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Authors
Cosco, Emily D
Caram, Justin R
Bruns, Oliver T
et al.

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Flavylium Polymethine Fluorophores for Near- and Shortwave Infrared Imaging

*Emily D. Cosco, Justin R. Caram, Oliver T. Bruns, Daniel Franke, Rachael A. Day, Erik P. Farr, Moungi G. Bawendi, and Ellen M. Sletten**

Abstract: *Bright fluorophores in the near-infrared and shortwave infrared (SWIR) regions of the electromagnetic spectrum are essential for optical imaging in vivo. In this work, we utilized a 7-dimethylamino flavylium heterocycle to construct a panel of novel red-shifted polymethine dyes, with emission wavelengths from 680 to 1045 nm. Photophysical characterization revealed that the 1- and 3-methine dyes display enhanced photostability and the 5- and 7-methine dyes exhibit exceptional brightness for their respective spectral regions. A micelle formulation of the 7-methine facilitated SWIR imaging in mice. This report presents the first polymethine dye designed and synthesized for SWIR in vivo imaging.*

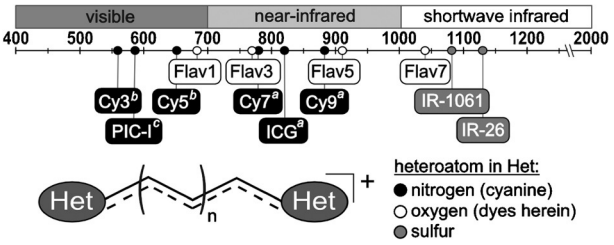


Figure 1. Regions of the electromagnetic spectrum and selected polymethine dyes, positioned by $\lambda_{max,em}$. The numbers in the Cy (structures in Chart S1) and Flav (dyes presented herein) nomenclature indicate the number of methine units in the chain.

Fluorophores emitting at near-infrared (NIR, $\lambda = 700$–1000 nm, Figure 1) wavelengths have been critical to the success of optical imaging in mammals.[1] Compared to visible light, NIR irradiation allows non-invasive, real-time analysis of biological processes due to decreased autofluorescence and scattering.[2] Optical imaging has the potential to become a cost-effective leading tool for clinical diagnoses and surgical guidance if bright, biocompatible, red-shifted fluorophores are developed.[3]

Clinical NIR imaging has been championed by indocyanine green (ICG).[4] The success of ICG is linked to low toxicity and favorable photophysical properties. These properties include the absorption coefficient (ε) and fluorescence quantum yield (Φ_F), which can be combined into a single quantum efficiency value ($QE = \varepsilon\Phi_F$).[5] ICG has a QE_{max} of $1350\,M^{-1}\,cm^{-1}$, providing a brightness benchmark for new NIR contrast agents.[6]

Moving beyond the traditional NIR portion of the electromagnetic spectrum to the shortwave infrared (SWIR, $\lambda = 1000$–2000 nm) region has emerged as a complementary strategy to enhance optical imaging.[7] The superior resolution and depth penetration attained with SWIR imaging were originally validated with carbon nanotubes,[8] rare-earth materials,[9] and quantum dots.[10] However, to translate the advantages of this region to the clinic, non-toxic SWIR fluorophores are necessary. Initial progress toward this goal entailed formulations of the SWIR polymethine dye IR-1061.[11] Dai and co-workers have explored donor-acceptor-donor (D-A-D) benzobisthiadiazole chromophores as SWIR emissive contrast agents.[12] Their work has shown that structural and formulation changes can enhance emission.[13] Despite impressive Φ_F values, the quantum efficiencies of the D-A-D chromophores are limited by their low absorption coefficients. Recently, the effect of high ε values has been highlighted by off-peak SWIR detection of ICG fluorescence surpassing the brightness of early-generation benzobisthiadiazoles.[14]

The high absorption coefficient of ICG is mirrored in other members of its chromophore class, the polymethine dyes. These dyes are charged molecules composed of heterocycles linked by a methine chain (Figure 1). Nitrogen-containing polymethines (cyanine dyes) with 1–5 methine units span the visible region, while 7-methines, such as ICG, reach NIR wavelengths.[15] Cyanine dyes are widely used in microarrays and live-cell microscopy.[16] Further engineering for sensing and photoinitiated drug delivery demonstrates the versatility of the polymethine scaffold.[17]

