 (d, J = 2.2 Hz, 2H), 4.73 (t, J = 8.0 Hz, 2H), 3.64 (t, J = 7.0 Hz, 2H), 3.28 (s, 11H), 1.91 (d, J = 16.0 Hz, 2H), 1.74 – 1.63 (m, 5H), 1.42 (qd, J = 9.7, 4.5 Hz, 2H). ^{13}C NMR (151 MHz, CDCl_3) δ 155.70, 143.09, 142.80, 133.40, 117.30, 114.23, 93.15, 48.46, 41.45, 33.13, 30.51, 26.26, 26.21, 7.78.



HAO-amine – 10-(6-aminohexyl)-3,6-bis(dimethylamino)acridin-10-ium.

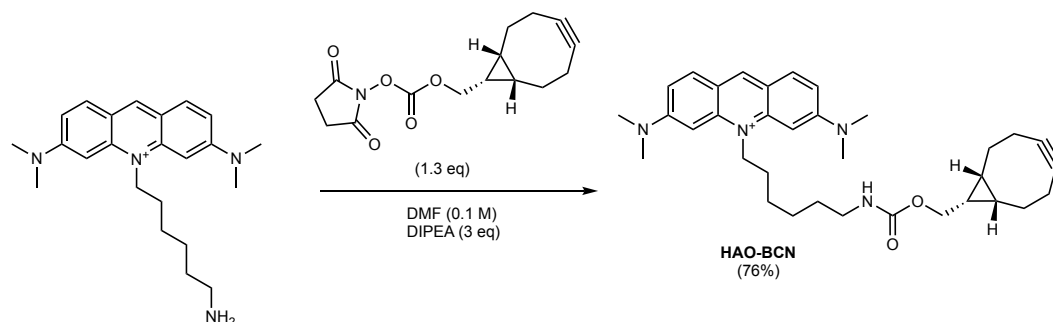
To a stirred solution of **HAO-phthalimide** (0.120 g, 0.242 mmol) in ethanol (min volume to dissolve), hydrazine hydrate—60% v/v—(0.048 g, 0.079 mL, 0.968 mmol, 4.0 equiv.) was added. The reaction was stirred overnight, and completion was confirmed by LCMS. Additionally, a white precipitate starting forming. The reaction mixture was filtered and purified directly via reverse phase HPLC (0-50% MeCN in Water, with 0.1% TFA additive), yielding the **HAO-amine** (36 mg, 40%) as an orange solid. ^1H NMR (600 MHz, MeOD) δ 8.66 (s, 1H), 7.91 (d, J = 9.3 Hz, 2H), 7.28 (dd, J = 9.3, 2.2 Hz, 2H), 6.68 (d, J = 2.4 Hz, 2H), 4.74 – 4.69 (m, 2H), 3.33 (s, 12H), 2.95 (t, J = 7.7 Hz, 2H), 2.08 – 2.00 (m, 2H), 1.77 – 1.66 (m, 4H), 1.57 (dddd, J = 14.8, 9.4, 6.6, 3.5 Hz, 2H). ^{13}C NMR (151 MHz, MeOD) δ 157.43, 144.35, 144.13, 134.39, 118.58, 115.57, 93.64, 48.34, 40.79, 40.57, 28.57, 27.63, 27.32, 26.97.



HAO-TCO – (E)-10-(6-(((cyclooct-3-en-1-yloxy)carbonyl)amino)hexyl)-3,6-bis(dimethylamino)acridin-10-ium.

HAO-amine (0.010 g, 0.021 mmol, 1.0 equiv.) was dissolved in anhydrous DMF (0.209 mL) and DIPEA (11 μL , 0.063 mmol, 3.0 equiv.) was added. The mixture was stirred and equilibrated for 5 min. TCO-PNB (8 mg, 0.027 mmol, 1.3 equiv.) was added to the reaction in one portion. The reaction was stirred at room temperature and monitored by LCMS until completion (between 1-3 hr). Upon completion, the mixture was concentrated under vacuum and purified via reverse phase HPLC (5-95% MeCN in Water, with 0.1% TFA additive), yielding the **HAO-TCO** (8 mg, 61%) as an orange solid. ^1H NMR (600 MHz, MeOD) δ 8.68 (s, 1H), 7.93 (d, J = 9.3 Hz, 2H), 7.30 (dd, J = 9.3, 2.2 Hz, 2H), 6.70 (d, J = 2.3 Hz, 2H), 5.71 – 5.52 (m, 1H), 5.44 (ddd, J = 15.7, 11.1, 3.6 Hz, 1H), 4.70 (d, J = 16.7 Hz, 1H), 4.29 (d, J = 8.3 Hz, 0H), 3.34 (s, 11H), 3.14 – 3.04 (m, 2H), 2.29 (qd, J = 10.9, 4.8 Hz, 2H), 2.07 – 1.98 (m, 3H), 1.98 – 1.90 (m, 3H), 1.85 (s, 1H), 1.74 – 1.63 (m, 5H), 1.60 – 1.49 (m, 6H). ^{13}C NMR (151 MHz, MeOD) δ 157.43, 144.35, 144.13, 134.39,

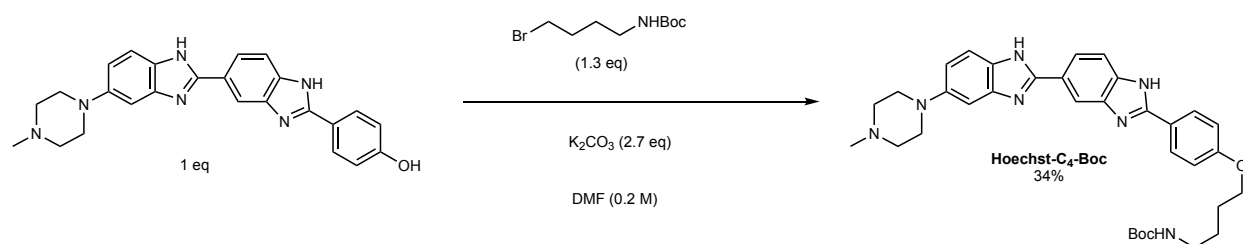
118.58, 115.57, 93.64, 48.34, 40.79, 40.57, 28.57, 27.63, 27.32, 26.97. HRMS (ES+) calcd for (C₃₂H₄₅N₄O₂⁺) [M⁺] 517.3538, found 517.34403



HAO-BCN – 10-(6-((((1R,8S,9S)-bicyclo[6.1.0]non-4-yn-9-yl)methoxycarbonyl)amino) hexyl) -3,6-bis(dimethylamino)acridin-10-ium.

HAO-amine (0.010 g, 0.021 mmol, 1.0 equiv.) was dissolved in anhydrous DMF (0.209 mL) and DIPEA (11 µL, 0.063 mmol, 3.0 equiv.) was added. The mixture was stirred and equilibrated for 5 min. BCN-NHS ester (8 mg, 0.027 mmol, 1.3 equiv.) was added to the reaction in one portion. The reaction was stirred at room temperature and monitored by LCMS until completion (1 hr). Upon completion, the mixture was concentrated under vacuum and purified via reverse phase HPLC (5-95% MeCN gradient in water), yielding the **HAO-BCN** (10 mg, 76%) as an orange solid. ¹H NMR (600 MHz, MeOD) δ 8.68 (s, 1H), 7.93 (d, *J* = 9.2 Hz, 2H), 7.32 – 7.27 (m, 2H), 6.70 (s, 2H), 4.76 – 4.63 (m, 2H), 4.12 (d, *J* = 8.2 Hz, 2H), 3.35 (s, 1H), 3.34 (s, 11H), 3.13 (q, *J* = 8.4 Hz, 2H), 2.91 (d, *J* = 13.2 Hz, 1H), 2.33 – 2.13 (m, 5H), 2.10 (d, *J* = 15.4 Hz, 2H), 2.02 (p, *J* = 7.9 Hz, 2H), 1.69 (p, *J* = 7.5 Hz, 2H), 1.61 – 1.51 (m, 4H), 1.41 – 1.24 (m, 2H), 0.90 (t, *J* = 9.6 Hz, 2H). ¹³C NMR (151 MHz, MeOD) δ 156.04, 142.93, 142.76, 132.98, 117.20, 114.20, 98.08, 92.24, 62.15, 39.91, 39.42, 29.42, 28.76, 26.08, 25.91, 25.49, 20.48, 19.96, 17.56. HRMS (ES+) calcd for (C₃₄H₄₅N₄O₂⁺) [M⁺] 541.3538, found 541.34378

Synthesis of Hoechst derivatives

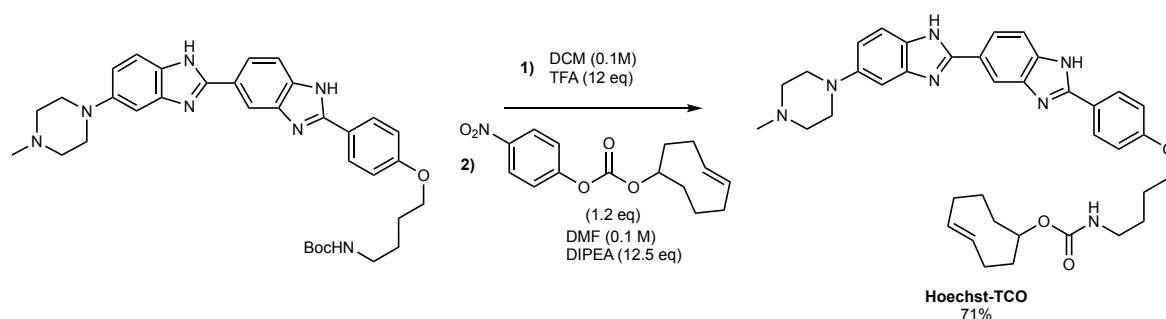


Hoechst-C₄-Boc – tert-butyl (4-(4-(5-(4-methylpiperazin-1-yl)-1H,1'H-[2,5'-bibenzo[d]imidazol]-2'-yl)phenoxy)butyl)carbamate

Following Hoechst-C₄-Boc was prepared according to the procedure published in Lukinavičius, G. *et al*⁴³. Hoechst 33258 hydrochloride salt was treated to isolate Hoechst free base—the free base was used to prepare Hoechst-C₄-Boc.

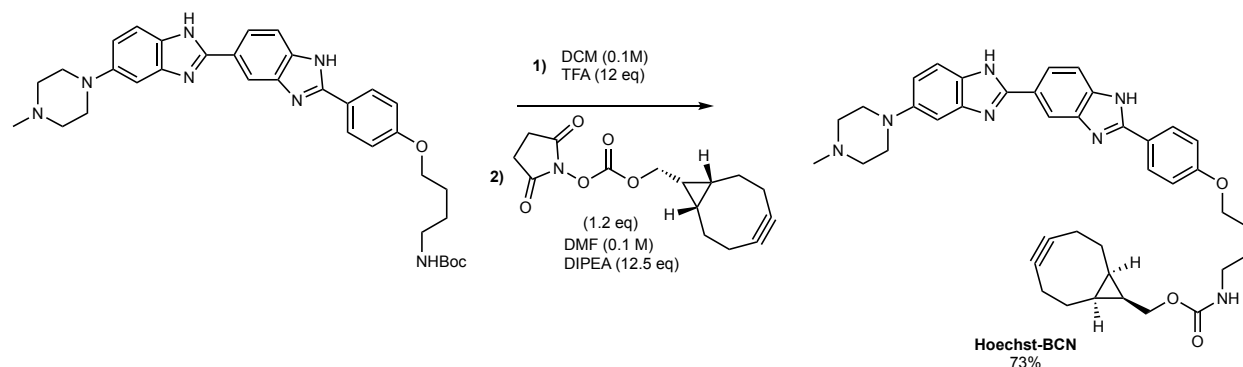
Hoechst free base (0.100 g, 0.236 mmol, 1.0 equiv.) and K₂CO₃ (0.088 g, 0.636 mmol, 2.7 equiv.) were combined in a reaction vial and sealed. The vial was filled with N₂, then anhydrous DMF (1.18 mL, 0.2 M) was added, and the mixture was stirred for 30 min to equilibrate. 4-(Boc-amino)butyl bromide (0.077 g, 0.306 mmol, 1.3 equiv.) was added to the vial in portions (sticky white solid which can start to melt by touch). The reaction was heated to 60°C, and stirred overnight. Reaction progress was monitored by LCMS. The crude was diluted in 5:5:1 MeCN:H₂O:AcOH, then purified by reverse phase HPLC, affording the desired product as a TFA salt (75 mg, 34%). ¹H NMR (500 MHz, MeOD) δ 8.38 – 8.34 (m, 1H), 8.11 – 8.02 (m, 3H), 7.91 (d, *J* = 8.6 Hz, 1H), 7.69 (d, *J* = 9.0 Hz, 1H), 7.36 (dd, *J* = 9.1, 2.3 Hz, 1H), 7.29 (d, *J* = 2.3 Hz, 1H), 7.12 (d, *J* = 8.6 Hz, 2H), 4.05 (t, *J* = 6.3 Hz, 2H), 3.94 (d, *J* = 13.1 Hz, 2H), 3.69 (d, *J* = 11.7 Hz, 2H), 3.34 (d, *J* = 9.6 Hz, 2H), 3.21 (d, *J* = 13.2 Hz, 2H), 3.12 (t, *J* = 6.9 Hz, 2H), 3.01 (s, 3H), 1.81 (dq, *J* = 12.0, 6.6 Hz, 2H), 1.65 (p, *J* = 7.1 Hz, 2H), 1.45 (s, 9H). ¹³C NMR (126 MHz, MeOD) δ 164.70, 162.30, 162.00, 158.62, 154.80, 150.74, 149.70, 138.49, 136.32, 134.60, 130.88, 128.07, 125.07, 120.53, 119.53, 118.85, 117.42, 116.75, 116.45, 115.65, 114.93, 100.91, 79.94, 69.34, 54.57, 49.63, 49.45, 49.28, 48.38, 43.57, 41.00, 28.80, 27.59, 27.46.

Note: trying to push the reaction to completion results in a side product with a double alkylation (N-alkylation is seen)—our preference was to stop the reaction while the desired produce was the dominant product. Unreacted Hoechst was reused for scale-ups



Hoechst-TCO – (E)-cyclooct-4-en-1-yl (4-(4-(5-(4-methylpiperazin-1-yl)-1H,1'H-[2,5'-bibenzo[d]imidazol]-2'-yl)phenoxy)butyl)carbamate

Hoechst-C₄-Boc (0.020 g, 0.021 mmol) was dissolved in dichloromethane (0.213 mL) and TFA (0.020 mL, 0.267 mmol, 12.5 equiv.). The reaction mixture was stirred at room temperature and monitored by LCMS to determine deprotection. Once completely deprotected, the solvent was evaporated, and the crude was dried under vacuum for 1-3 hrs. The deprotected amine intermediate was dissolved in DMF (0.213 mL, 0.1 M), and DIPEA (46 μ L, 0.267 mmol, 12.5 equiv.) was added, and the solution was stirred for 5 min to equilibrate. TCO-PNB ester (7 mg, 0.026 mmol, 1.2 equiv.) was dissolved in DMF (0.213 mL) and added dropwise via syringe. The reaction was stirred at room temperature and monitored by LCMS until completion (4 h). The mixture was concentrated under vacuum, dissolved in 50:50 Water/Acetonitrile with 0.1% Formic acid, and purified via reverse phase HPLC (5-95% MeCN in water with 0.1% TFA additive) to yield **Hoechst-TCO** as a yellow solid (15 mg, 71%). ¹H NMR (600 MHz, MeOD) δ 8.42 – 8.39 (m, 1H), 8.13 – 8.08 (m, 2H), 8.06 (dd, J = 8.5, 1.8 Hz, 1H), 7.91 (dd, J = 8.5, 0.7 Hz, 1H), 7.72 (d, J = 9.0 Hz, 1H), 7.39 (dd, J = 9.0, 2.3 Hz, 1H), 7.31 (d, J = 2.2 Hz, 1H), 7.19 – 7.14 (m, 2H), 5.60 (ddd, J = 21.1, 10.1, 5.3 Hz, 1H), 5.47 (ddd, J = 15.9, 11.0, 3.6 Hz, 1H), 4.34 – 4.28 (m, 1H), 4.11 (t, J = 6.3 Hz, 2H), 3.95 (s, 2H), 3.69 (s, 2H), 3.35 (s, 2H), 3.17 (dt, J = 8.2, 4.1 Hz, 2H), 3.02 (s, 3H), 2.33 (dh, J = 15.5, 4.9 Hz, 3H), 1.99 (s, 1H), 2.00 – 1.88 (m, 2H), 1.84 (dq, J = 8.9, 6.4 Hz, 2H), 1.78 – 1.64 (m, 4H), 1.59 (ddd, J = 15.8, 12.1, 5.7 Hz, 1H). ¹³C NMR (126 MHz, MeOD) δ 164.70, 162.30, 162.00, 158.62, 154.80, 150.74, 149.70, 138.49, 136.32, 134.60, 130.88, 128.07, 125.07, 120.53, 119.53, 118.85, 117.42, 116.75, 116.45, 115.65, 114.93, 100.91, 79.94, 69.34, 54.57, 49.63, 49.45, 49.28, 48.38, 43.57, 41.00, 28.80, 27.59, 27.46. . HRMS (ES⁺) calcd for (C₃₈H₄₅N₇O₃) [M+H⁺] 648.36566, found 648.35196

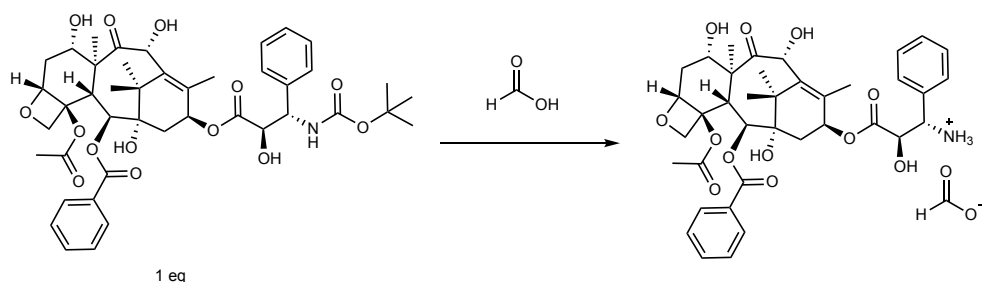


Hoechst-BCN – ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (4-(4-(5-(4-methylpiperazin-1-yl)-1H,1'H-[2,5'-bibenzo[d]imidazol]-2'-yl)phenoxy)butyl)carbamate

Hoechst-C₄-Boc (0.022 g, 0.023 mmol) was dissolved in dichloromethane (0.250 mL) and TFA (0.022 mL, 0.293 mmol, 12.5 equiv.). The reaction mixture was stirred at room temperature and monitored by LCMS to determine deprotection. Once completely deprotected, the solvent was evaporated, and the crude was dried under vacuum for 1-3 hrs. The deprotected amine intermediate was dissolved in DMF (0.235 mL, 0.1 M), and DIPEA (51 μ L, 0.293 mmol, 12.5 equiv.) was added, and the solution was stirred for 5 min to equilibrate. BCN-NHS ester (8 mg, 0.028 mmol, 1.2 equiv.) was dissolved in DMF (0.235 mL) and added dropwise via syringe. The reaction was stirred at room temperature and monitored by LCMS until completion (4 h). The mixture was concentrated under vacuum, dissolved in 50:50 Water/Acetonitrile with 0.1% Formic acid, and purified via reverse phase HPLC (5-95% MeCN in water with 0.1% TFA additive) to yield **Hoechst-BCN** as a yellow solid (18 mg, 73%). ¹H NMR (600 MHz, MeOD) δ 8.34 (d, J = 1.7 Hz, 1H), 8.09 – 8.02 (m, 3H), 7.88 (d, J = 8.5 Hz, 1H), 7.68 (d, J = 9.0 Hz, 1H), 7.35 (dd, J = 9.0, 2.2 Hz, 1H), 7.28 (d, J = 2.2 Hz, 1H), 7.11 (d, J = 8.5 Hz, 2H), 4.14 (d, J = 8.1 Hz, 2H), 4.06 (t, J = 6.4 Hz, 2H), 3.99 – 3.86 (m, 2H), 3.68 (s, 2H), 3.64 (d, J = 7.7 Hz, 2H), 3.43 – 3.32 (m, 2H), 3.19 (t, J = 6.9 Hz, 4H), 3.01 (s, 3H), 2.29 – 2.18 (m, 7H), 2.14 (ddt, J = 15.9, 4.5, 2.2 Hz, 4H), 1.86 – 1.77 (m, 3H), 1.72 – 1.64 (m, 3H), 1.64 – 1.53 (m, 4H), 1.38 (p, J = 8.7 Hz, 1H), 1.27 (tt, J = 9.2, 7.7 Hz, 1H), 0.97 – 0.84 (m, 3H). ¹³C NMR (126 MHz, MeOD) δ 164.70, 162.30, 162.00, 158.62, 154.80, 150.74, 149.70, 138.49, 136.32, 134.60, 130.88, 128.07, 125.07, 120.53, 119.53, 118.85, 117.42, 116.75, 116.45, 115.65, 114.93, 100.91, 79.94, 69.34, 54.57, 49.63, 49.45, 49.28, 48.38, 43.57, 41.00, 28.80, 27.59, 27.46. HRMS (ES⁺) calcd for (C₄₀H₄₅N₇O₃) [M+H⁺] 672.36566, found 672.35274

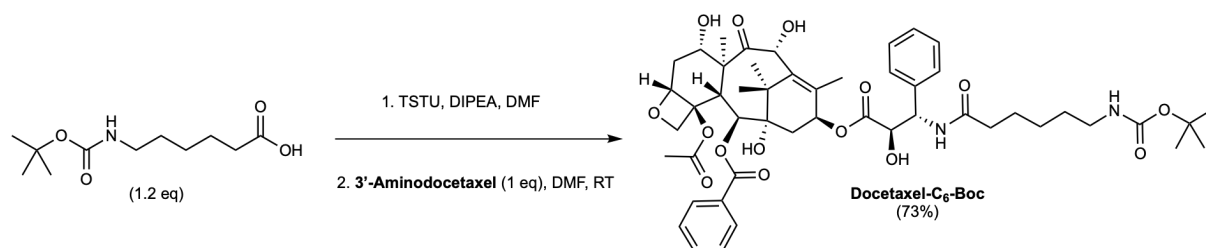
Synthesis of Docetaxel Ligands

Synthesis of Docetaxel ligands was adapted from Beliu, G. *et al*⁷⁶.



3'-Aminodocetaxel

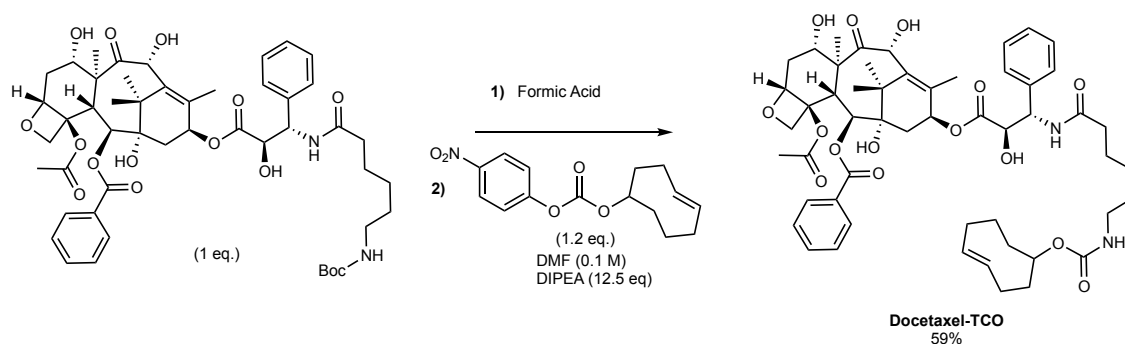
No inert conditions required. Docetaxel (100 mg, 0.124 mmol) was dissolved in Formic Acid (2 mL, 0.060 M). The vial was agitated till all the solid was dissolved, then allowed to sit at room temperature. Deprotection was monitored by LC/MS, and total conversion was achieved in 30 min. Formic acid was removed by evaporation, then the crude was dissolved in water, flash frozen and lyophilized. The formate salt was isolated as a white solid (quantitative yield), and used without further purification.



Docetaxel-C₆-Boc

Boc-aminocaproic acid (0.019 g, 0.084 mmol, 1.2 equiv.) and TSTU (0.027 g, 0.091 mmol, 1.3 equiv.) were combined in a dry microwave vial. DMF (0.702 mL, 0.100 M) and DIPEA (53 μ L, 0.302 mmol, 4.3 equiv.) were added, and the reaction mixture stirred under nitrogen. Formation of the NHS-ester intermediate was monitored by LCMS (complete conversion in 30 min). **3'-Aminodocetaxel** (0.053 g, 0.070 mmol, 1.0 equiv.) was dissolved separately in DMF (250 μ L), then added dropwise to the NHS-ester mixture and stirred at room temperature. LCMS monitoring showed complete conversion after 1 hour. The reaction mixture was concentrated under vacuum, dissolved in 50/50 MeCN/H₂O with 0.1% formic acid, and purified by reverse-phase HPLC (gradient: 5–95% acetonitrile in water with 0.1% TFA), providing the **Docetaxel-C₆-Boc** (43 mg, 71% yield) as a white solid. ¹H NMR (500 MHz, MeOD) δ 8.14 – 8.06 (m, 2H), 7.70 – 7.62 (m, 1H), 7.60 – 7.53 (m, 2H), 7.45 – 7.35 (m, 4H), 7.28 (qt, J = 6.5, 1.7 Hz, 1H), 6.16 (td, J = 9.1, 1.7 Hz, 1H), 5.65 (d, J = 7.2 Hz, 1H), 5.52 – 5.42 (m, 1H), 5.27 (s, 1H), 4.99 (dd, J = 9.7, 2.2 Hz, 1H), 4.59 (dd, J = 7.7, 4.5 Hz, 1H), 4.26 – 4.18 (m, 2H), 4.19 (d, J = 8.4 Hz, 1H), 3.87 (d, J = 7.2 Hz, 1H), 2.98 (t, J = 7.1 Hz, 2H), 2.44 (ddd, J = 14.4, 9.7, 6.5 Hz, 1H), 2.33 (d, J = 4.1

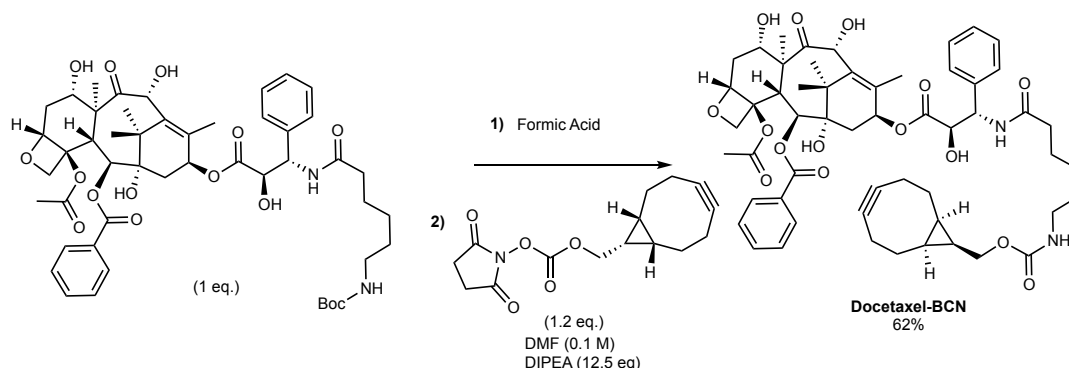
Hz, 3H), 2.32 – 2.14 (m, 3H), 2.05 – 1.93 (m, 1H), 1.90 (t, $J = 1.8$ Hz, 3H), 1.83 (ddd, $J = 13.9, 11.3, 2.3$ Hz, 1H), 1.70 (s, 3H), 1.68 – 1.53 (m, 3H), 1.44 (d, $J = 7.4$ Hz, 1H), 1.42 (s, 10H), 1.31 (dt, $J = 14.9, 7.2, 4.1$ Hz, 2H), 1.19 (s, 3H), 1.13 (d, $J = 3.8$ Hz, 3H). ^{13}C NMR (126 MHz, MeOD) δ 209.67, 174.50, 173.03, 170.40, 166.27, 159.25, 158.94, 157.12, 138.73, 137.81, 136.58, 133.13, 130.03, 129.79, 128.29, 128.25, 127.47, 127.01, 84.51, 80.93, 78.41, 77.68, 76.19, 75.02, 74.24, 73.45, 71.26, 71.13, 57.46, 55.46, 48.21, 48.04, 47.87, 46.46, 43.13, 39.76, 39.10, 36.09, 35.49, 35.41, 29.22, 27.38, 26.01, 25.67, 25.47, 25.34, 21.81, 20.26, 13.00, 9.05.



Docetaxel-TCO

Docetaxel-C₆-Boc (0.010 g (mass calc. for TFA salt), 0.010 mmol, 1 equiv.) was dissolved in formic acid (0.5 mL) and the reaction stirred at room temperature until LCMS confirmed deprotection. The crude mixture was concentrated and dried overnight under high vacuum. The deprotected amine intermediate was dissolved in DMF (97 μL , 0.1 M) and DIPEA (21 μL , 0.121 mmol, 12.5 equiv.) was added—the solution was equilibrated for 15 min. TCO-PNB ester (3 mg, 0.012 mmol, 1.2 equiv.) in DMF (97 μL) was then added dropwise via syringe. The reaction was stirred at room temperature and monitored by LCMS until complete (4 h). The reaction mixture was concentrated under vacuum. The crude was resuspended in 1:1:1 MeOH/H₂O/MeCN, and purified via reverse phase HPLC (5-95% MeCN in water with 0.1% TFA additive), yielding **Docetaxel-TCO** (5.6 mg, 59%) as a white solid. ^1H NMR (600 MHz, MeOD) δ 8.14 – 8.09 (m, 2H), 7.69 – 7.63 (m, 1H), 7.56 (t, $J = 7.8$ Hz, 2H), 7.44 – 7.34 (m, 4H), 7.28 (tt, $J = 6.9, 1.7$ Hz, 1H), 6.16 (t, $J = 9.0$ Hz, 1H), 5.65 (d, $J = 7.2$ Hz, 1H), 5.58 (ddd, $J = 15.3, 9.9, 4.9$ Hz, 1H), 5.51 – 5.42 (m, 2H), 5.27 (s, 1H), 4.99 (dd, $J = 9.7, 2.2$ Hz, 1H), 4.58 (d, $J = 4.6$ Hz, 1H), 4.28 (s, 1H), 4.25 – 4.17 (m, 3H), 3.88 (d, $J = 7.2$ Hz, 1H), 3.00 (dt, $J = 10.8, 6.6$ Hz, 2H), 2.44 (ddd, $J = 14.4, 9.7, 6.5$ Hz, 1H), 2.34 (d, $J = 0.8$ Hz, 3H), 2.33 – 2.21 (m, 7H), 2.05 – 1.96 (m, 2H), 1.96 – 1.88 (m, 5H), 1.83 (ddd, $J = 13.9, 11.3, 2.3$ Hz, 1H), 1.70 (s, 3H), 1.68 – 1.62 (m, 1H), 1.59 (dd, $J = 16.2, 8.9$ Hz, 3H), 1.48 – 1.39 (m, 2H), 1.35 – 1.26 (m, 2H), 1.19 (s, 3H), 1.13 (s, 3H). ^{13}C NMR (151 MHz, MeOD) δ 209.73, 170.42, 166.31, 137.82, 136.62, 134.68, 133.14, 132.37, 130.06, 129.80, 128.30, 128.26, 127.47, 127.02, 84.54, 80.98, 80.15, 77.71, 76.23, 75.06, 74.26, 73.44, 71.28, 71.18, 57.50, 55.44, 46.49, 43.16,

40.83, 38.24, 36.12, 35.47, 33.77, 32.09, 30.71, 29.24, 25.96, 25.72, 25.29, 21.83, 20.29, 13.01, 9.07. HRMS (ES⁺) calcd for (C₅₃H₆₈N₂O₁₅) [M+H⁺] 973.46924, found 973.45095



Docetaxel-BCN

Docetaxel-C₆-Boc (0.010 g (mass calc. for TFA salt), 0.010 mmol, 1 equiv.) was dissolved in formic acid (0.5 mL) and the reaction stirred at room temperature until LCMS confirmed deprotection. The crude mixture was concentrated and dried overnight under high vacuum. The deprotected amine intermediate was dissolved in DMF (97 μ L, 0.1 M) and DIPEA (21 μ L, 0.121 mmol, 12.5 equiv.) was added—the solution was equilibrated for 15 min. BCN-NHS ester (3 mg, 0.012 mmol, 1.2 equiv.) in DMF (97 μ L) was then added dropwise via syringe. The reaction was stirred at room temperature and monitored by LCMS until complete (4 h). The reaction mixture was concentrated under vacuum. The crude was resuspended in 1:1:1 MeOH/H₂O/MeCN, and purified via reverse phase HPLC (5-95% MeCN in water with 0.1% TFA additive), yielding **Docetaxel-BCN** (6.0 mg, 59%) as a white solid. ¹H NMR (600 MHz, MeOD) δ 8.14 – 8.09 (m, 2H), 7.66 (ddt, J = 7.9, 6.9, 1.3 Hz, 1H), 7.60 – 7.53 (m, 2H), 7.44 – 7.36 (m, 4H), 7.31 – 7.25 (m, 1H), 6.16 (t, J = 8.9 Hz, 1H), 5.65 (d, J = 7.2 Hz, 1H), 5.46 (d, J = 4.6 Hz, 1H), 5.27 (s, 1H), 4.99 (dd, J = 9.8, 2.2 Hz, 1H), 4.59 (d, J = 4.5 Hz, 1H), 4.25 – 4.17 (m, 3H), 4.11 (d, J = 8.1 Hz, 2H), 3.88 (d, J = 7.1 Hz, 1H), 3.05 (t, J = 7.0 Hz, 2H), 2.44 (ddd, J = 14.4, 9.7, 6.5 Hz, 1H), 2.34 (s, 3H), 2.33 – 2.24 (m, 4H), 2.22 (ddt, J = 14.0, 10.3, 2.1 Hz, 5H), 2.20 – 2.13 (m, 3H), 2.08 – 1.99 (m, 2H), 1.90 (d, J = 1.4 Hz, 3H), 1.83 (ddd, J = 13.9, 11.3, 2.4 Hz, 1H), 1.70 (s, 3H), 1.62 (p, J = 7.4 Hz, 4H), 1.49 – 1.42 (m, 2H), 1.42 – 1.28 (m, 3H), 1.19 (s, 3H), 1.13 (s, 3H), 0.95 – 0.89 (m, 2H). ¹³C NMR (151 MHz, MeOD) δ 211.12, 174.45, 171.83, 167.71, 140.18, 139.23, 138.03, 134.54, 131.47, 131.21, 129.70, 129.66, 128.87, 128.42, 99.53, 85.94, 82.39, 79.11, 77.63, 76.48, 75.67, 74.85, 72.68, 72.60, 63.57, 58.90, 56.85, 49.57, 47.90, 44.56, 41.53, 37.52, 36.89, 30.16, 27.36, 27.10, 26.70, 23.22, 21.93, 21.69, 21.38, 18.97, 14.41, 10.46. HRMS (ES⁺) calcd for (C₅₅H₆₈N₂O₁₅) [M+H⁺] 997.46924, found 997.44931

Synthesis of HD555

HD555 was prepared according to the previously published procedure in Werther, P. *et al.*⁸⁴

Characterization data matched with previously published data: ¹H NMR (400 MHz, MeOD) δ 7.98 (dd, J = 7.7, 1.2 Hz, 1H), 7.81 – 7.68 (m, 2H), 7.20 (d, J = 9.5 Hz, 2H), 7.02 (dd, J = 9.5, 2.5 Hz, 2H), 6.92 (d, J = 2.5 Hz, 2H), 5.65 (s, 2H), 3.30 (s, 11H), 2.94 (s, 3H). HRMS (ES+) calcd for (C₂₈H₂₇N₆O₄) [M⁺] 511.20883, found 511.20933

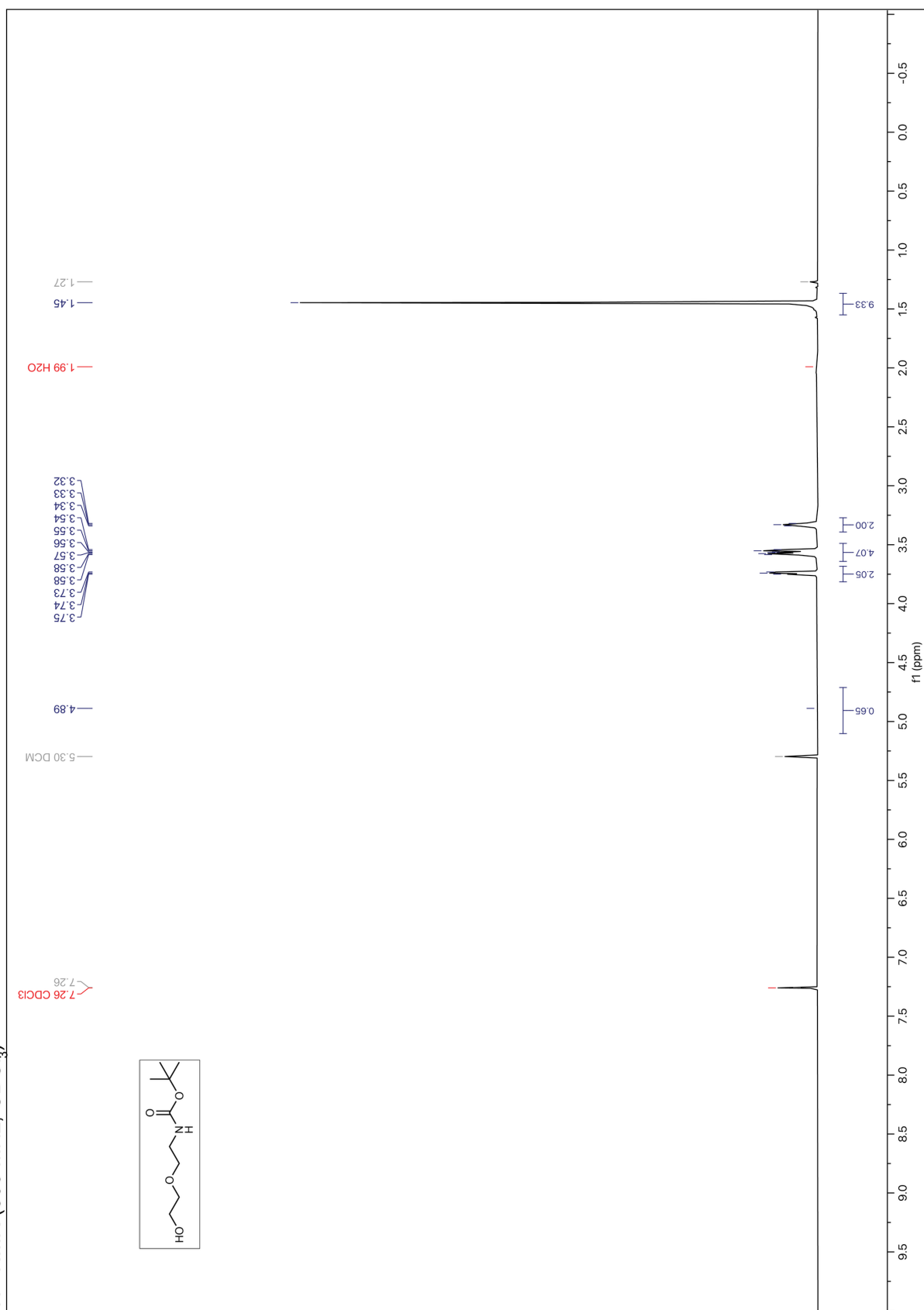
References

82. Singh, V., Wang, S. & Kool, E. T. Genetically Encoded Multispectral Labeling of Proteins with Polyfluorophores on a DNA Backbone. *Journal of the American Chemical Society* **135**, 6184–6191 (2013).
83. Chen, W., Guo, C., Ding, H., Yang, X. & Zhang, K. Controlled Ring-Opening Polymerization of Macrocyclic Monomers Based on Ring-Opening/Ring-Closing Cascade Reaction. *J. Am. Chem. Soc.* jacs.3c10765 (2023) doi:10.1021/jacs.3c10765.
84. Werther, P. *et al.* Bio-orthogonal Red and Far-Red Fluorogenic Probes for Wash-Free Live-Cell and Super-resolution Microscopy. *ACS Central Science* **7**, 1561–1571 (2021).

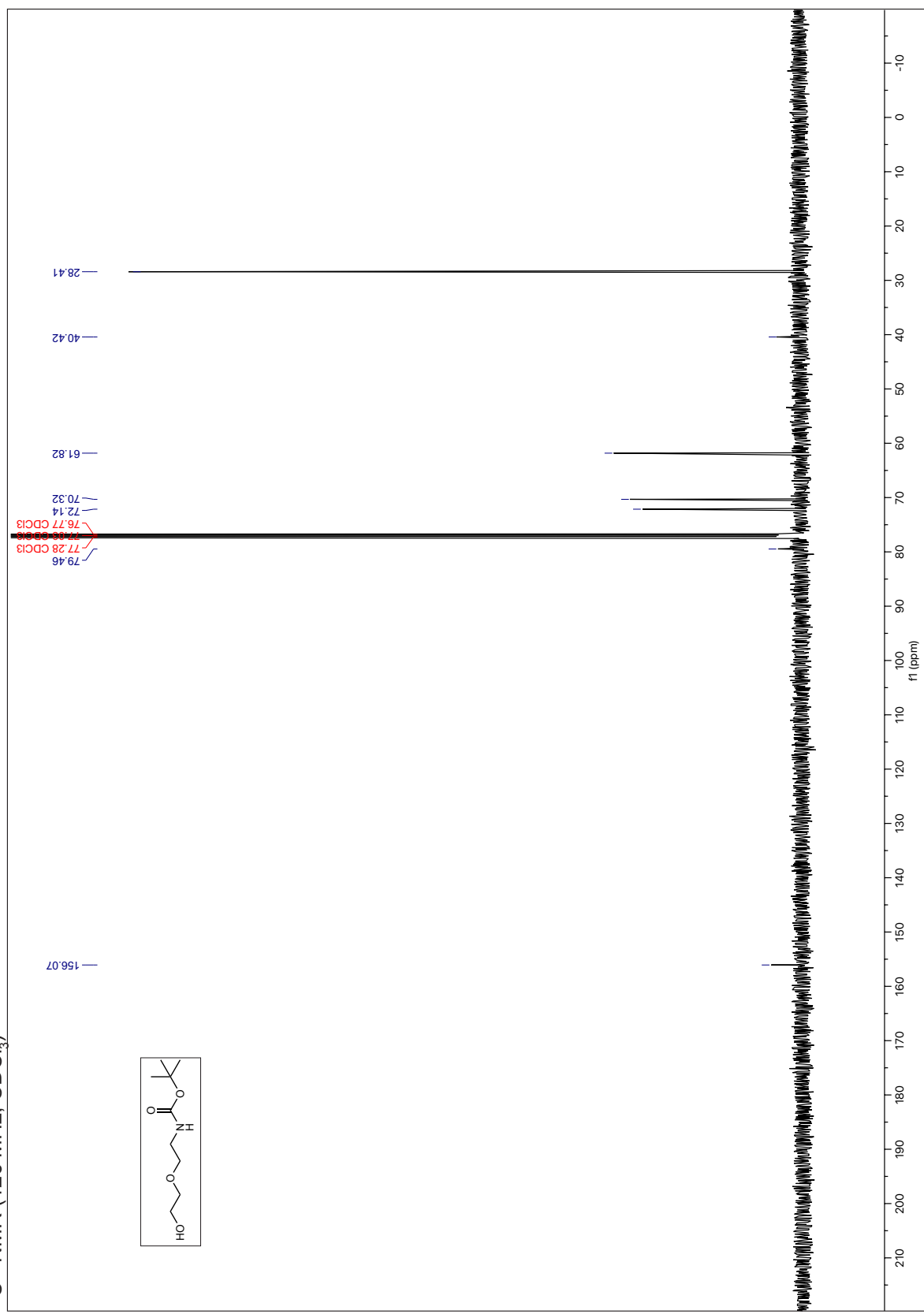
NMR Spectra

(See below)

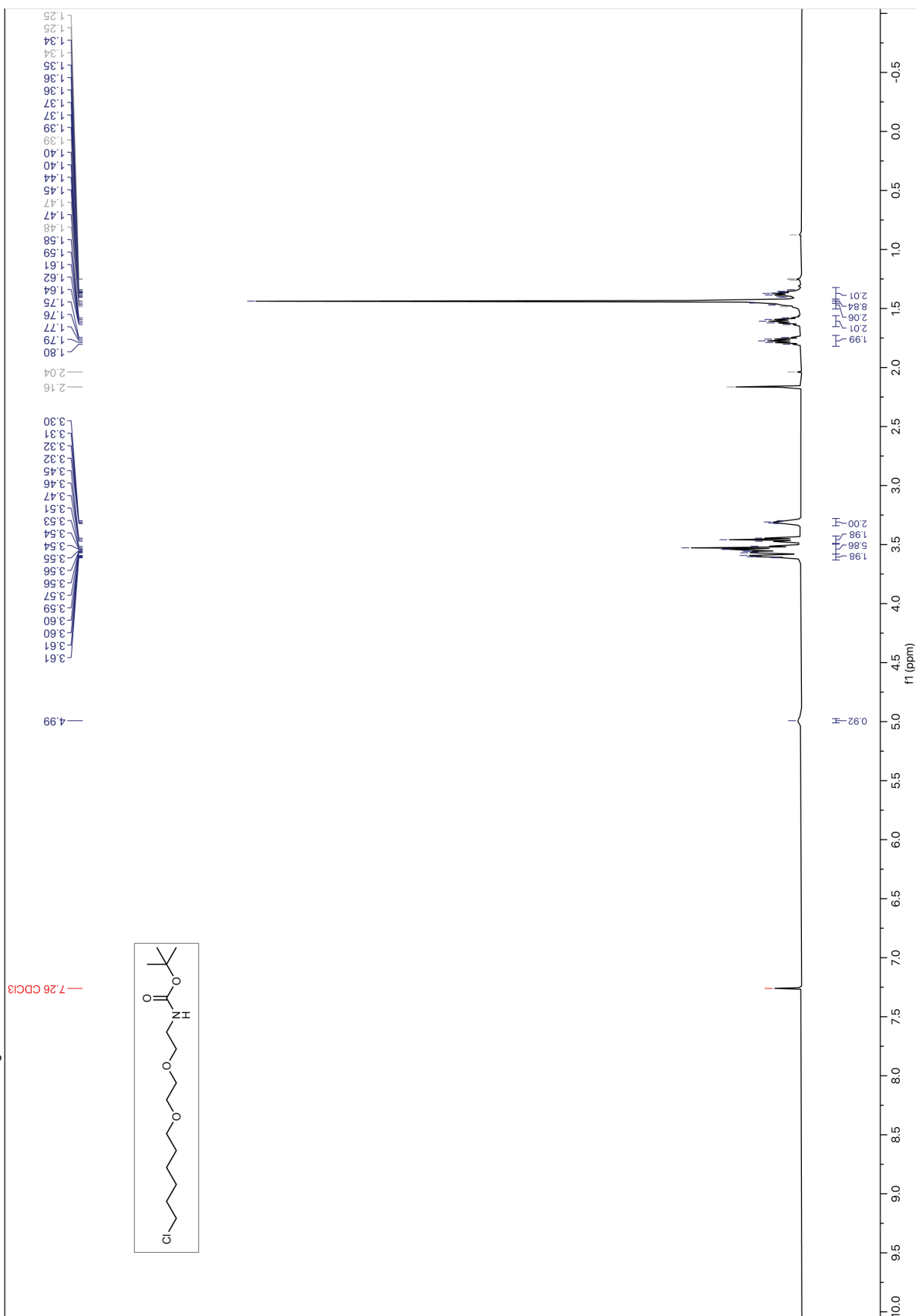
¹H - NMR (500 MHz, CDCl₃)