Despite their promise as bright, tunable fluorophores, polymethines have yet to be optimized as SWIR contrast agents. Lengthening the polymethine chain, a classic method to red-shift cyanine dyes, can compromise Φ_F, decrease stability, and lead to the loss of symmetric electron delocalization.[18] Heterocycle modification is an alternate approach and represents a promising avenue toward stable, SWIR-emissive polymethines.[19] Extending heterocycle conjugation or adding electron-donating groups has been shown to bathochromically shift polymethine dyes.[18,20] Varying the heteroatom from oxygen to other chalcogens results in red-shifted absorption, although increased intersystem crossing due to the heavy-atom effect can compromise Φ_F.[21]

[*] E. D. Cosco, R. A. Day, E. P. Farr, Prof. E. M. Sletten
Department of Chemistry and Biochemistry
University of California, Los Angeles, Los Angeles, CA 90095 (USA)
E-mail: sletten@chem.ucla.edu

Dr. J. R. Caram, Dr. O. T. Bruns, D. Franke, Prof. M. G. Bawendi
Department of Chemistry, Massachusetts Institute of Technology
Cambridge, MA 02139 (USA)

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201706974.

Collectively, these fundamental studies give insight into fluorophore design, which we looked to apply to the benchmark SWIR polymethine, the thiaflavylium dye IR-26.[22] We hypothesized that exchange of the sulfur atom for oxygen would enhance the Φ_F, although it would blue-shift the absorbance. To compensate for this shift, we proposed the addition of an electron-donating dimethylamino group. Thus, we directed our attention to dimethylamino flavylium polymethine fluorophores.

Polymethine dyes are prepared through the introduction of an activated heterocycle to an aldehyde or bis(aldehyde) equivalent. We prepared the requisite 7-*N*,*N*-dimethylamino-4-methyl-flavylium (**1**; Scheme S1 in the Supporting Information)[23] and explored the photophysical scope of the polymethine dyes that can be accessed with this heterocycle. Combining **1** with bis(phenylimine) **2**, malonaldehyde bis(phenylimine), or paraformaldehyde under basic conditions yielded the series of flavylium dyes **3**, **4**, and **5**, respectively (Scheme 1). The synthesis of **3** originally yielded a mixture of highly colored products, which included **5** and **6**. Further investigation revealed that treatment of **1** with only base in ethanol gave a mixture of **5** and **6**. We determined this transformation to be oxygen-dependent (Figure S1 in the Supporting Information) and hypothesize that radical addition of O_2 to the flavylium generates a peroxide that combines with deprotonated **1** to yield **6** and an equivalent of formaldehyde (Scheme S2). This provides unique access to less common monomethine dyes through a single heterocycle.

The dimethylamino flavylium heterocycle resulted in stable polymethine dyes spanning the electromagnetic spectrum from far-red to SWIR wavelengths (Figure 2, Table 1). The flavylium dyes are bathochromically shifted from classic cyanine dyes by approximately 200 nm (Figure 1). The photostability of dyes **3**–**6** were measured under irradiation at 532 nm (530 mW cm^{-2}) and the results indicate that all of the fluorophores display reasonable to excellent photostability (Table S1, Figure S2). Dye **6** absorbs at 650 nm, similar to a 5-cyanine, but has lower ε and Φ_F values, which are consistent with the short polymethine chain.[19] Compound **5** has similar absorption properties to the standard 7-cyanine HITCI. While HITCI has an approximately 10-fold higher Φ_F than **5**,[24] it is 4-fold less photostable (Figure S3 and Table S2). The most intriguing dyes of this series are **4** and **3** due to their far-NIR and SWIR fluorescence. Dye **4** emits at 908 nm, a unique wavelength for polymethines, with high QE_{max} (10000 M^{-1} cm^{-1}) and photostability. Finally, **3** is a true SWIR fluorophore with emission at 1045 nm, an impressive SWIR QE_{max} of 1200 M^{-1} cm^{-1}, and a 10^3 slower per-photon photobleaching rate when irradiated at 1050 nm (16 mW cm^{-2}) compared to 532 nm (Table S3, Figure S4). As a result of its advantageous SWIR photophysical properties, **3** was named Flav7 and further investigated as a contrast agent.