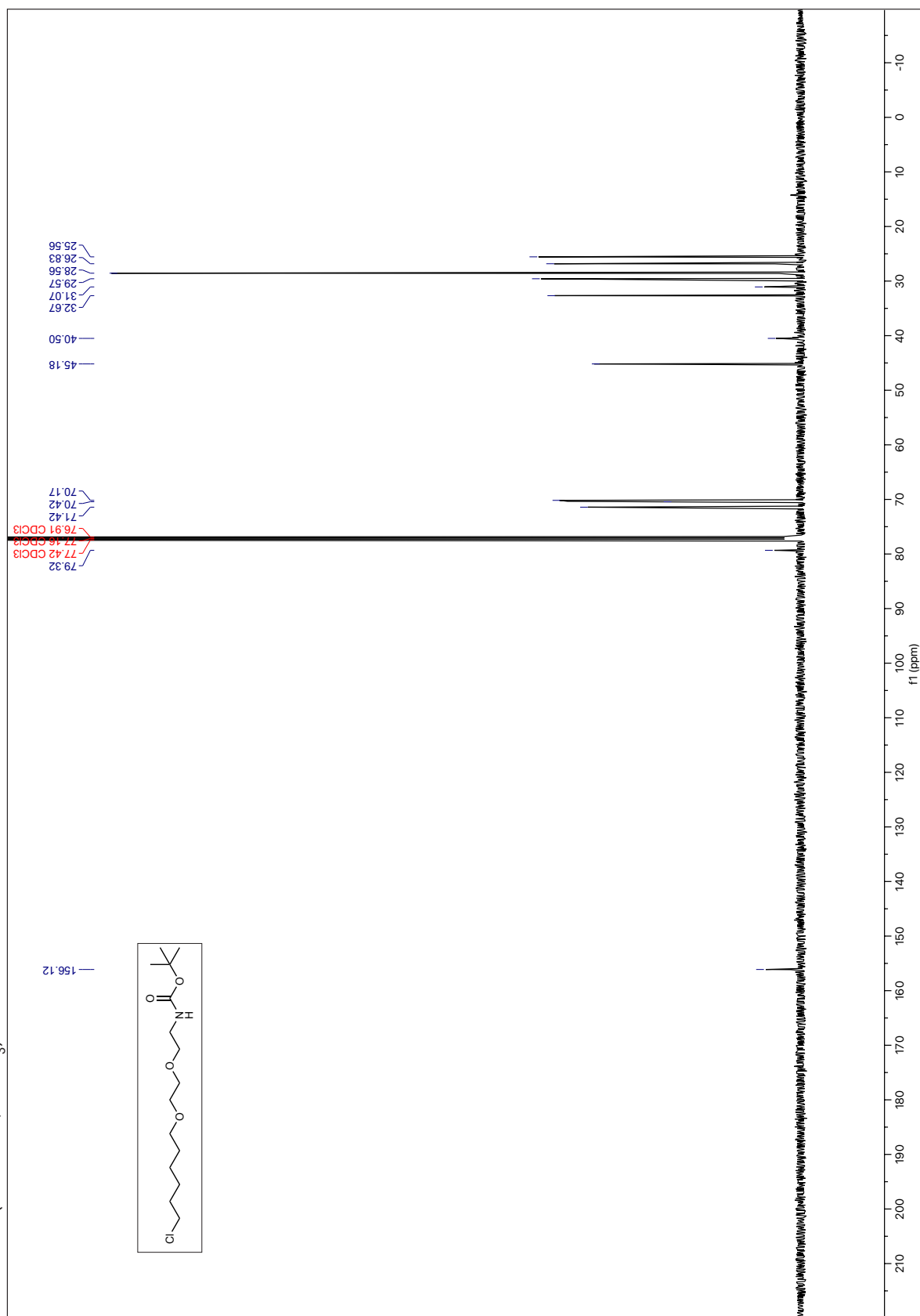
¹³C - NMR (126 MHz, CDCl₃)



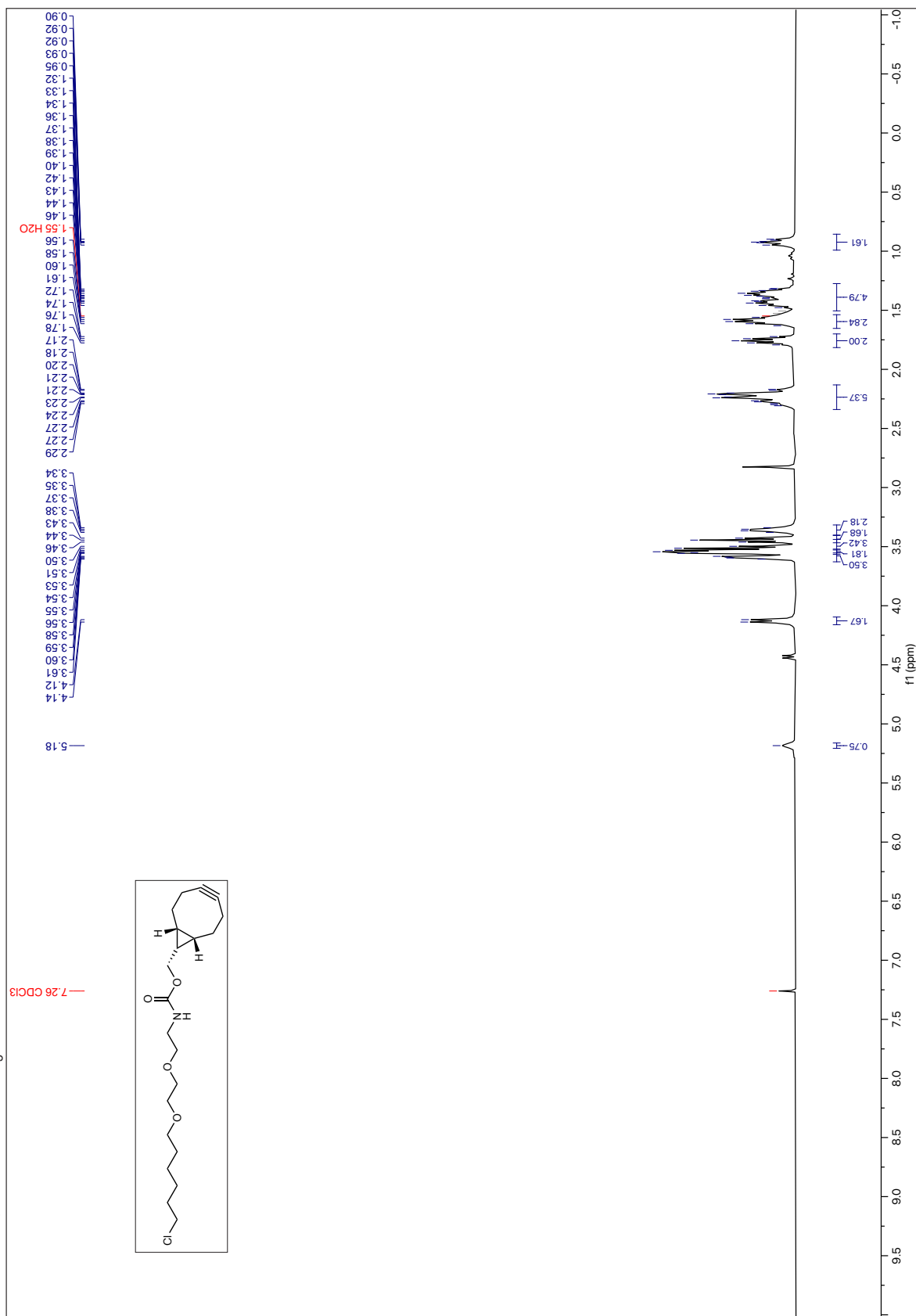
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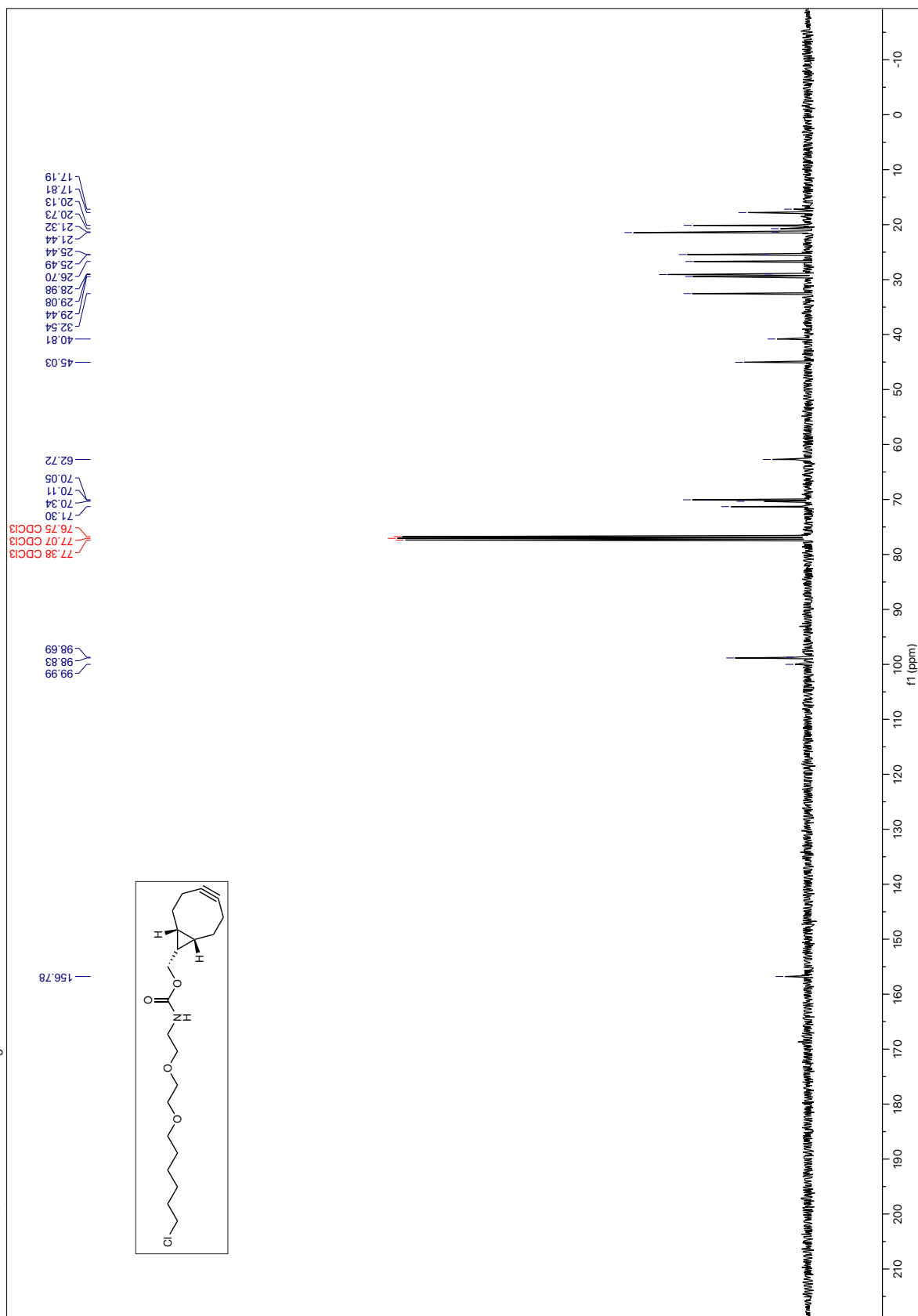
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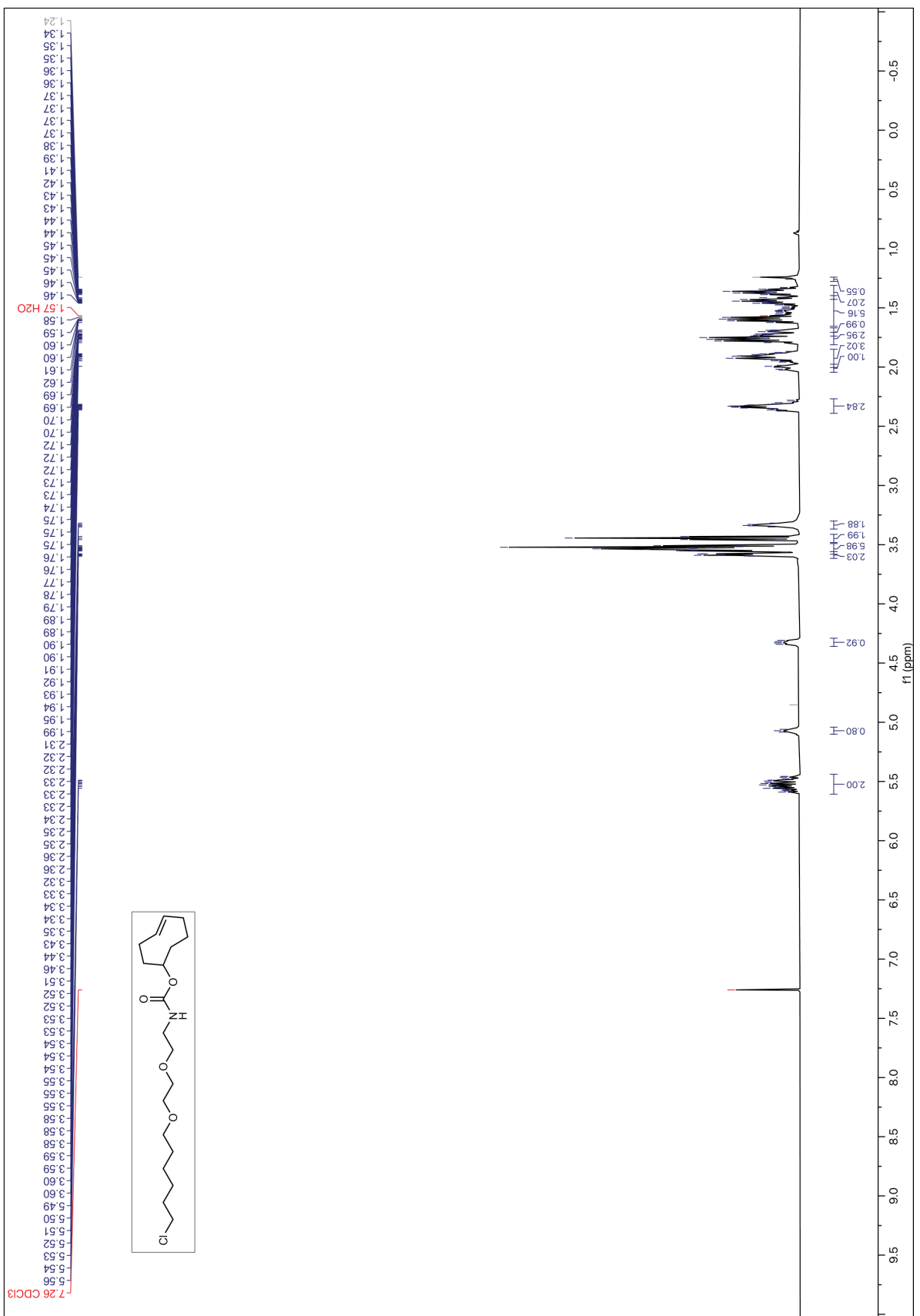
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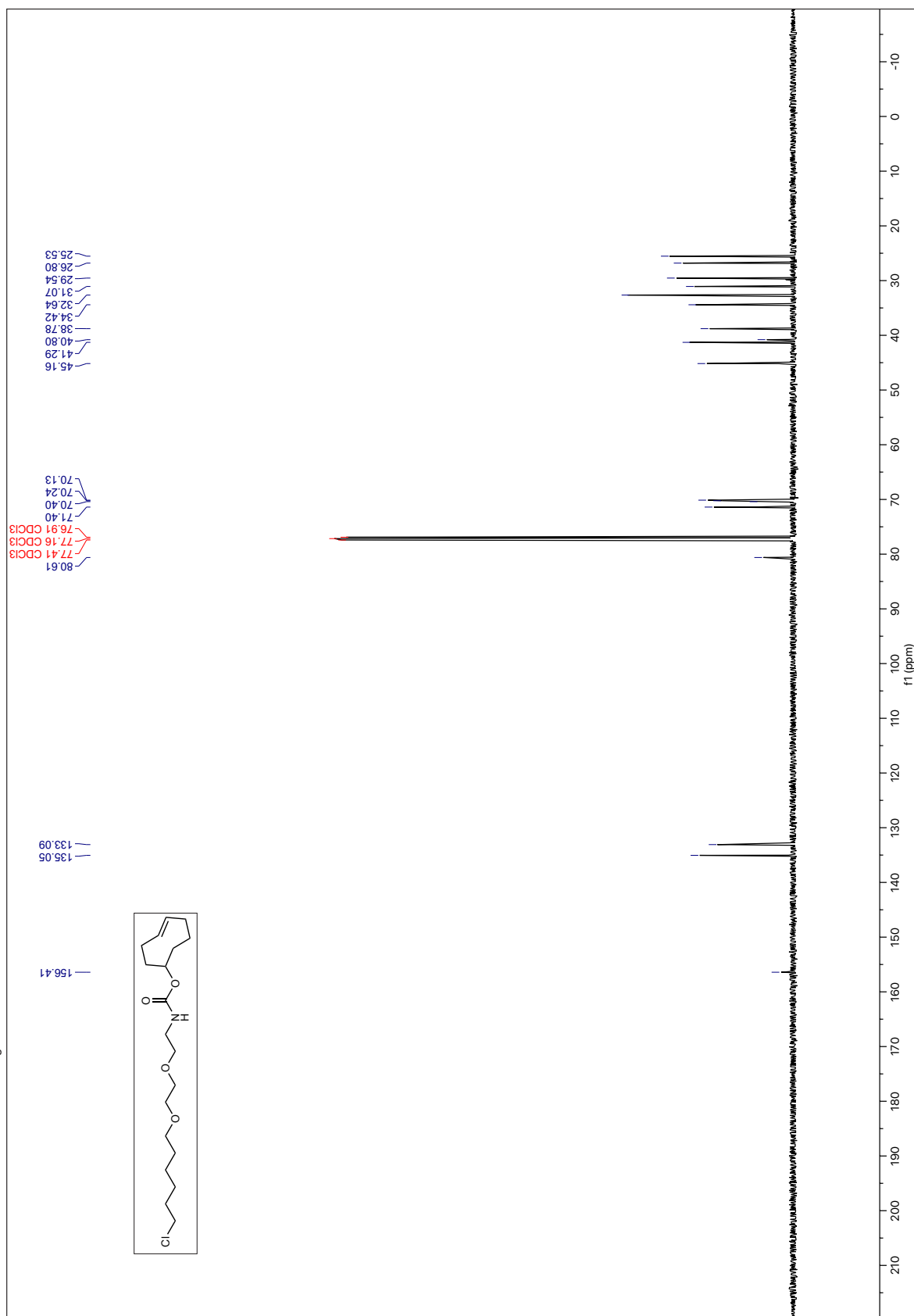
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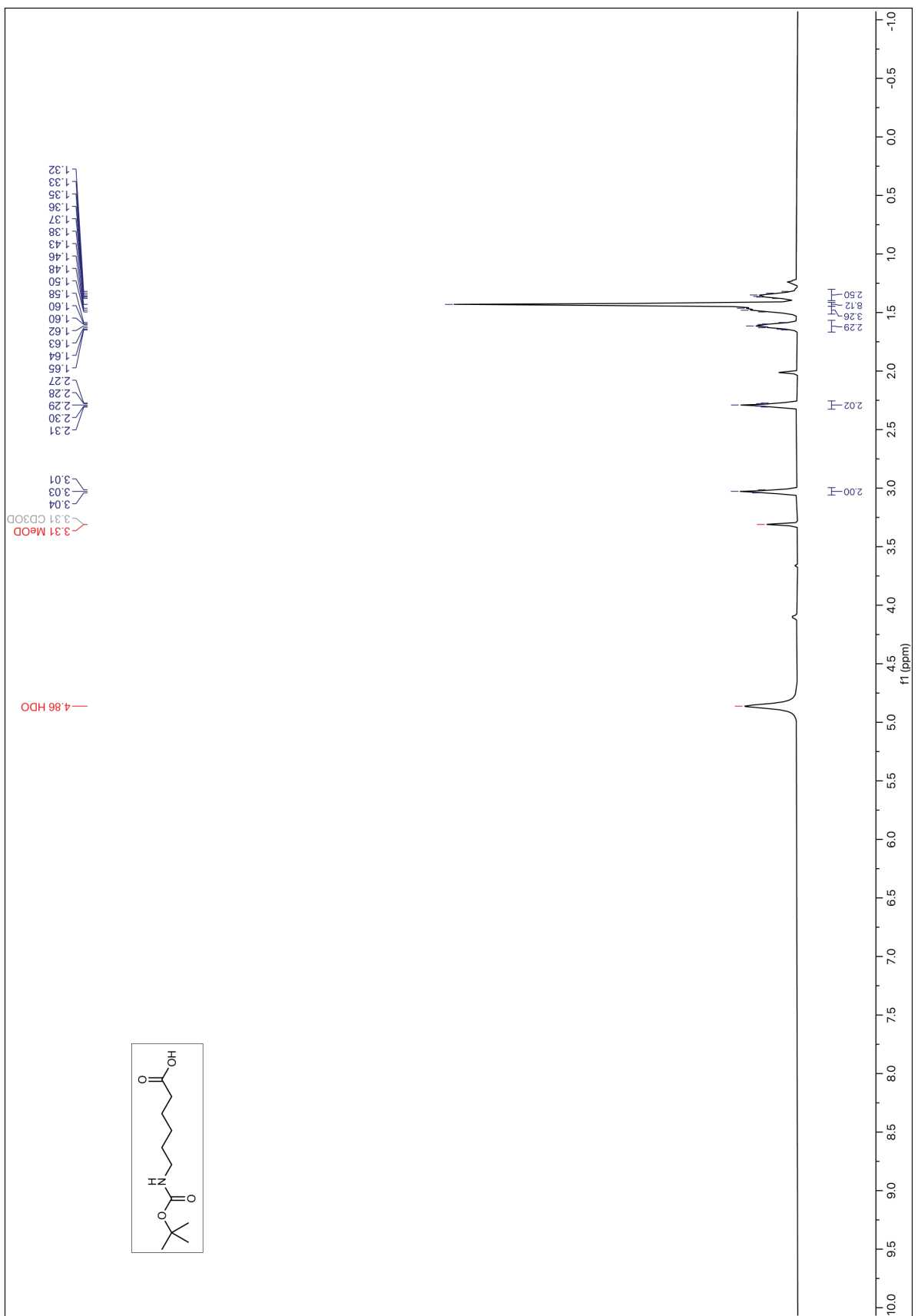
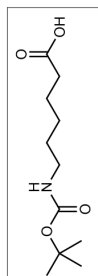
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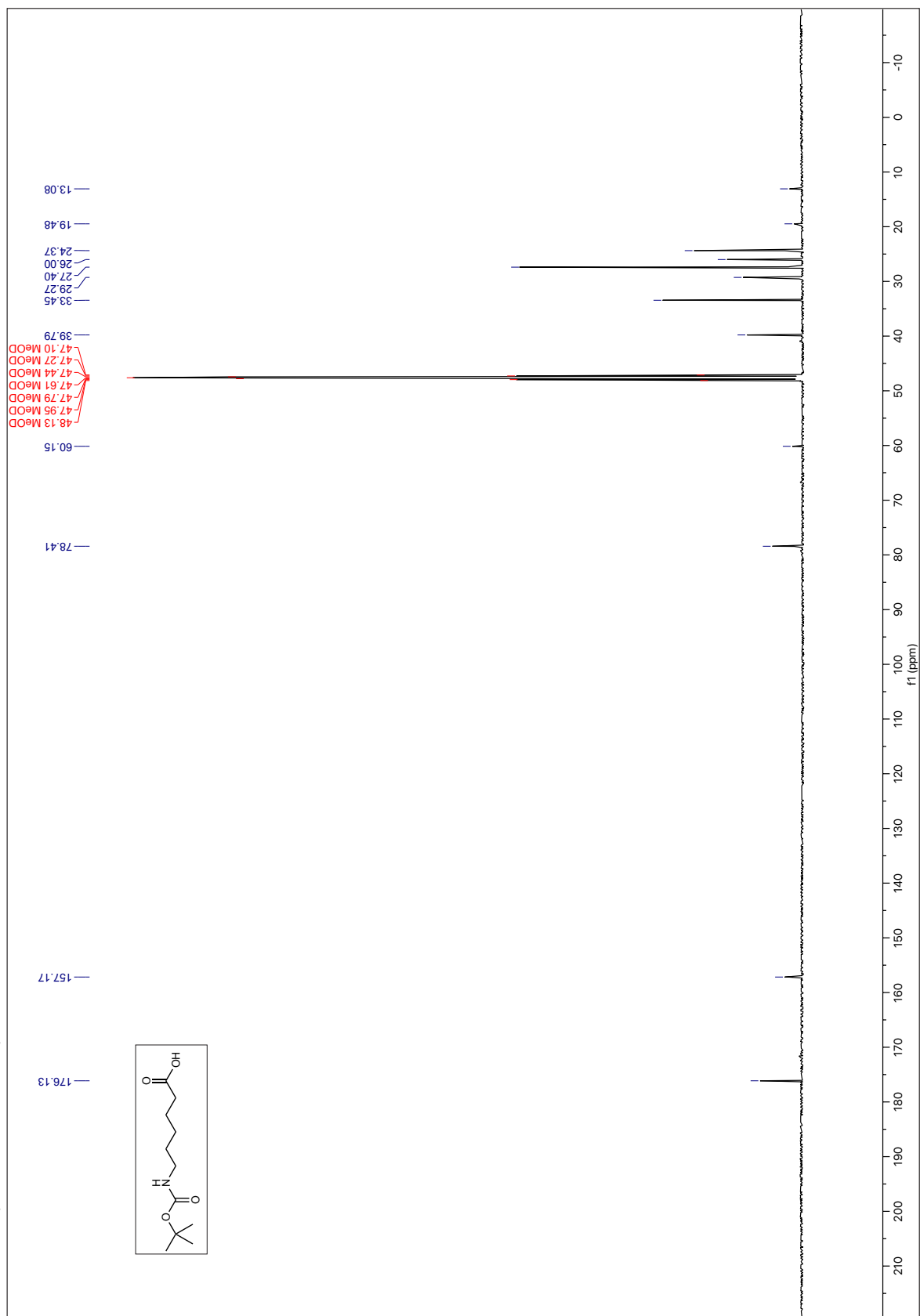
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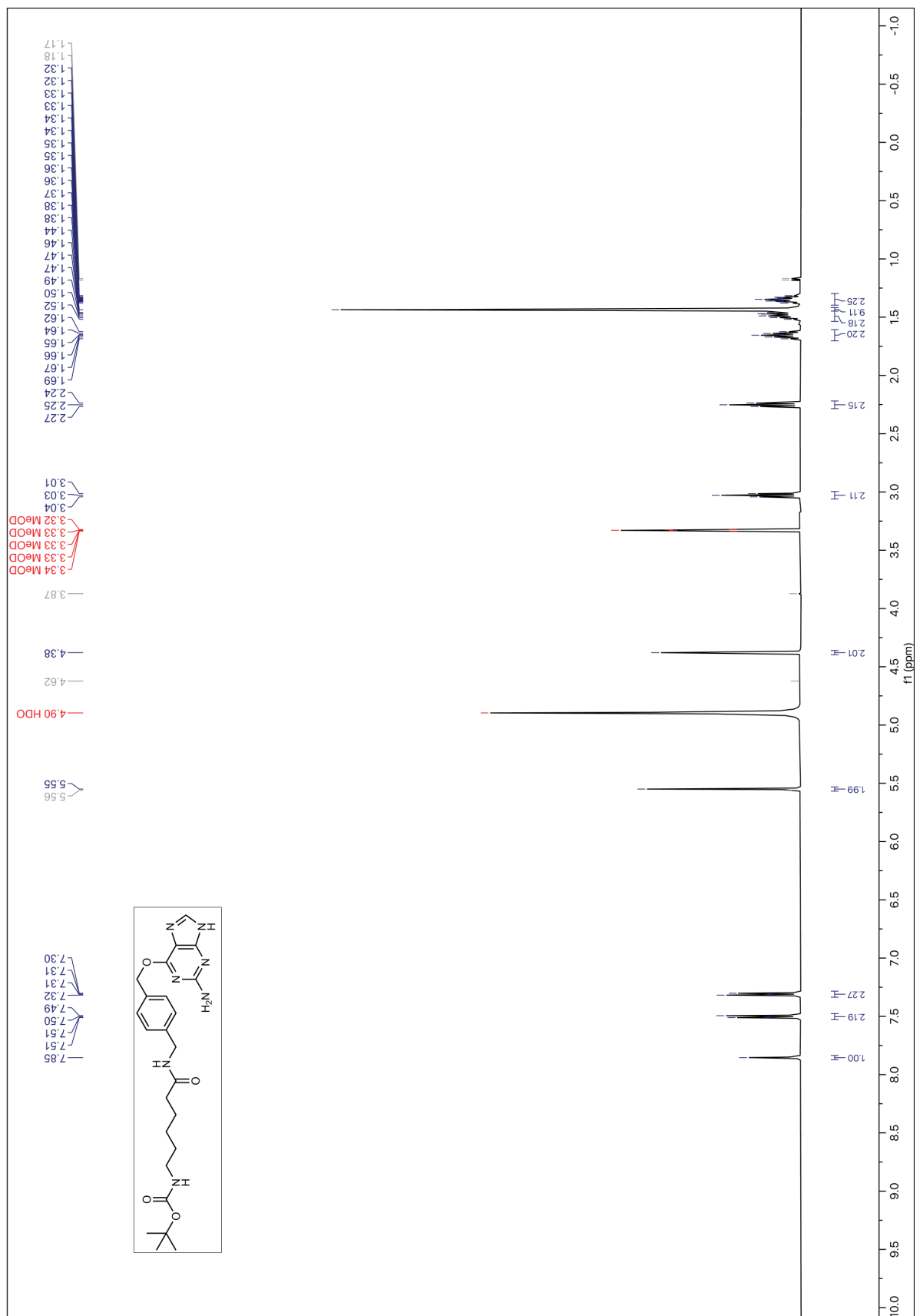
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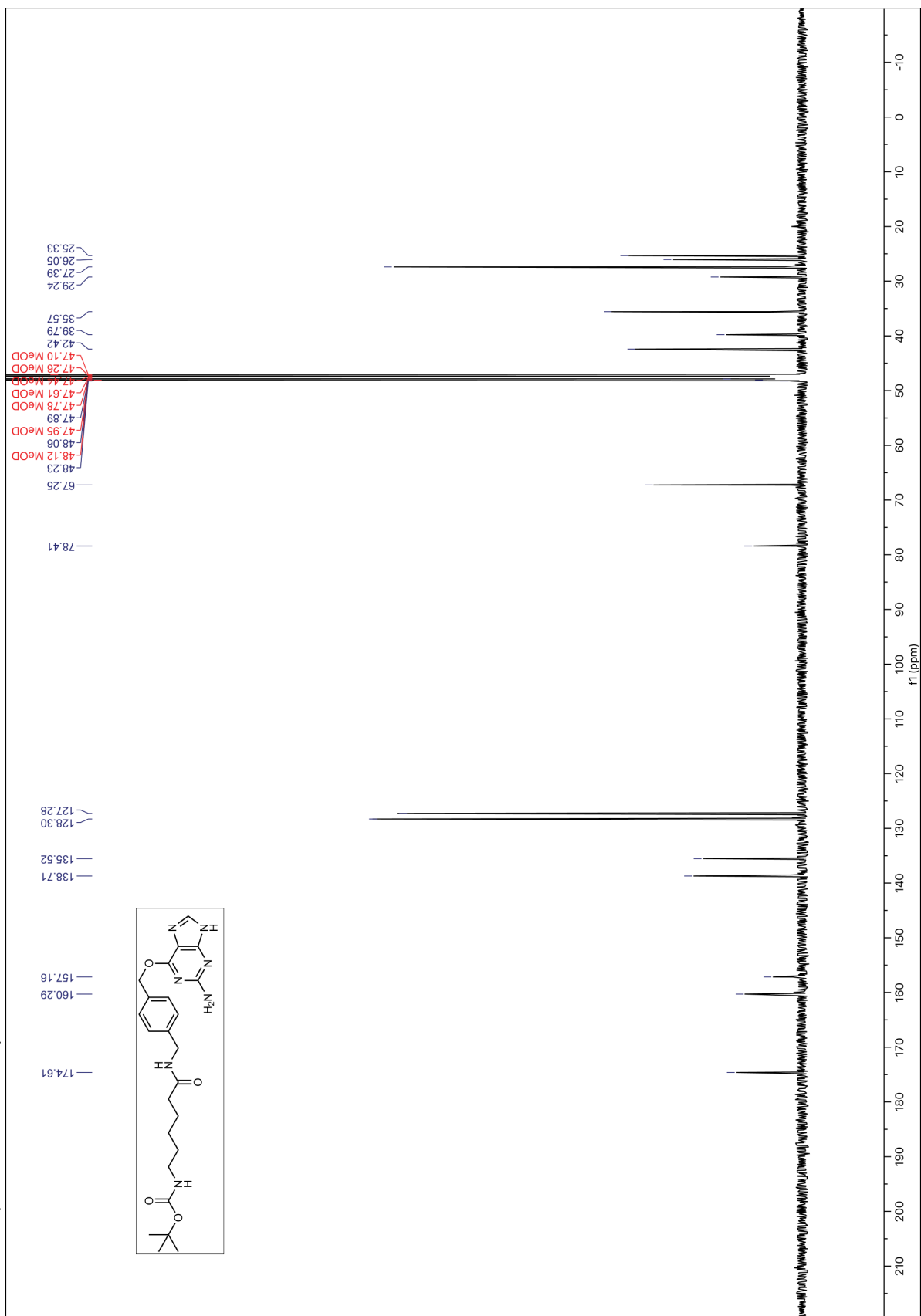
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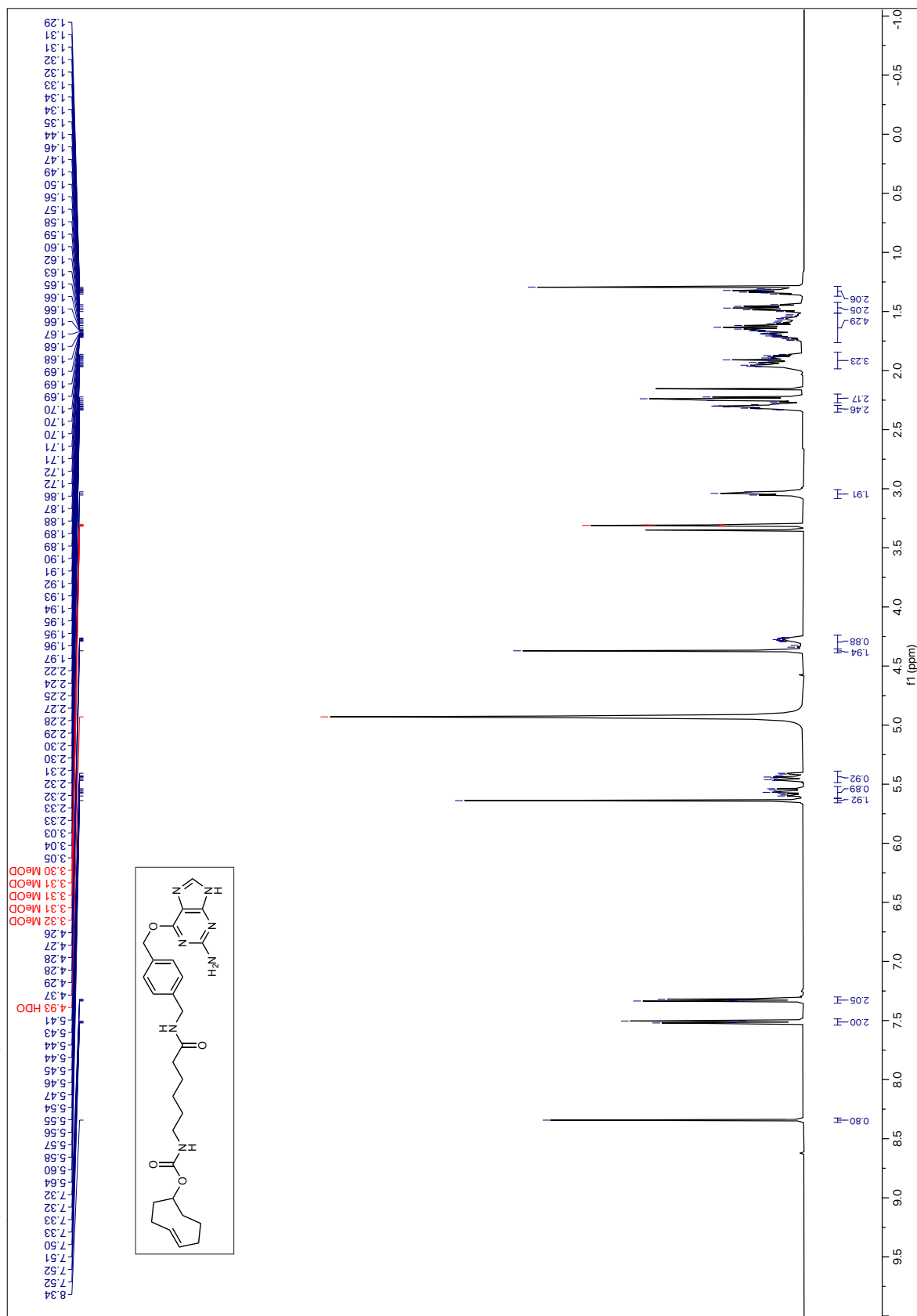