First, we evaluated the emission of Flav7 (**3**) against the SWIR dyes IR-26 and IR-1061. The reported Φ_F values for IR-26 have been inconsistent,[24,26] and the Φ_F of IR-1061 has yet to be thoroughly characterized.[11,27] To establish that Flav7 is more emissive than existing SWIR polymethine dyes, we directly compared the photoluminescence of each dye with a SWIR camera. Solutions of Flav7, IR-26, and IR-1061

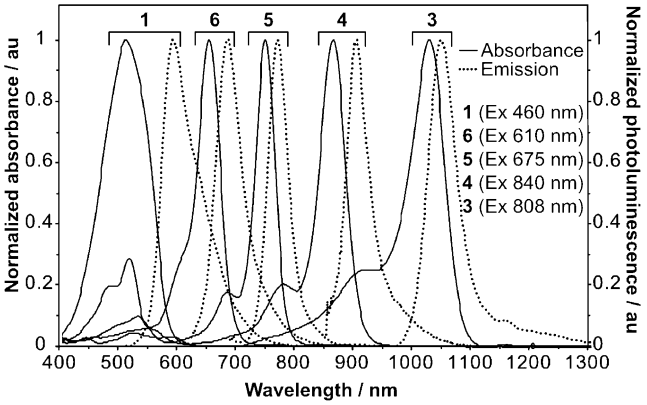
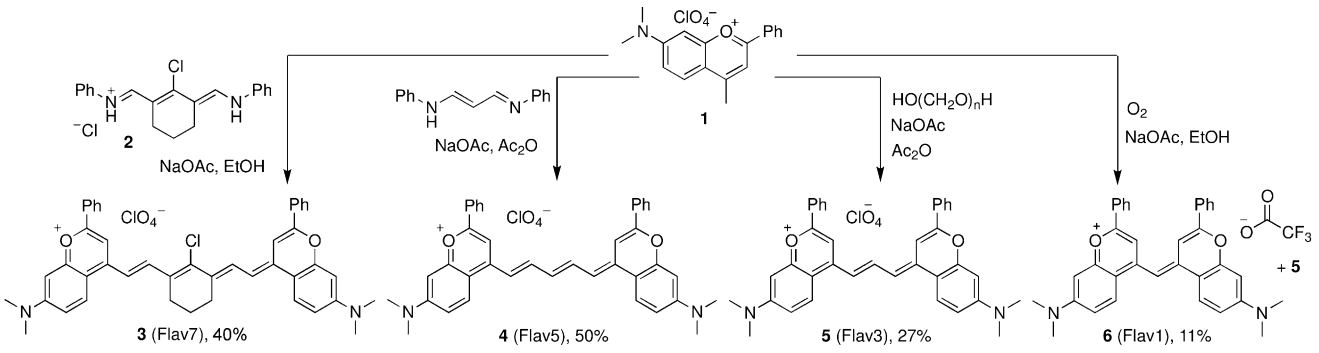


Figure 2. Normalized absorbance (solid) and photoluminescence (dotted) of **1**, **3**–**6** in dichloromethane.[25]

Table 1: Photophysical characterization of **1**, **3**–**6** in dichloromethane.

Compound	$\lambda_{max,abs}$ [nm]	$\varepsilon(\lambda_{max})$ [M^{-1} cm^{-1}][a]	$\lambda_{max,em}$ [nm]	Φ_F [%][a]	QE_{max}[6] [M^{-1} cm^{-1}]
1	510	17000	587	–	–
6	650	16000	684	0.7	100
5	746	220000	766	2.9	6600
4	862	240000	908	5	10000
3	1026	236000	1045	0.53	1200

[a] See the Supporting Information for ε and Φ_F errors.



Scheme 1. Synthesis of dimethylamino flavylium polymethine dyes **3**–**6**.

in dichloromethane with identical absorbance at 808 nm (Figure S5) were excited and imaged over 1000–1600 nm (Figure 3 A). The average intensity was quantified (Figure 3 B) and revealed that Flav7 has the highest Φ_F of the three dyes. These data correlate with absolute Φ_F values determined using an integrating sphere (Figure 3 C).