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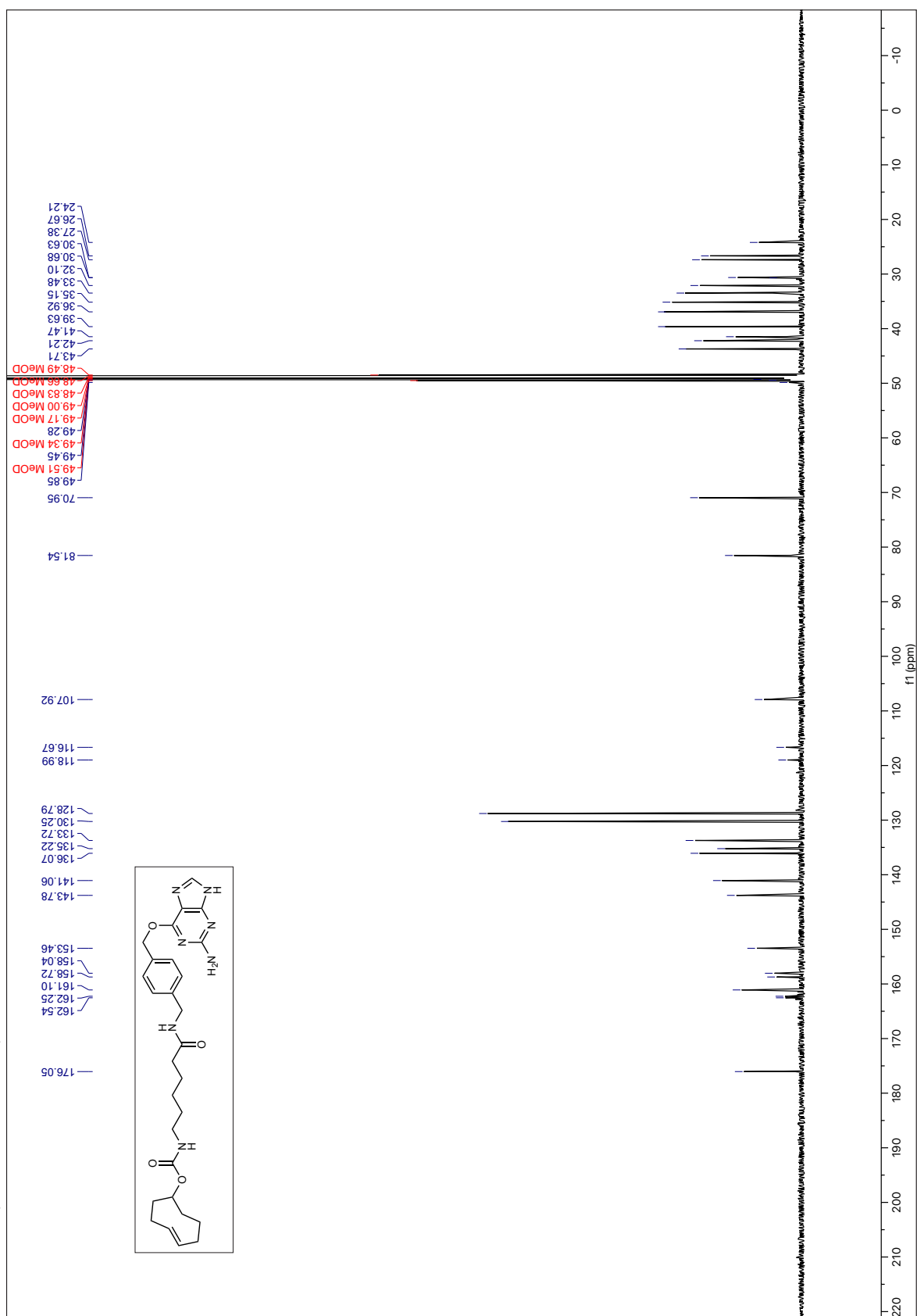
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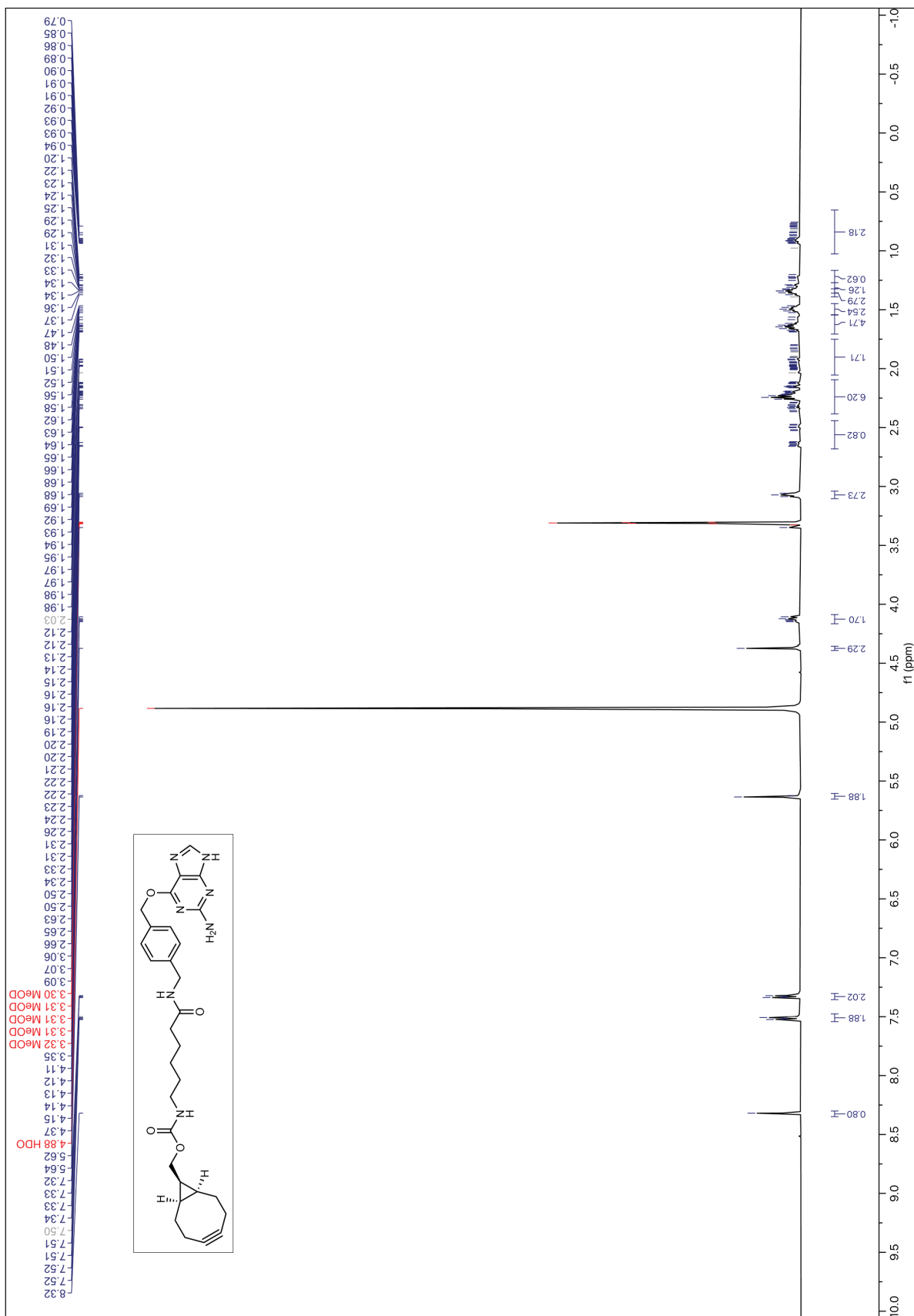
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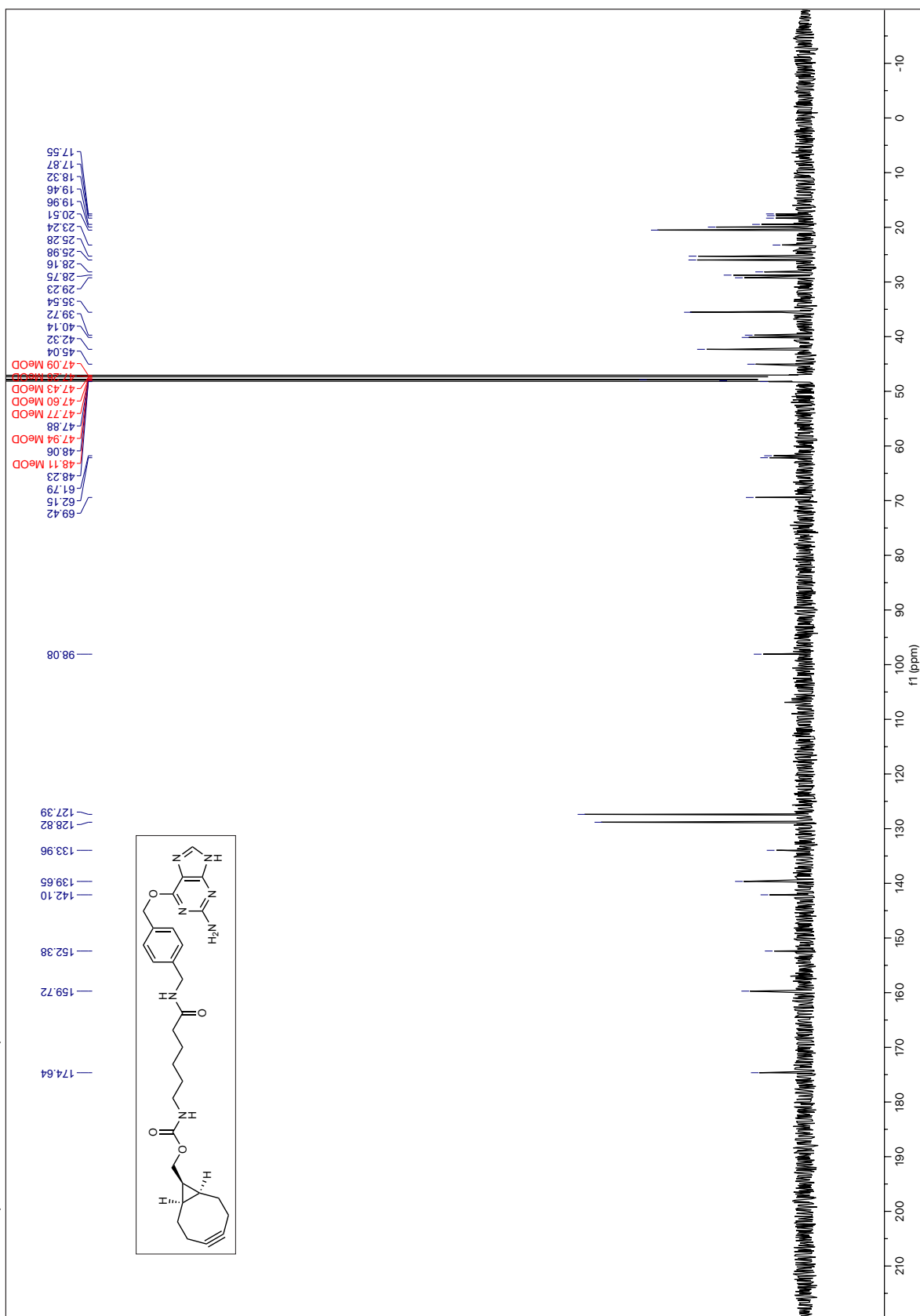
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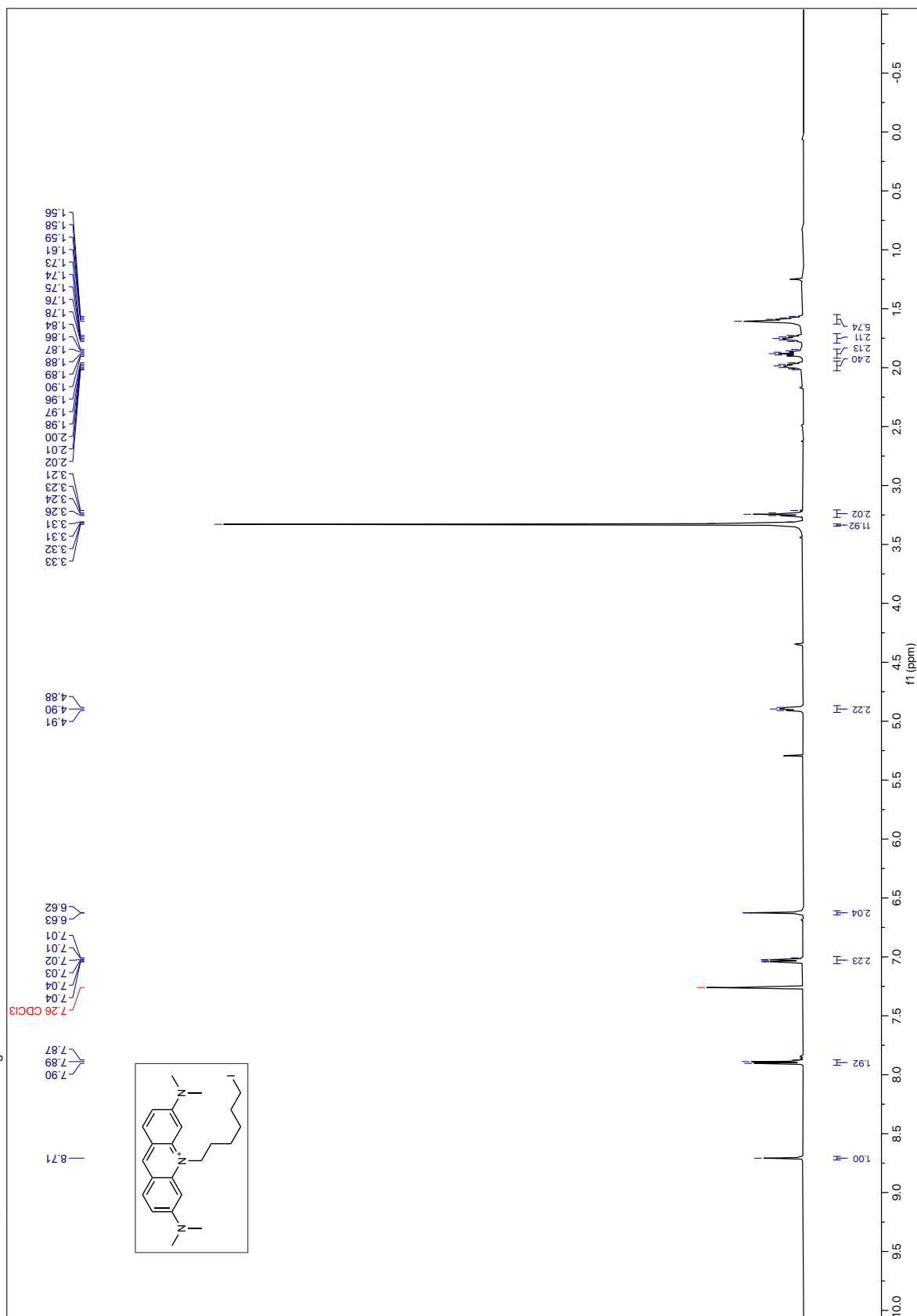
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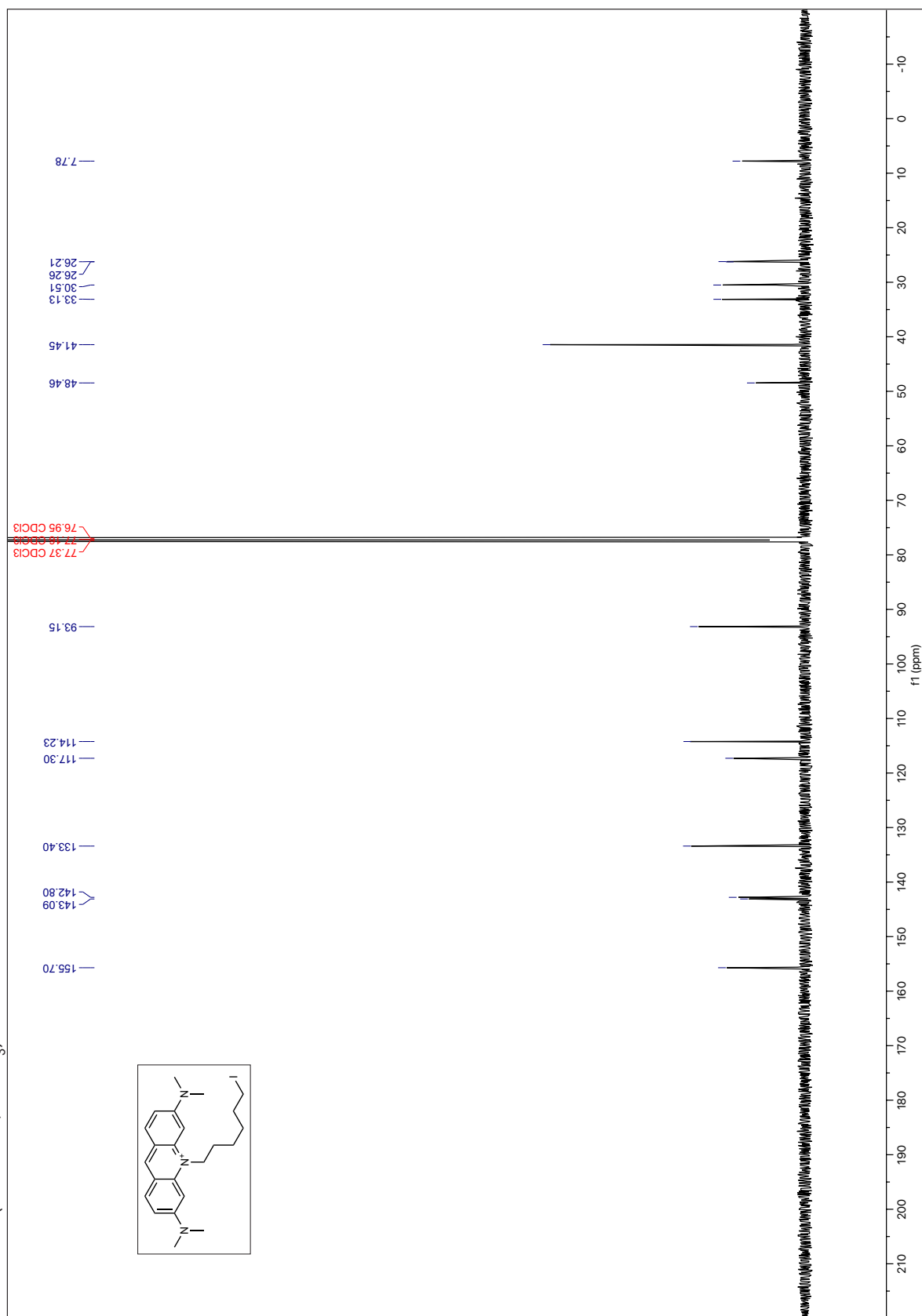
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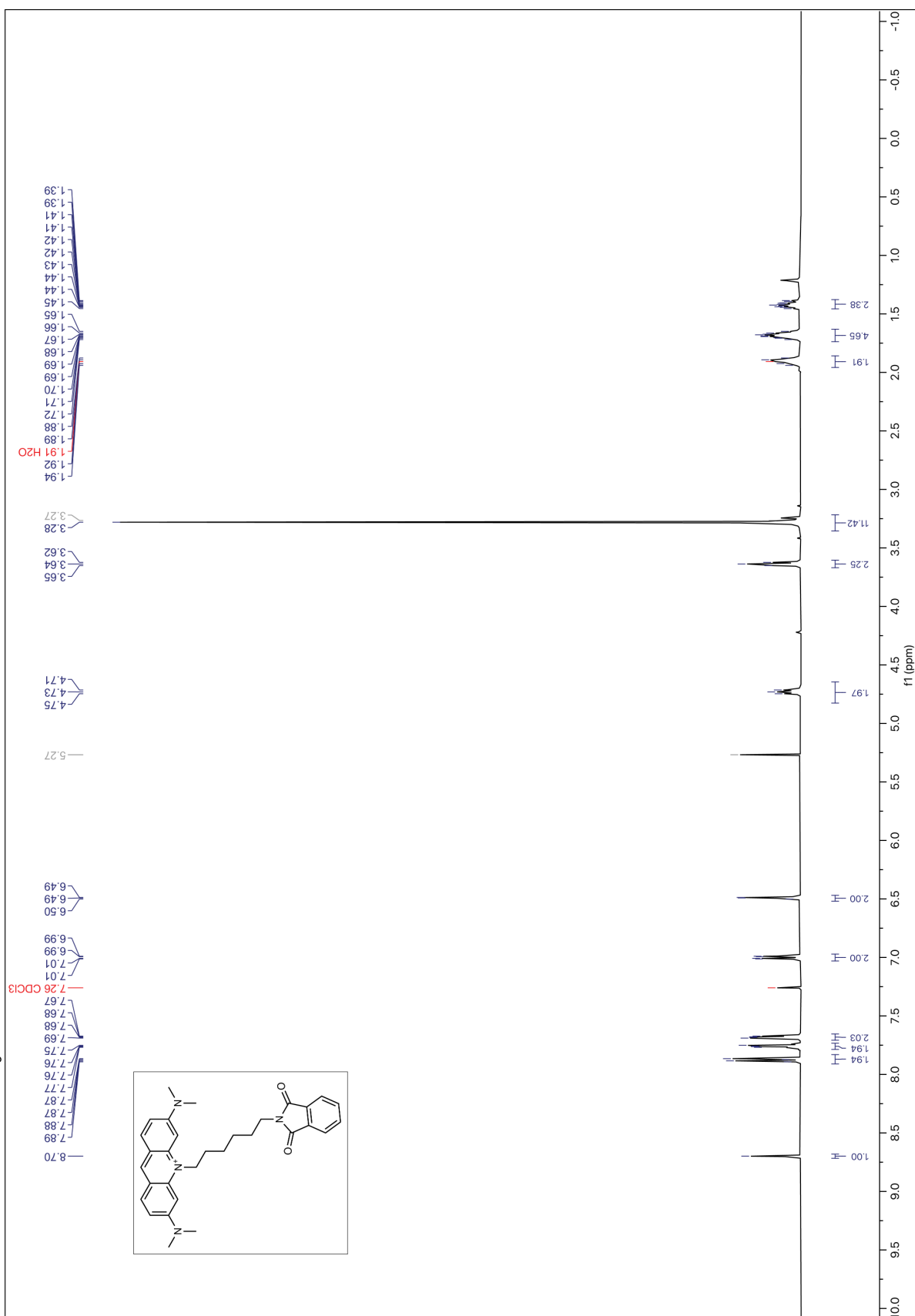
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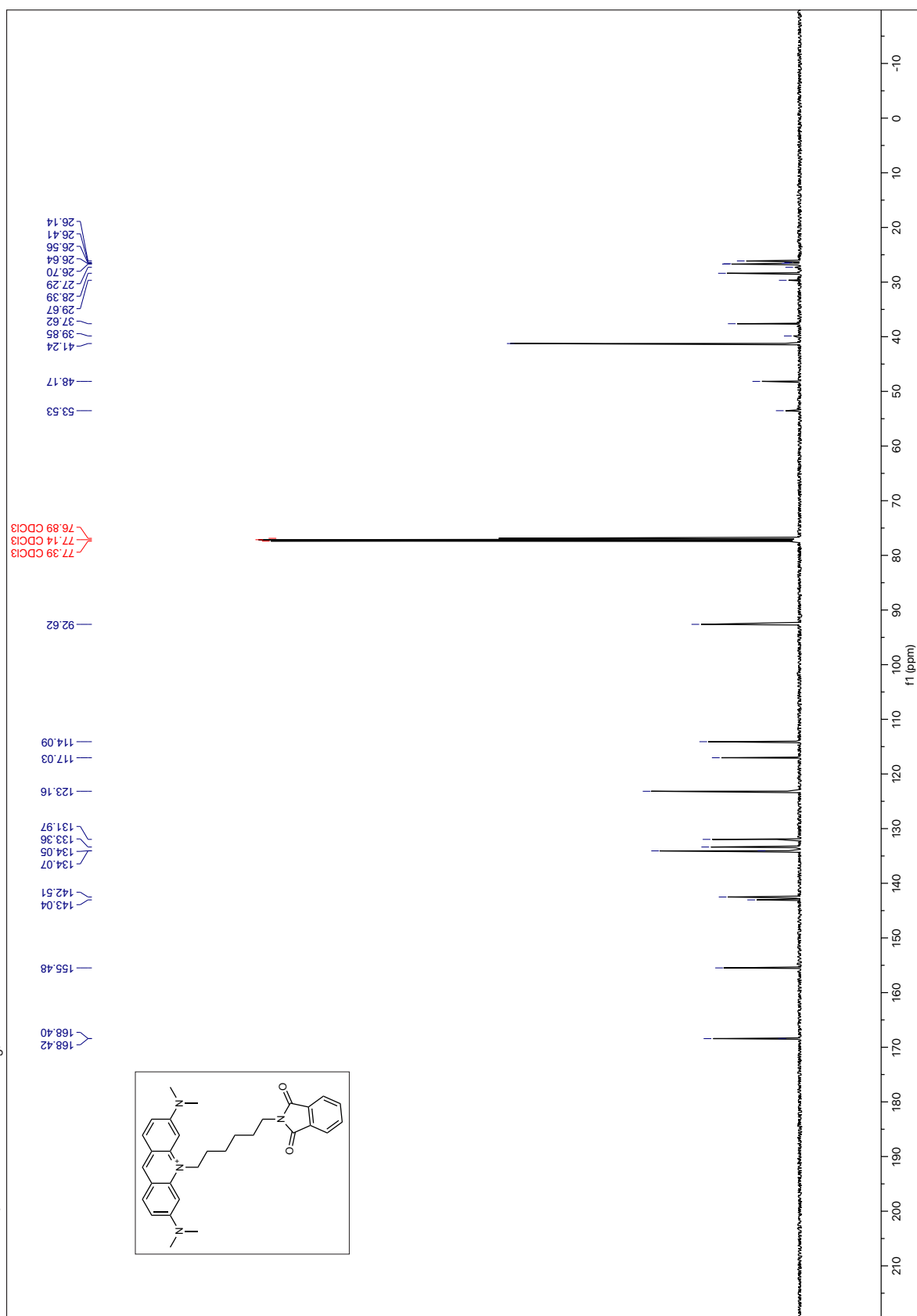
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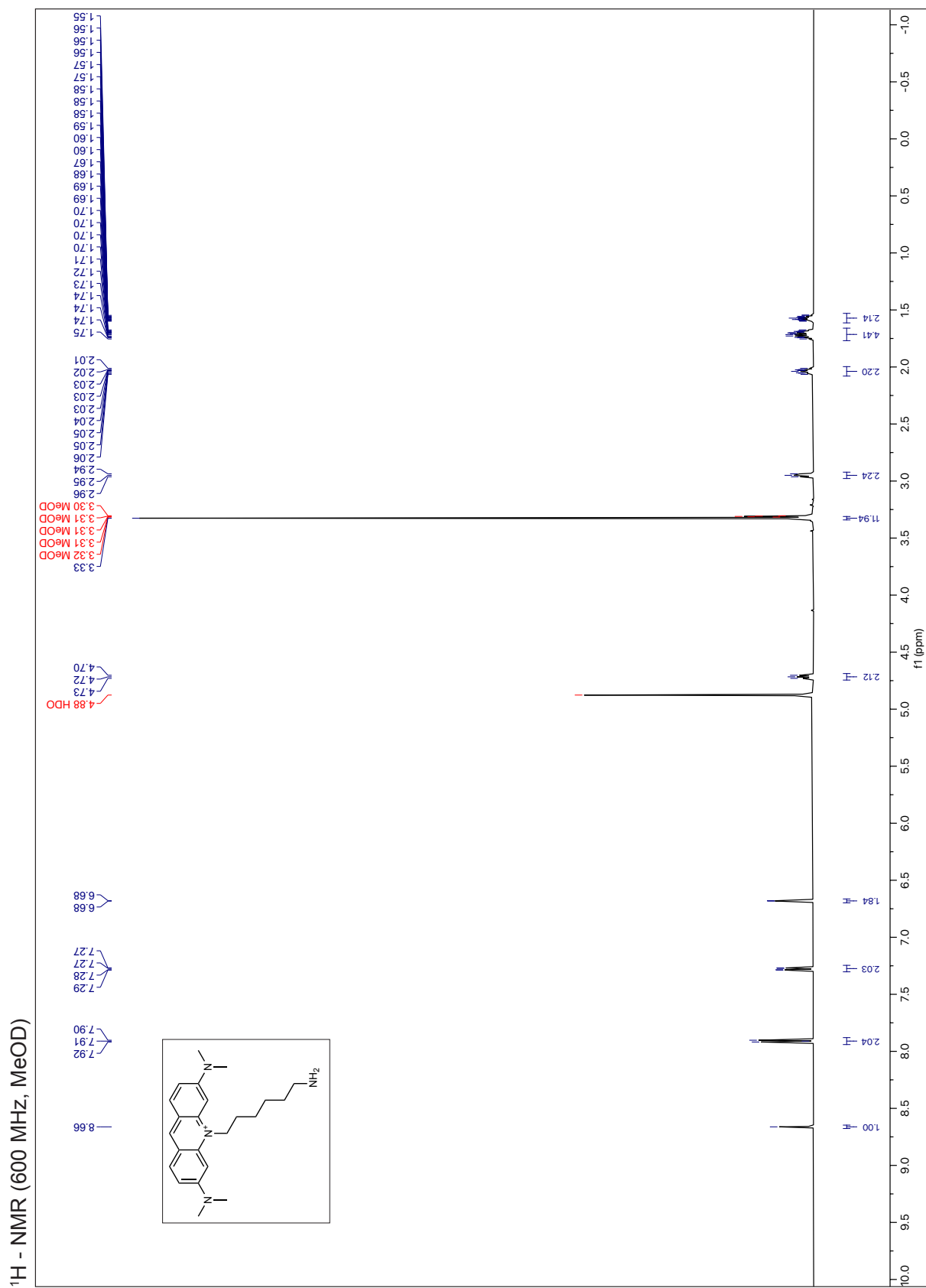