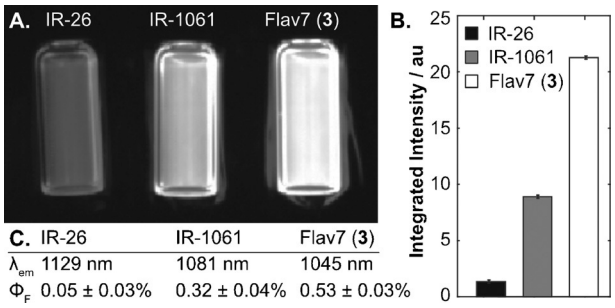


Figure 3. A) Image of vials of IR-26, IR-1061, and **3** with matched optical density at 808 nm in dichloromethane, excited at 808 nm and collected using an InGaAs camera (λ = 1000–1600 nm). B) Average background-subtracted intensity for 10 frames normalized to exposure. C) Absolute Φ_F of the three dyes.

To employ Flav7 for in vivo imaging, a formulation in phosphate buffered saline (PBS) was necessary. We first assayed the stability of Flav7 in water/acetonitrile mixtures. These studies indicated that in the presence of water, Flav7 undergoes aggregation, but the monomer appears stable (Figures S6–S9),[28] which suggests that Flav7 is suitable for micelle encapsulation. Building upon prior work with mPEG-DSPE lipids,[11] we prepared micelles containing Flav7 in PBS. The formulations displayed broad absorption from 700–1100 nm, yet sharp emission at 1075 nm from monomeric Flav7 (Figure S10).[29] We assayed the biocompatibility of the formulations in mammalian cell culture and found no significant difference between empty and Flav7-loaded micelles (Figure S11).

SWIR imaging in mice was performed with the fluorescent formulation of Flav7. Nude mice were intravenously injected with Flav7 micelles and immediately imaged with 808 nm excitation and 1000–1600 nm detection (Figure 4 A,B, Videos S1–S4, Figures S12–S15). The characteristic high spatial and temporal resolution of the SWIR region[8–11] is evident in the vasculature of the hind limb and the ability to quantify the heart rate of the anesthetized mouse (Figure 4 C,D). These results illustrate that the flavylium scaffold is a promising new addition to SWIR contrast agents.

In summary, we have designed a new class of polymethine dyes with dimethylamino flavylium heterocycles. These dyes are notably red-shifted compared to prevalent cyanine dyes, and expand the opportunities for imaging and detection at NIR and SWIR wavelengths. The hallmark fluorophore is the 7-methine (Flav7) which is 13 times brighter than IR-26, the current SWIR benchmark. The in vivo performance of this new contrast agent was assessed through the detection of dye-containing micelles. With these studies, we have achieved the brightest SWIR polymethine employed for imaging to date.

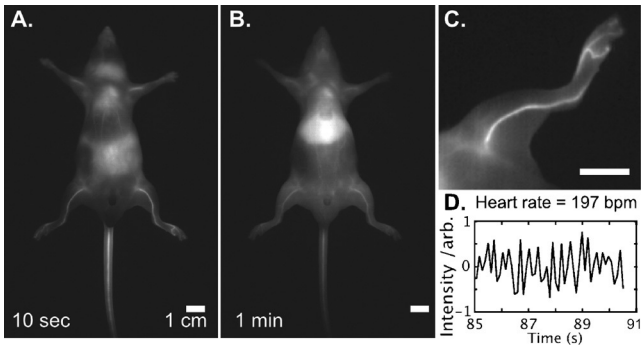


Figure 4. Images of nude mouse following tail-vein injection of Flav micelles with diffuse 808 nm excitation and collection on an InGaAs camera (λ = 1000–1600 nm) at 9.17 frames s^{-1} (see Videos S1–S4). Average background-subtracted images were taken for 10 frames at 10 s (A) and 1 min (B) after injection. C) Close-up image of the hindlimb from (A). D) Intensity versus time plot of signal from the heart region. mPEG-DSPE = methoxy-poly(ethylene glycol)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N.

As a class, polymethine dyes show distinct advantages as organic SWIR contrast agents due to their high absorption coefficients, which lead to large quantum efficiencies. Furthermore, small Stokes shifts provide potential for both SWIR excitation and detection. These qualities, coupled with the clinical success of ICG and the results herein, suggest that polymethine fluorophores are poised to translate SWIR diagnostics into the clinic.

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Conflict of interest

The authors declare no conflict of interest.

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