¹H - NMR (500 MHz, CDCl₃)

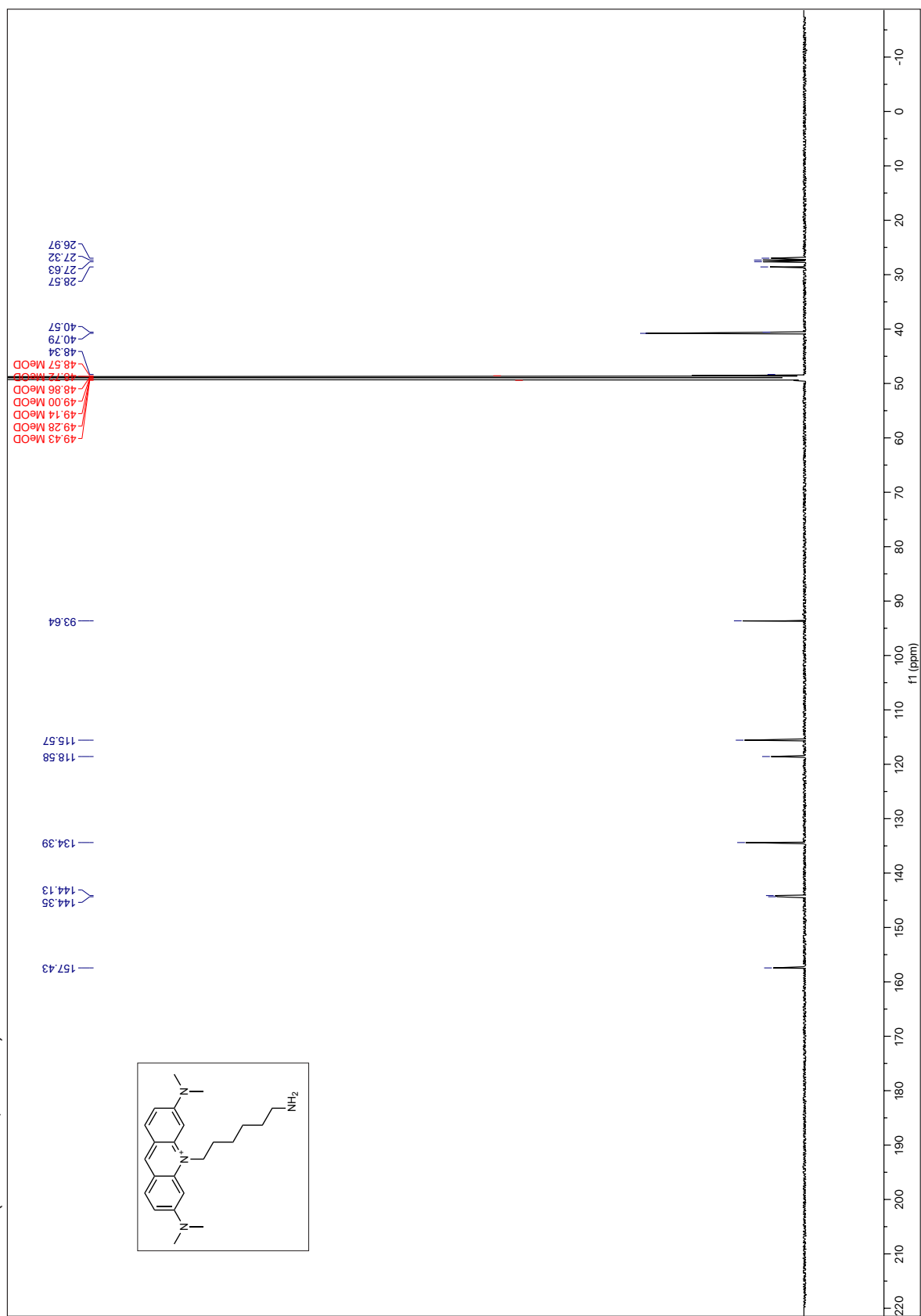


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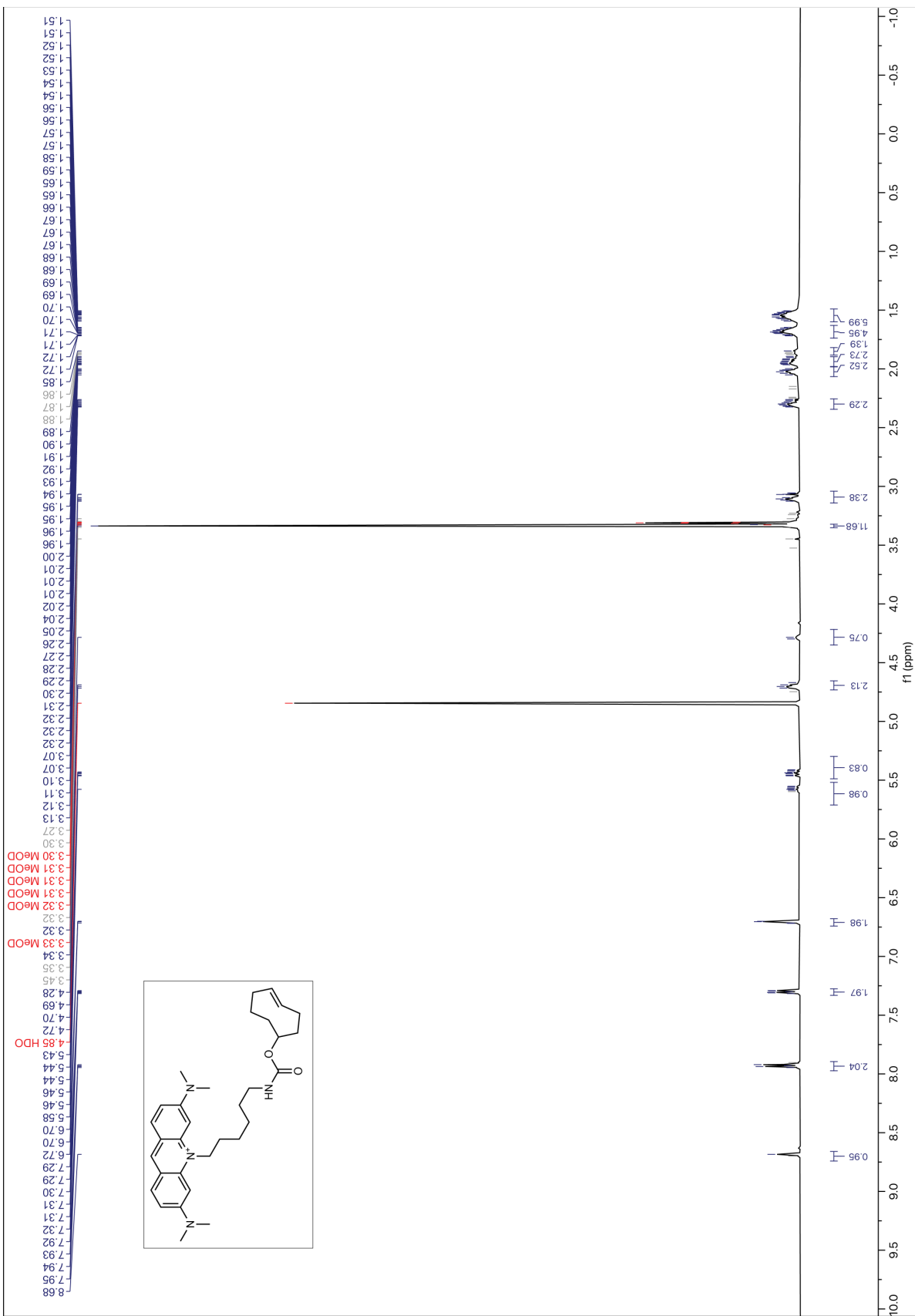




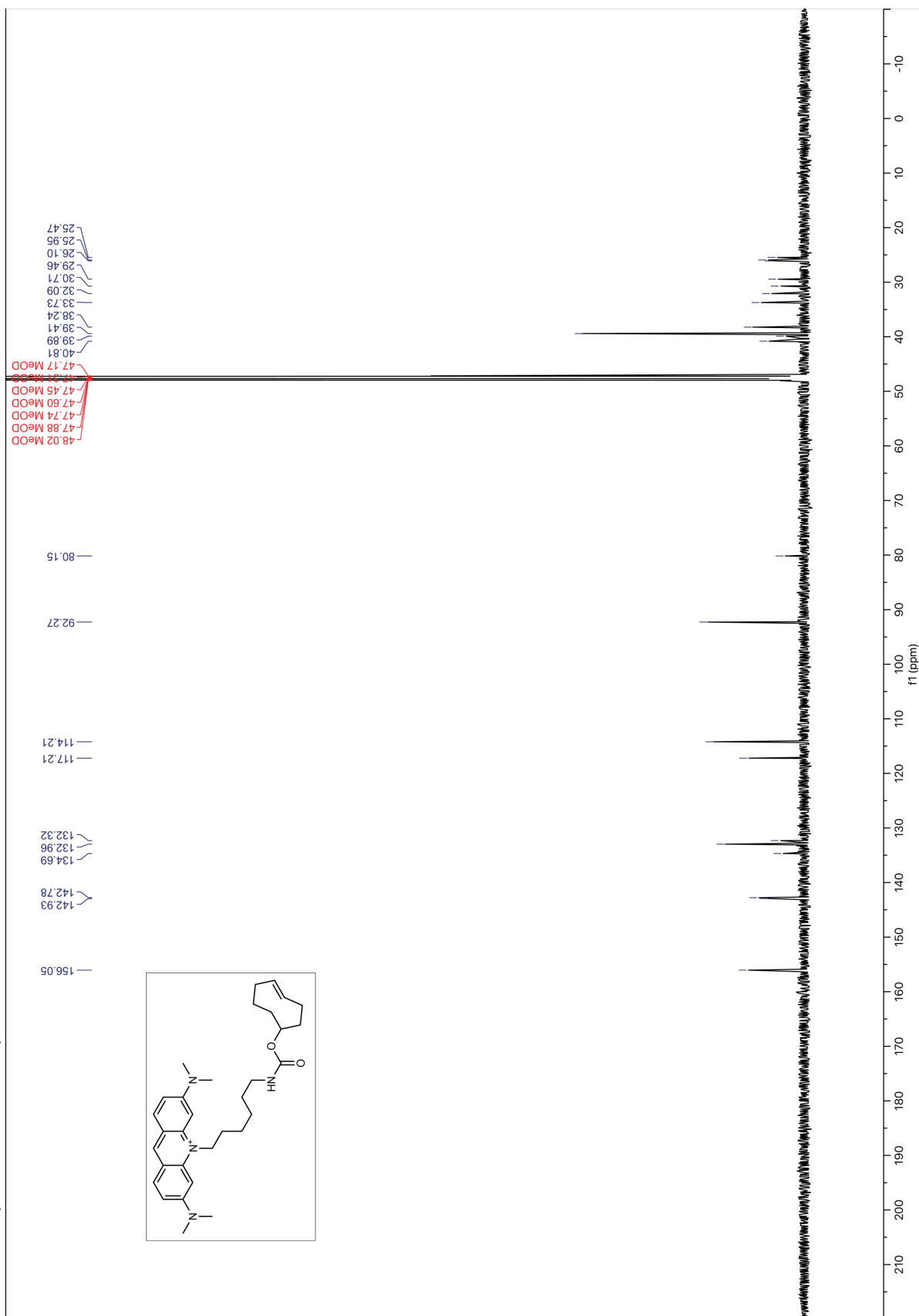
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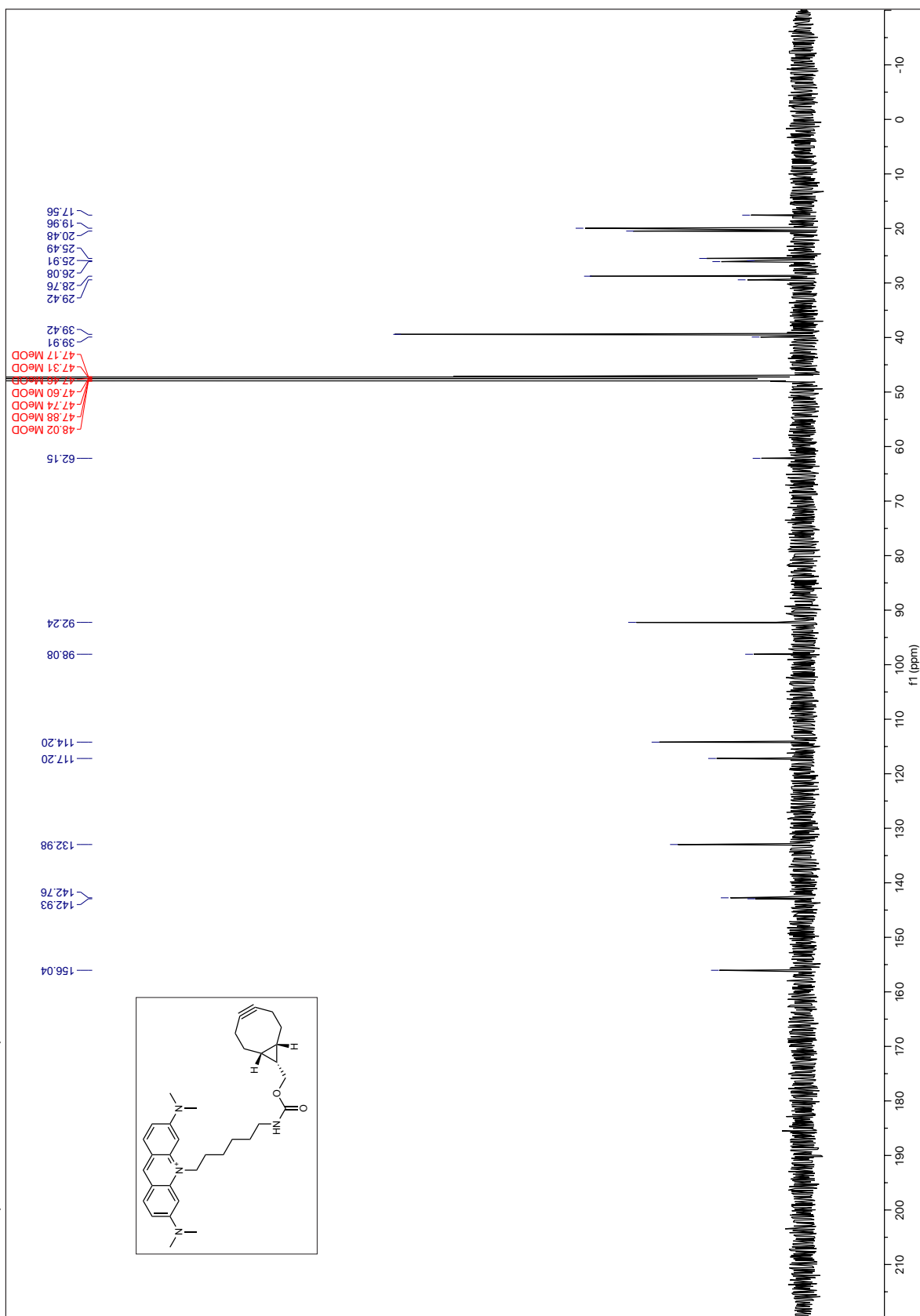


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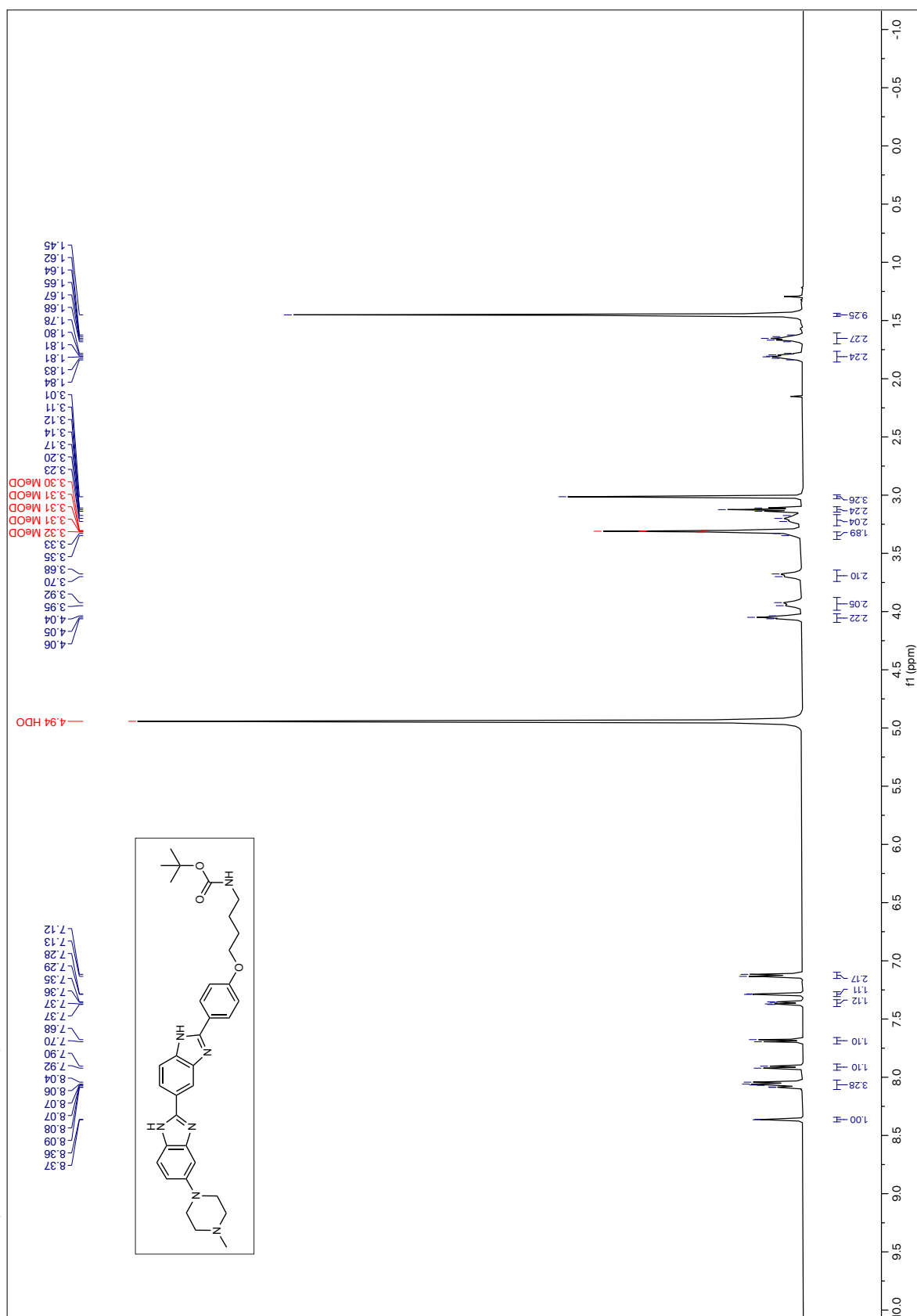




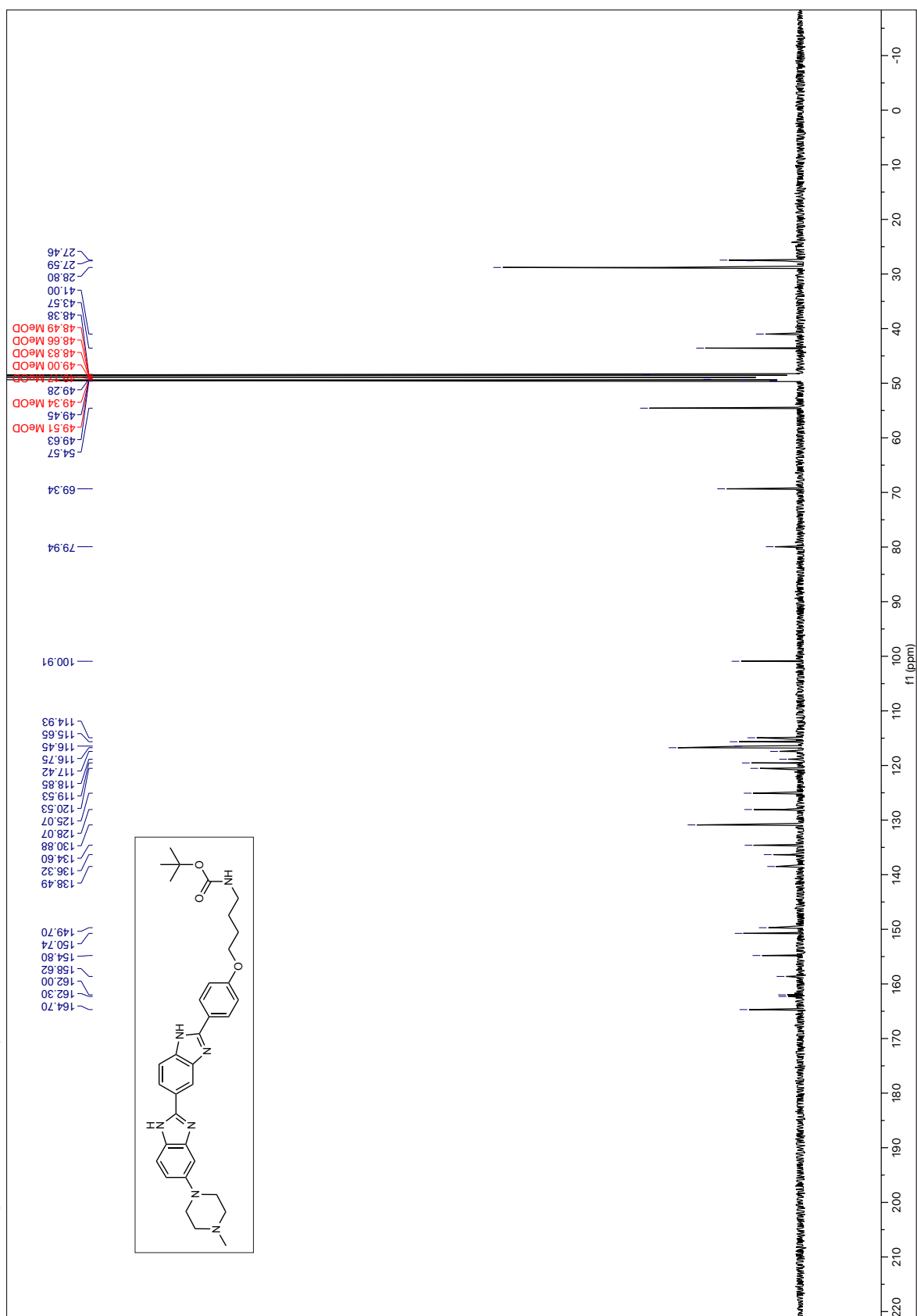
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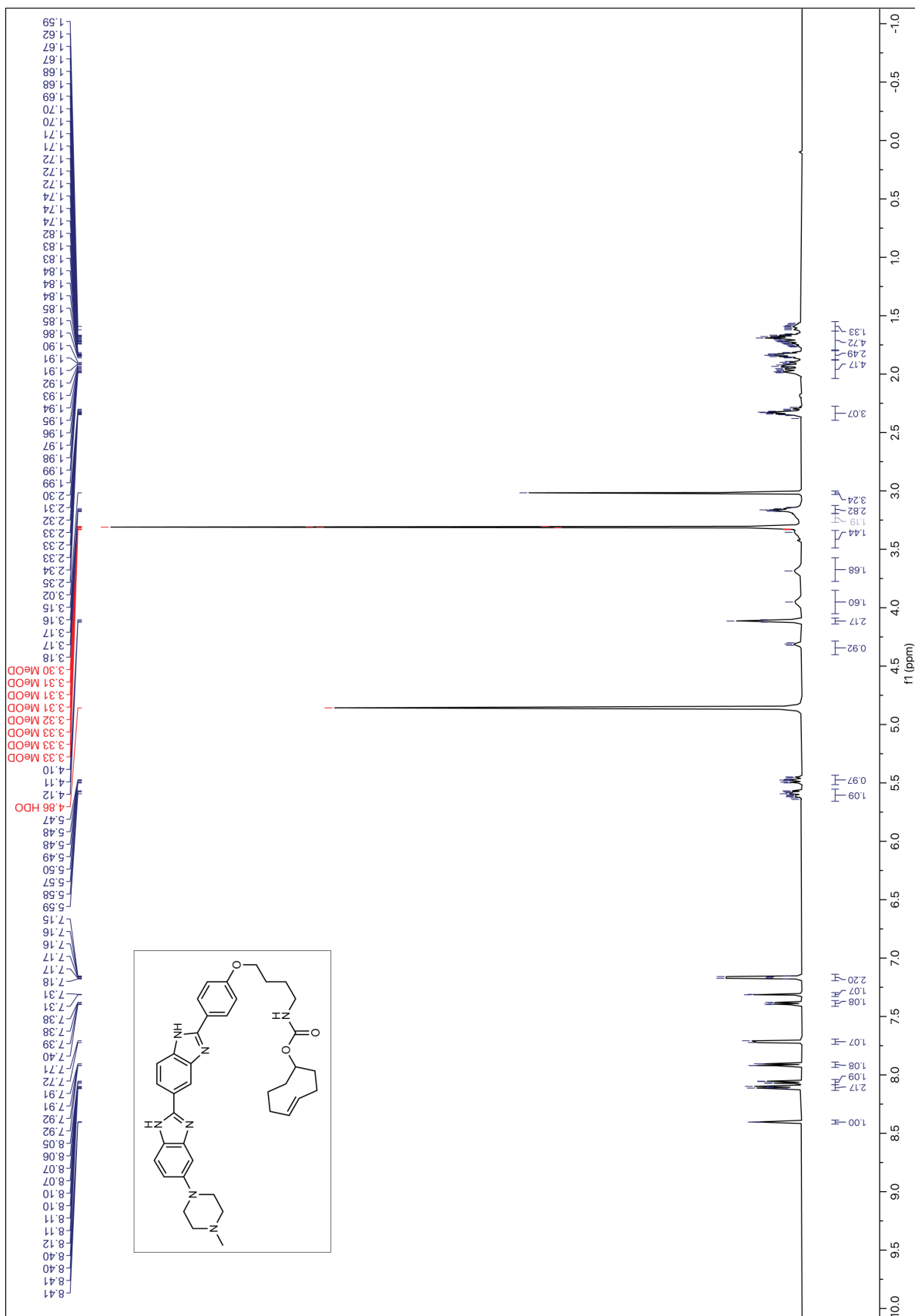
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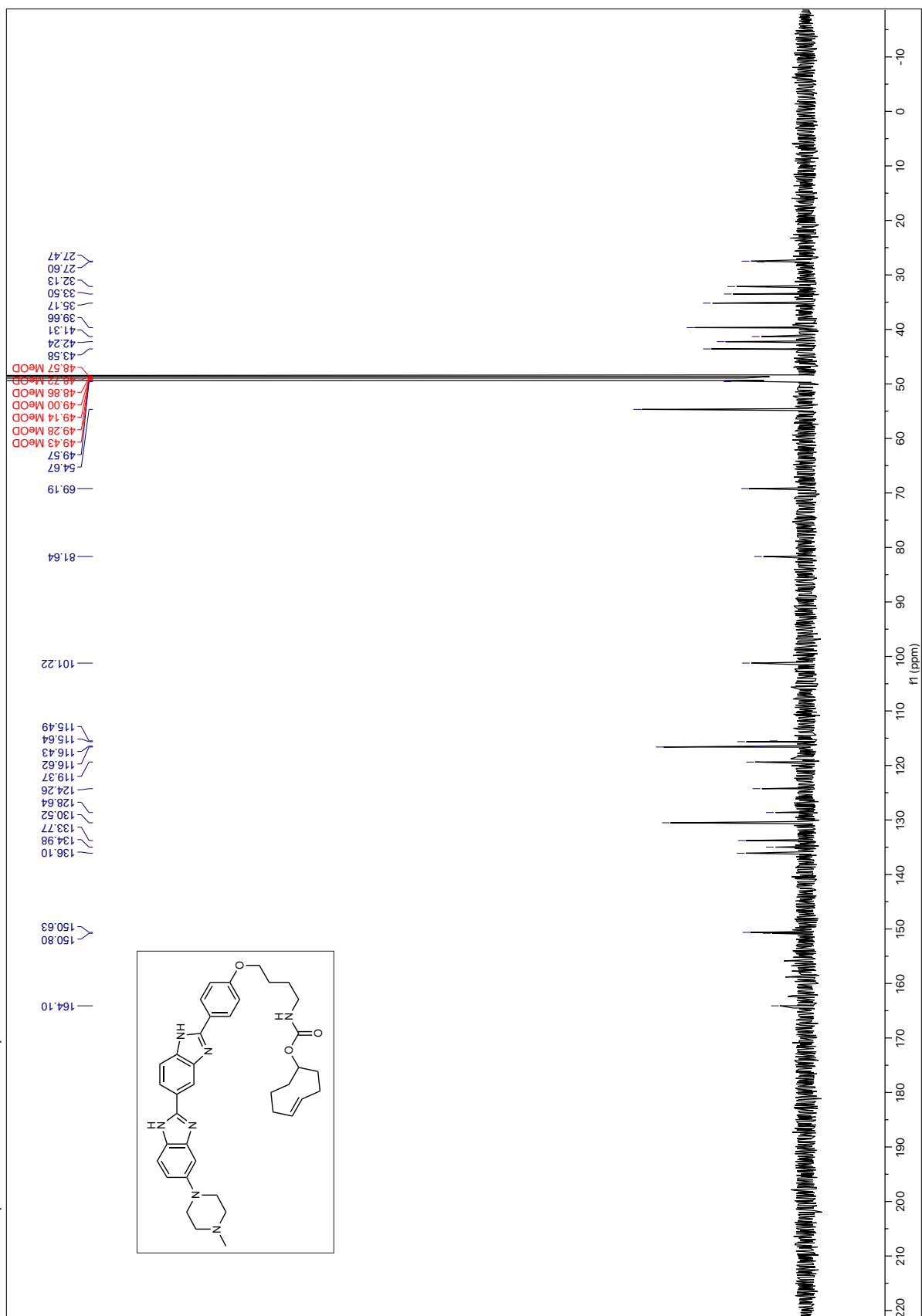
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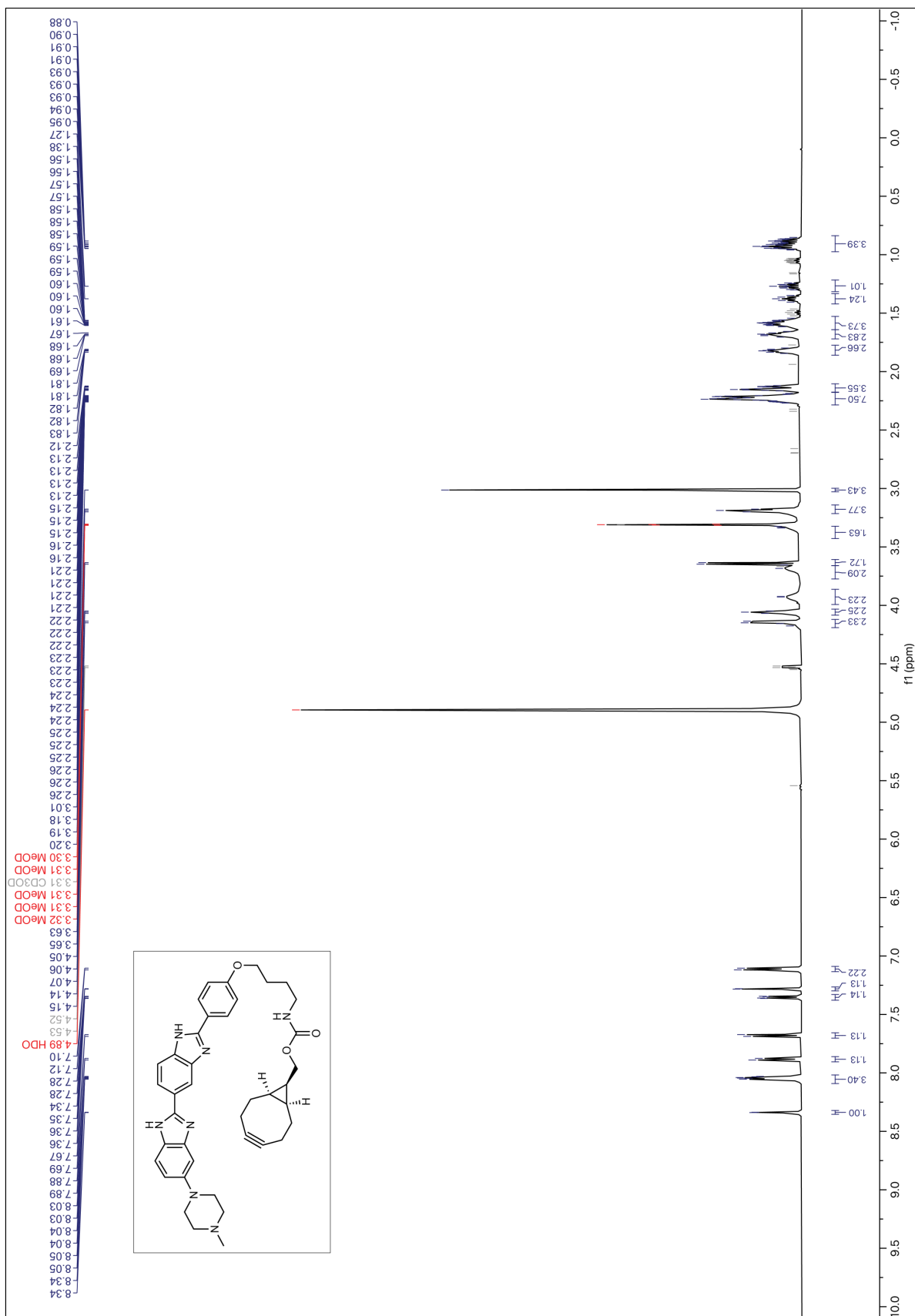
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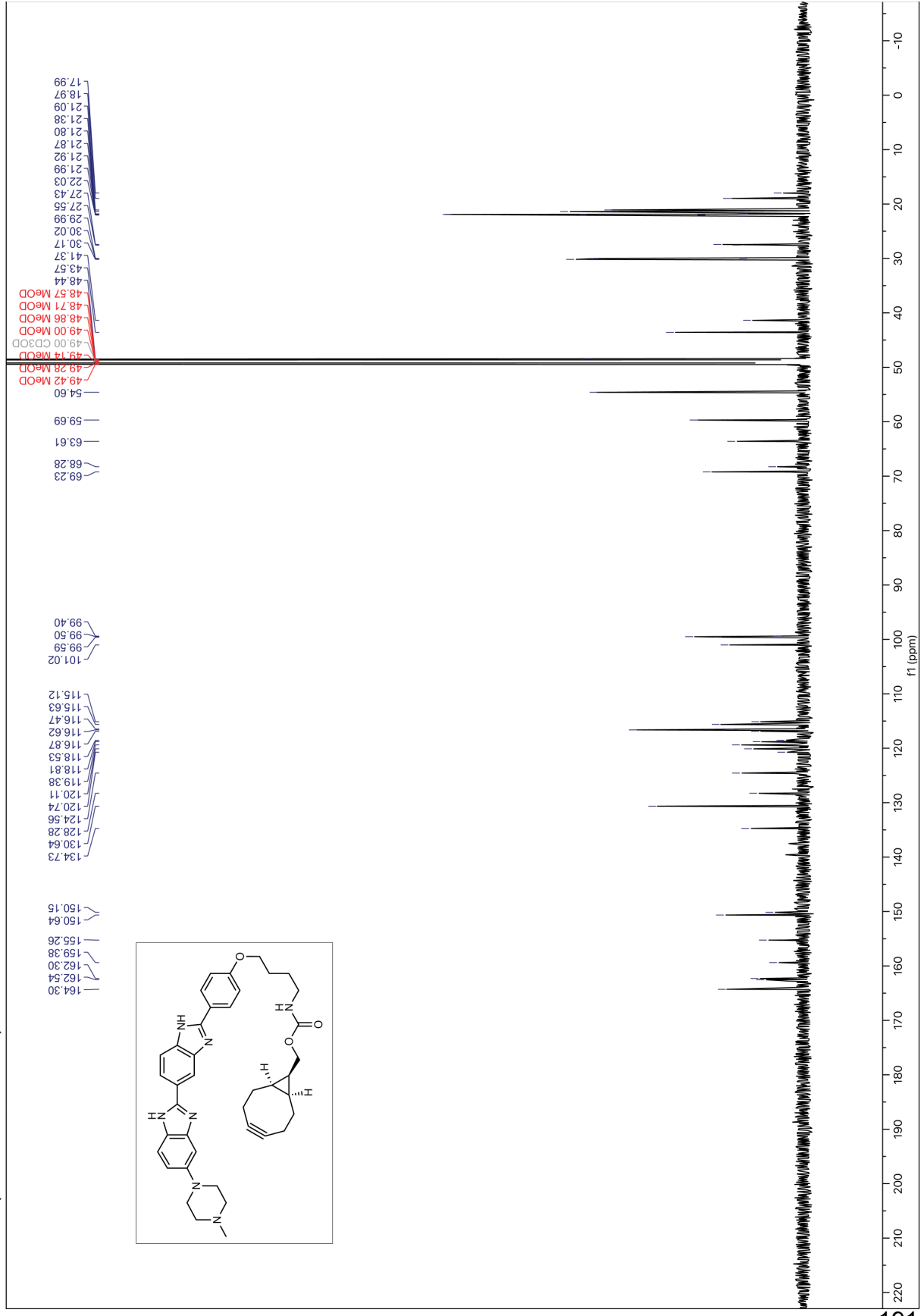
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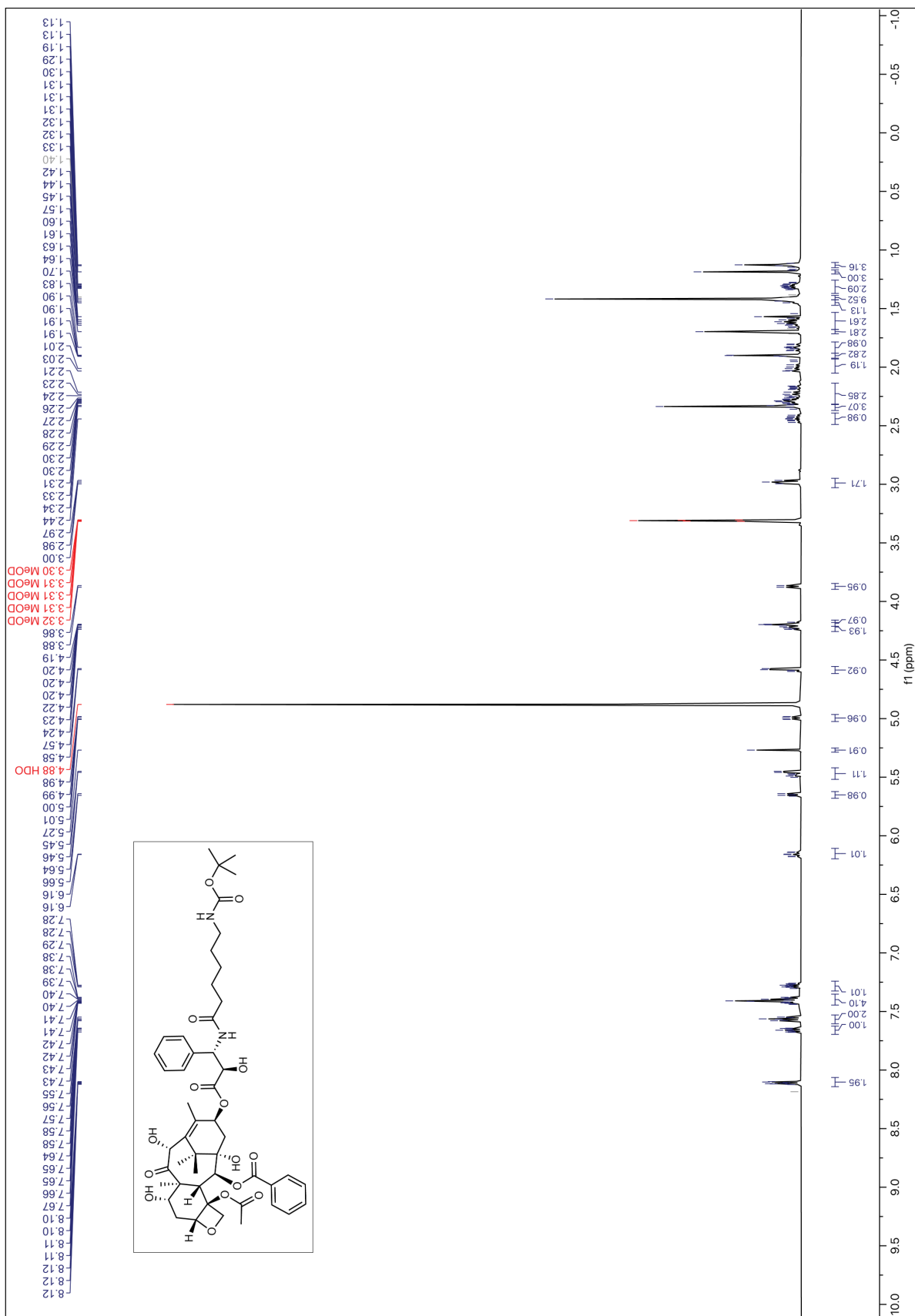
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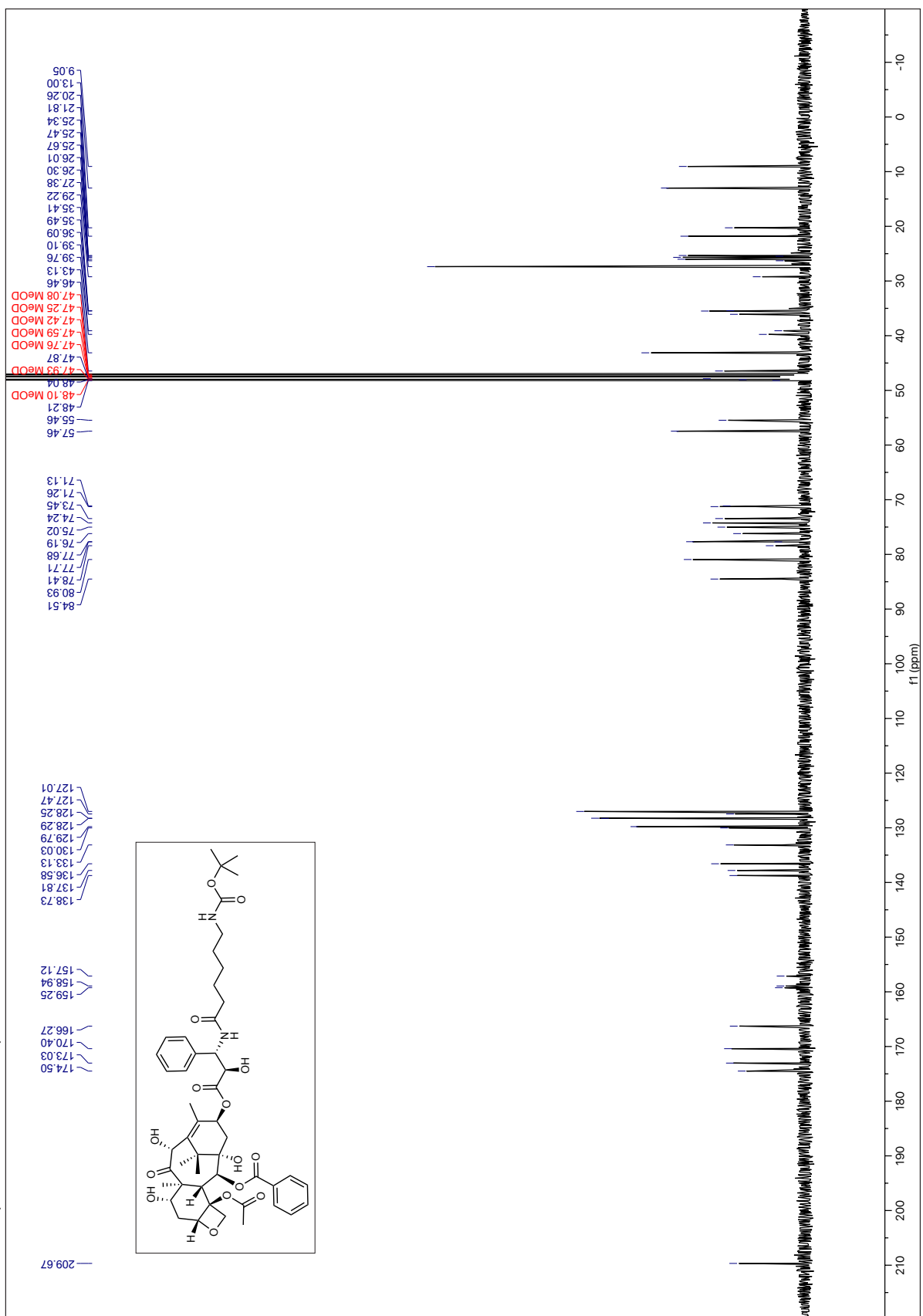
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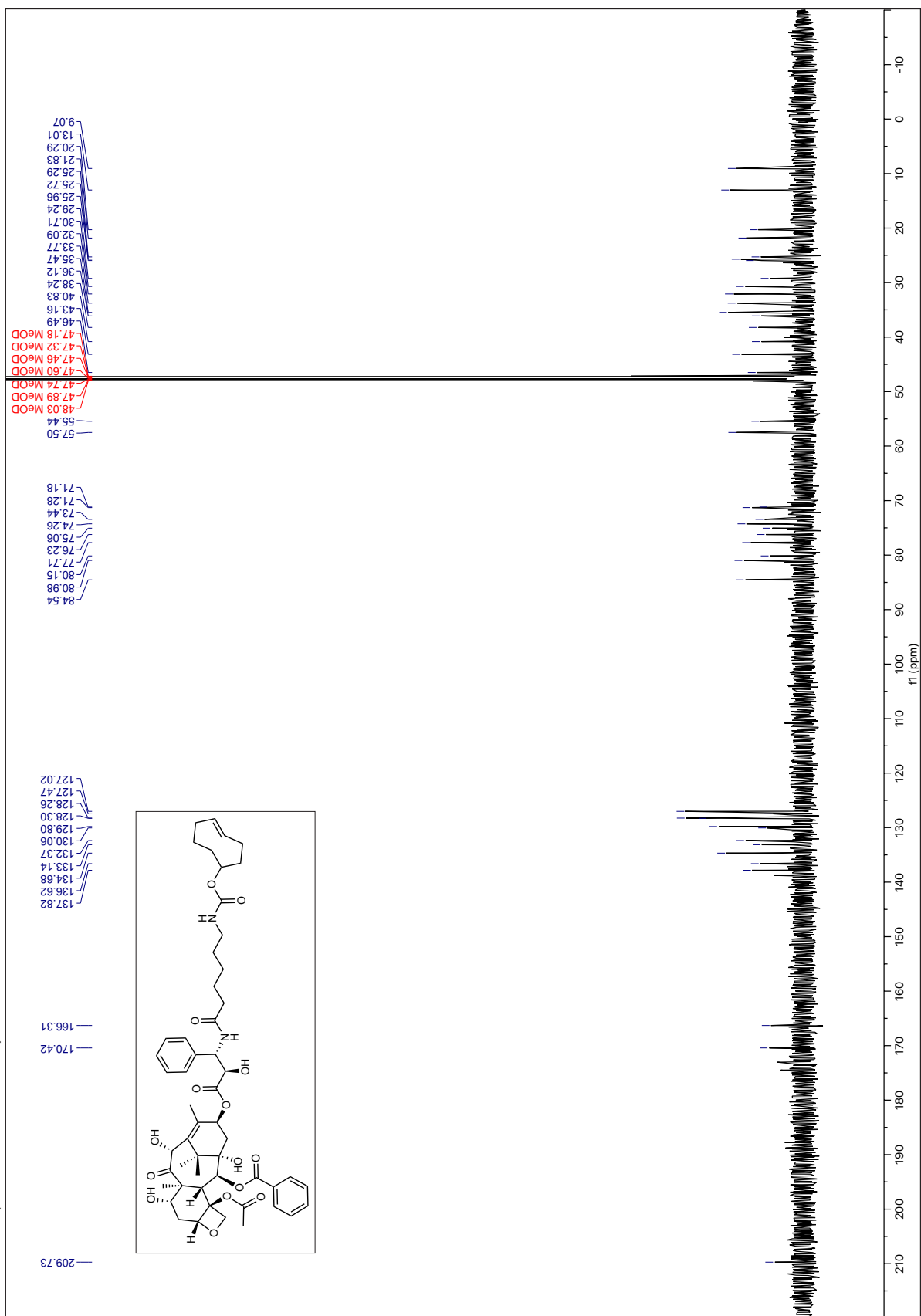


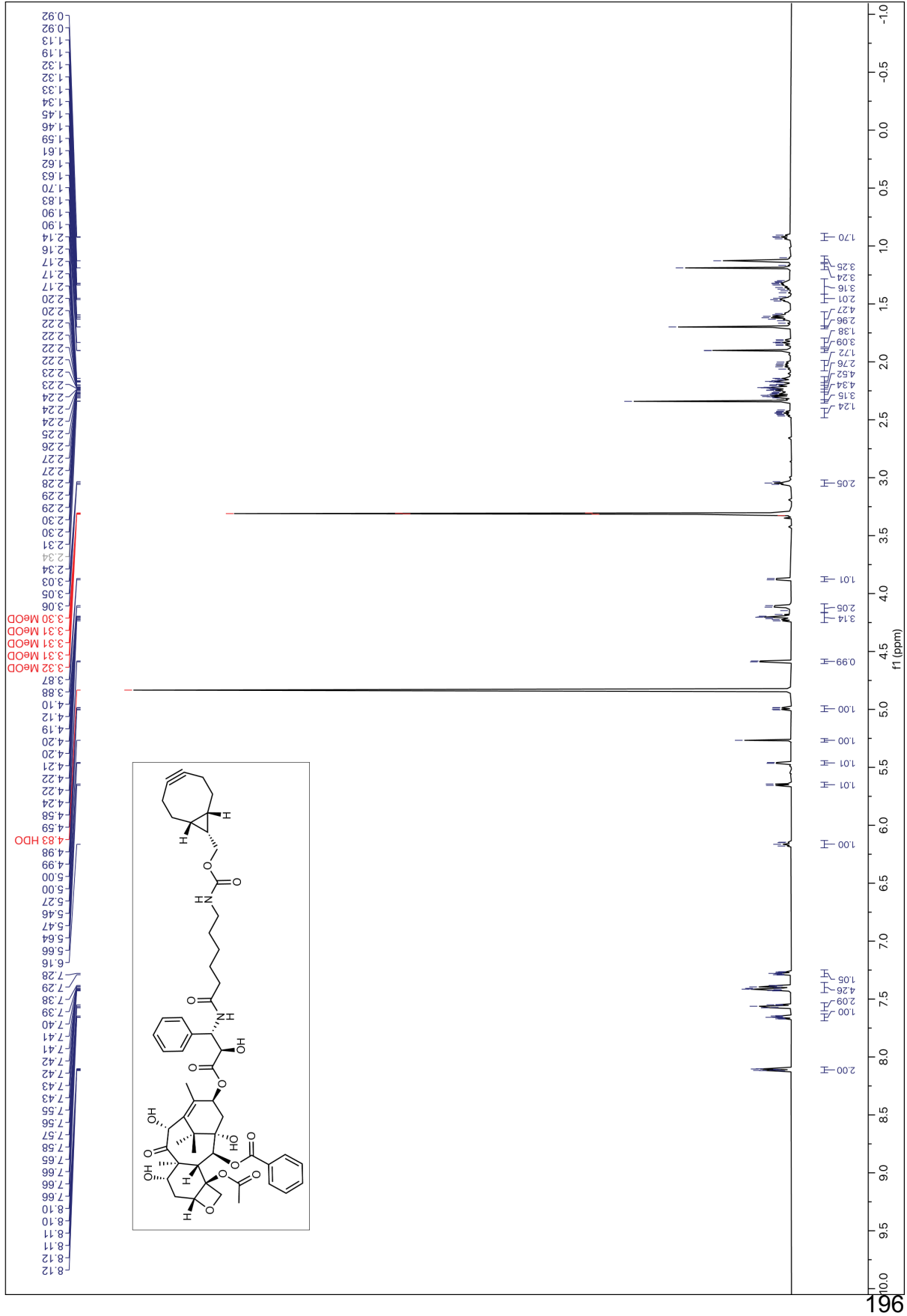
¹³C - NMR (126 MHz, MeOD)



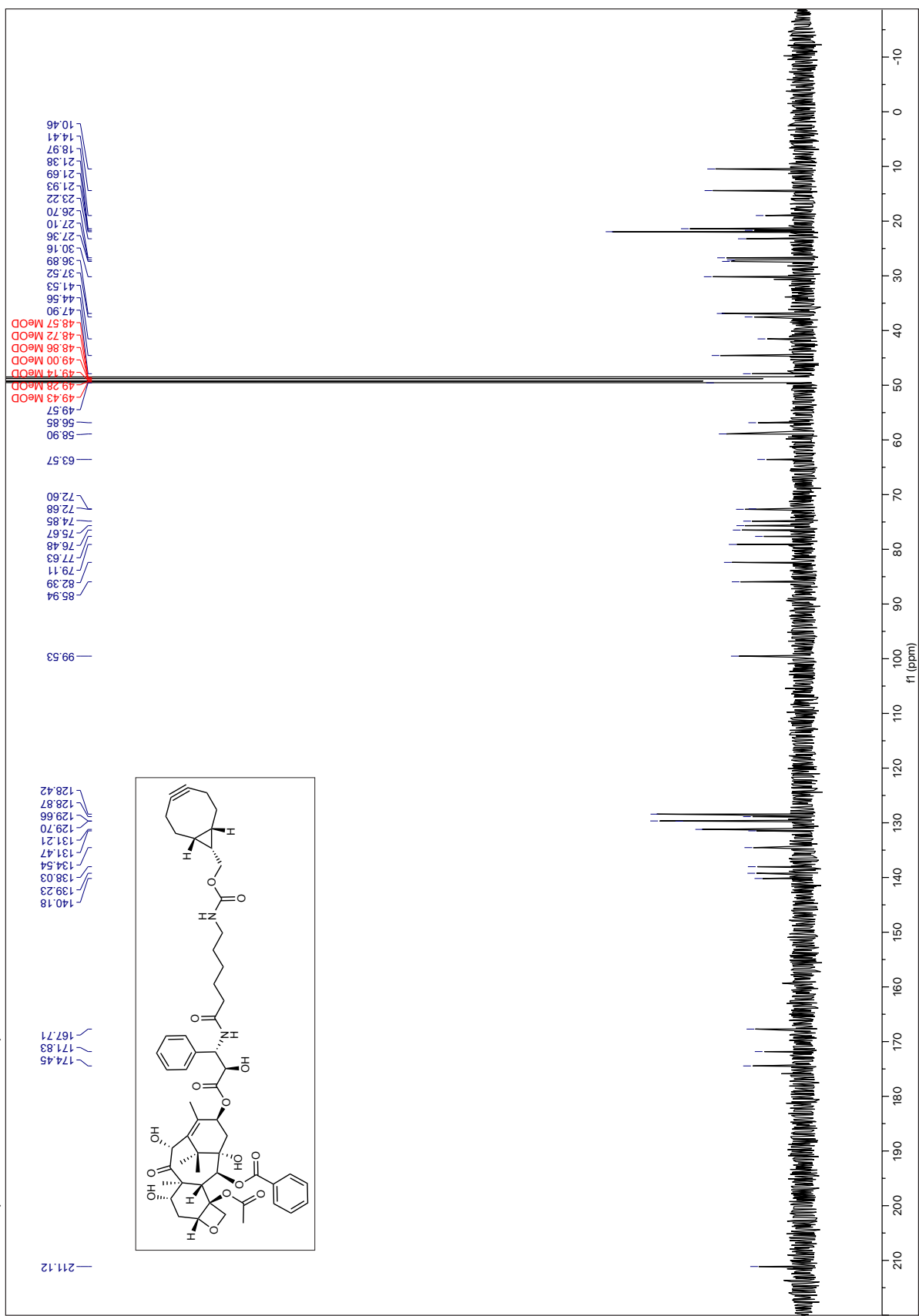


¹³C - NMR (151 MHz, MeOD)

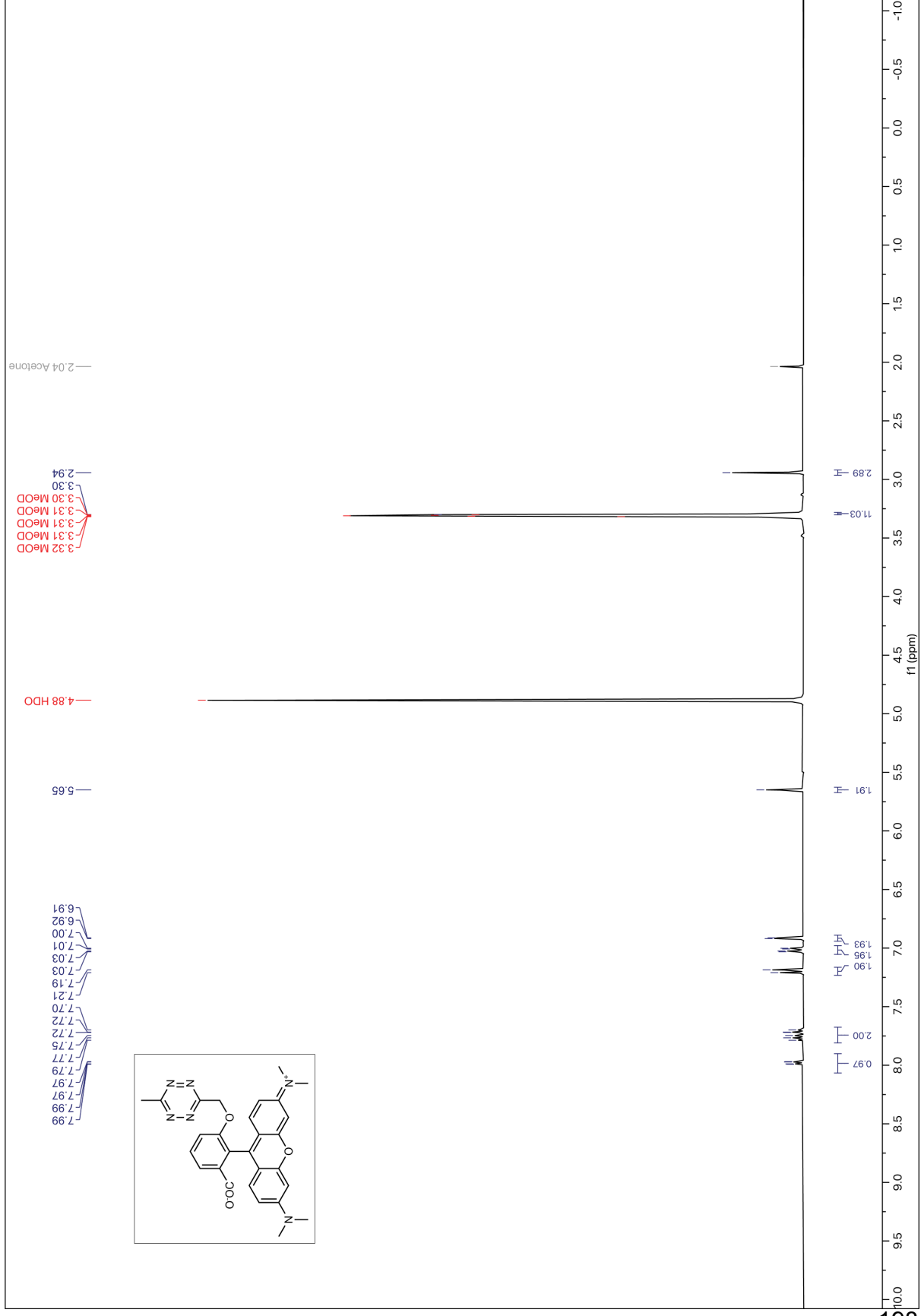




¹³C - NMR (151 MHz, MeOD)



¹H - NMR (400 MHz, MeOD)



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Chapter 4: Conclusions and Outlook

Our ability to visualize a microscopic, dynamic, and complex world drives biological insights. Fluorescence microscopy is a standard tool in any cell biologist's arsenal—even a complex system like a confocal microscope is commonplace in most research labs and organizations. Excitingly, a wave of optical and computational innovation has driven technologies such as super-resolution microscopy^{1,2}, light-sheet microscopy³, and fluorescence lifetime imaging (FLIM)⁴. These technologies allow one to visualize cellular dynamics at unprecedented resolutions, both spatially and temporally. Furthermore, technologies such as FLIM allow one to measure pH⁵, viscosity⁶, and membrane potential⁷, all in a living cell. Central to the success of these technologies are fluorophores—the magic molecules that allow us to visualize this microscopic world.

Fluorophores are the silent champions of cell biology studies—allowing us to highlight and visualize specific organelle or biomolecules. Breakthroughs in fluorophore tagging, such as the evolution of GFP by Roger Tsien⁸, allowed researchers to label and tag biomolecules in living cells, thereby facilitating the study of cell and organelle dynamics. However, with a wave of new microscopy innovations—such as multiplexing, spectral detection, and super-resolution microscopy—fluorophores needed an update to their photostability and targeting strategies.

Enter a wave of fluorophore development and new chemistries—the development of Silicon rhodamine (SiR)⁹ and JaneliaFluors (JF dyes)^{10–12} invigorated the microscopy community. These groundbreaking innovations introduced fluorophores that had tunable spectral properties, far-red excitation, and could withstand high photon budgets; all this to say, they were better for biological experiments. Coupled with self-labeling enzymes, HaloTag and SNAPTag, these new enabled the expansion of the scope of imaging experiments, from time-lapse imaging to live-cell super-resolution imaging. However, genetically encoding these self-labeling enzymes still meant that the fluorophores had to rely on biologically driven targeting strategies.

Small-molecule targeting probes offer an alternative: one that aims to affect intrinsic biology as little as possible. Chapter One details small-molecule and lipid-like tools for fluorescence microscopy. Tools such as HIDE probes^{13–18}, IMPACT¹⁹, turn-on fluorophores^{20–23}, and MemBright²⁴ probes enables to label biomolecules or organelle membranes without the need for transient transfection or protein-based labeling strategies. Such tools make it possible to study primary cell lines and hard-to-transfect cells. Adopting these tools allows biologists to study organelle dynamics and interactions in living cells without interfering with native biology.

Chapter two details the development of one such lipid-like imaging tool, MAO-SiR¹⁸. MAO-SiR is a HIDE probe targeting the inner mitochondrial membrane (IMM). The IMM

poses three challenges: 1) it is a highly dynamic structure requiring high-temporal resolution to track morphological changes, 2) its structure is below the diffraction limit of light, and 3) it is susceptible to environmental changes, especially the generation of reactive oxygen species (ROs) typically encountered when imaging. These challenges seemed apt for the development of a new HIDE probe. We developed and synthesized MAO-SiR and demonstrated that it has an intrinsic ability to target the IMM, is non-toxic, and does not impede mitochondrial function. Furthermore, we used MAO-SiR to image the IMM at super-resolution for extended periods, and we outperformed HaloTag and SNAP-Tag approaches in labeling the IMM. Lastly, we showed that the MAO-SiR was compatible with primary cells such as cardiomyocytes. These data show that MAO-SiR is a preferred alternative to other small-molecule and protein labeling strategies.

HIDE probes demonstrate the advantages of small-molecule and chemically-driven organelle targeting over protein and biologically-driven approaches. In Chapter Three, we adapt this approach to FLIM and demonstrate that it is possible to carry out FLIM multiplexing using all small-molecule tools. FLIM multiplexing overcomes limitations of spectral imaging and allows biologists to image several more targets within a cell simultaneously^{25,26}. Here, we describe the development of a platform that uses bioorthogonal chemistry to induce changes in the tetrazine-triggered fluorophore HD555^{23,27} lifetimes. We show that bioorthogonal ligation partners can predictably change the lifetime of HD555. Then we leverage these changes to image up to three organelles simultaneously, with a single dye and no transfection. These data open a realm of possibilities in terms of dye development and biorthogonal reaction development, potentially ushering in a new wave of small-molecule tool development or tweaks.

Looking forward, these works provide opportunities in across multiple realms. Probe development and application to biological contexts should go hand-in-hand. A simple yet powerful expansion of these technologies, is to simply apply them to new and interesting biological systems that demand them. DiIC₁₆-SiR was an example of using small molecule probes to image organelle dynamics in Nieman-Picks disease cell lines. Using a tool such as MAO-SiR to study organelle dynamics in neurons or cardiomyocytes, would be incredibly powerful.

Beyond simply applying these probes to biological problems, they enable the development and implementation of new tracking and segmentation algorithms—further enabling development of microscopy tools. Additionally, new data from FLIM multiplexing with small-molecules could spurn on a new effort to develop a suite of turn-on probes with higher turn-on differences (which would translate to better lifetime separation). Computational studies could also be employed to determine the correlation between bioorthogonal partners and turn-on.

This dissertation details efforts in the field of developing tools for biologists and microscopists. We've leveraged chemical intuition and a rational design to develop useful and cutting-edge tools for the biological community. We hope that over time, these tools serve that community, and lead to a more equitable, and just future.

References

1. Betzig, E. *et al.* Imaging Intracellular Fluorescent Proteins at Nanometer Resolution. *Science* **313**, 1642–1645 (2006).
2. Hell, S. W. & Wichmann, J. Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. *Opt. Lett.* **19**, 780 (1994).
3. Chen, B.-C. *et al.* Lattice light-sheet microscopy: Imaging molecules to embryos at high spatiotemporal resolution. *Science* **346**, 1257998 (2014).
4. Digman, M. A., Caiolfa, V. R., Zamai, M. & Gratton, E. The Phasor Approach to Fluorescence Lifetime Imaging Analysis. *Biophysical Journal* **94**, L14–L16 (2008).
5. Rennick, J. J., Nowell, C. J., Pouton, C. W. & Johnston, A. P. R. Resolving subcellular pH with a quantitative fluorescent lifetime biosensor. *Nature Communications* **13**, 6023 (2022).
6. Yang, Z. *et al.* A Nile Red/BODIPY-based bimodal probe sensitive to changes in the micropolarity and microviscosity of the endoplasmic reticulum. *Chem. Commun.* **50**, 11672–11675 (2014).
7. Lazzari-Dean, J. R., Gest, A. M. & Miller, E. W. Optical estimation of absolute membrane potential using fluorescence lifetime imaging. *eLife* **8**, e44522 (2019).
8. Heim, R., Cubitt, A. B. & Tsien, R. Y. Improved green fluorescence. *Nature* **373**, 663–664 (1995).
9. Lukinavičius, G. *et al.* A near-infrared fluorophore for live-cell super-resolution microscopy of cellular proteins. *Nature Chemistry* **5**, 132–139 (2013).
10. Grimm, J. B. *et al.* A general method to improve fluorophores for live-cell and single-molecule microscopy. *Nature Methods* **12**, 244–250 (2015).
11. Grimm, J. B. *et al.* A general method to optimize and functionalize red-shifted rhodamine dyes. *Nature methods* **17**, 815–821 (2020).
12. Grimm, J. B. *et al.* A general method to fine-tune fluorophores for live-cell and in vivo imaging. *Nature Methods* **14**, e04236 14 (2017).
13. Erdmann, R. S. *et al.* Super-Resolution Imaging of the Golgi in Live Cells with a Bioorthogonal Ceramide Probe. *Angewandte Chemie-International Edition* **53**, 10242–10246 (2014).

14. Thompson, A. D. *et al.* Long-Term Live-Cell STED Nanoscopy of Primary and Cultured Cells with the Plasma Membrane HIDE Probe Dil-SiR. *Angewandte Chemie International Edition* **56**, 10408–10412 (2017).
15. Takakura, H. *et al.* Long time-lapse nanoscopy with spontaneously blinking membrane probes. *Nature Biotechnology* **35**, 773–780 (2017).
16. Gupta, A., Rivera-Molina, F., Xi, Z., Toomre, D. & Schepartz, A. Endosome motility defects revealed at super-resolution in live cells using HIDE probes. *Nature Chemical Biology* **16**, 408+ (2020).
17. Chu, L. *et al.* Two-color nanoscopy of organelles for extended times with HIDE probes. *Nature Communications* **11**, 4271 (2020).
18. Zheng, S. *et al.* Long-term super-resolution inner mitochondrial membrane imaging with a lipid probe. *Nat Chem Biol* **20**, 83–92 (2024).
19. Liang, D. *et al.* A real-time, click chemistry imaging approach reveals stimulus-specific subcellular locations of phospholipase D activity. *Proceedings of the National Academy of Sciences* **116**, 15453–15462 (2019).
20. Wieczorek, A., Buckup, T. & Wombacher, R. Rigid tetrazine fluorophore conjugates with fluorogenic properties in the inverse electron demand Diels–Alder reaction. *Organic & Biomolecular Chemistry* **12**, 4177–4185 (2014).
21. Wieczorek, A., Werther, P., Euchner, J. & Wombacher, R. Green- to far-red-emitting fluorogenic tetrazine probes – synthetic access and no-wash protein imaging inside living cells. *Chemical Science* **8**, 1506–1510 (2016).
22. Werther, P. *et al.* Live-Cell Localization Microscopy with a Fluorogenic and Self-Blinking Tetrazine Probe. *Angewandte Chemie International Edition* **59**, 804–810 (2020).
23. Werther, P. *et al.* Bio-orthogonal Red and Far-Red Fluorogenic Probes for Wash-Free Live-Cell and Super-resolution Microscopy. *ACS Cent. Sci.* **7**, 1561–1571 (2021).
24. Collot, M. *et al.* MemBright: A Family of Fluorescent Membrane Probes for Advanced Cellular Imaging and Neuroscience. *Cell Chemical Biology* **26**, 600+ (2019).
25. Frei, M. S., Koch, B., Hiblot, J. & Johnsson, K. Live-Cell Fluorescence Lifetime Multiplexing Using Synthetic Fluorescent Probes. *ACS Chem. Biol.* **17**, 1321–1327 (2022).
26. Frei, M. S. *et al.* Engineered HaloTag variants for fluorescence lifetime multiplexing. *Nat Methods* **19**, 65–70 (2022).
27. Hild, F., Werther, P., Yserentant, K., Wombacher, R. & Hertel, D.-P. A dark intermediate in the fluorogenic reaction between tetrazine fluorophores and trans-cyclooctene. *Biophysical Reports* **2**, 100084 (2022).