Tips on Making Tiny Tips: Secrets to Submicron Nanoelectrospray Emitters

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Abstract

Nanoelectrospray ionization emitters with submicron tip diameters have significant advantages for use in native mass spectrometry, including the ability to produce resolved charge-state distributions for proteins and macromolecular complexes from standard biochemical buffers that contain high concentrations of nonvolatile salts and to prevent non-specific aggregation that can occur during droplet evaporation. We report on various factors affecting the tip morphology and provide tips to producing and using emitters with submicron tips. Effects of pulling parameters for a Sutter Instrument P-87 tip puller on the resulting tip diameter and morphology are shown. The "Pull" parameter has the largest effect on tip diameter, followed by "Velocity", "Pressure", and "Heat", whereas the "Time" parameter has minimal effect beyond a lower threshold. High "Pull" values generate emitters with multiple tapers, whereas high "Velocity" values generate a tip with only a single tapered region. A protocol for producing reproducible emitters in the submicron size range, as well as guidelines and tips for using these emitters with standard biochemical buffers that contain high concentrations of non-volatile salts is presented with the aim of expanding their use within the native MS community.

Introduction

Nanoelectrospray ionization (nESI) is widely used in native mass spectrometry to produce gaseous ions of biomolecules and intact macromolecular complexes from aqueous solutions. The majority of nESI emitters that are currently used have tip diameters substantially larger than a micron. Electrospray emitters with submicron tip diameters have several demonstrated advantages, including the ability to desalt macromolecular ions from biochemical buffers consisting of high concentrations of nonvolatile salts,[1–6] prevent aggregation inside electrospray droplets to identify complexes that exist in solution,[7–9] and improve measurements of dissociation constants of metal ions or ligands bound to biopolymers.[6,10–14] Narrow bore emitters have been used to sample nanoliters of solution and to rapidly mix two solutions to investigate fast processes on the 1 to 100 μs timescale.[15,16]

Despite these advantages, emitters with submicron tips are not widely used, in part due to reported issues with clogging and complications with obtaining reproducible tip diameters and ion currents. Differences in performance between labs may in part be related to the lack of a standard protocol for tip pulling and effects of ambient conditions, which may lead to different emitter morphologies. Detailed here is a guide to producing nESI emitters with tips that have submicron diameters as well as recommendations on using these emitters for native MS. This guide is in response to questions from many in the native MS community and incorporates notes from the Sutter Instrument manual,[17] published tip puller parameters,[13,18] and a helpful guide on tip pulling by Erin M. Panczyk.[19]

Methods

Thin-walled, filamented borosilicate capillaries (1.0 mm outer diameter, 0.78 mm inner diameter, Sutter Instrument, Novato, CA, BF100-78-10) were pulled using a Flaming/Brown P-87 micropipette puller (Sutter Instrument) equipped with a box-filament (FB255B). Tip pulling parameters were systematically varied to determine the effects of each parameter on tip size with the goal of producing a range of small tip sizes. The cooling gas (dry air) pressure was varied between 100 and 350. For each set of parameters, four tips were imaged using a Hitachi TM-1000 scanning electron microscope (Schaumberg, IL) in the Electron Microscopy Lab at University of California, Berkeley. The resulting images were analyzed in ImageJ (v. 1.53k) using the "Find Edges" function to enhance the contrast between the inner and outer diameters of the tip.

Results and Discussion

Effects of Tip Puller Parameters

In the following discussion, the words capillary, emitter, and tip are used to refer to the borosilicate tube prior to pulling, the pulled electrospray emitter, and the pulled end of the emitter, respectively. Five pulling parameters were varied independently to determine their effects on the final tip diameter. The starting parameters (Heat = 545, Pull = 0, Vel = 70, Time = 235, Pressure = 300) were chosen because they produce highly reproducible tips with an inner diameter of 2.33 ± 0.02 μm. Readers may find Figure 10 in the P-87 user manual (Rev. 0299c (20081016)) helpful for understanding each of the following parameters,[17] which are briefly described here. The "Pull" parameter controls the force applied to the pulley arms and has the largest effect on tip diameter. An increase from 0 to 150 results in a reduction in the tip diameter from 2.33 ± 0.02 μm to 350 ± 50 nm, respectively. Both low and high values produce a similar

initial taper (Figure 1a inset, blue box), but higher values produce a distinctly different, narrower taper closer to the end of the tip (Figure 1a inset, orange box). The "Velocity" parameter controls when the filament is turned off during the initial capillary deformation and initiates the flow of the cooling gas 40 ms prior to the start of the "Pull" event. This parameter has the second largest effect. An increase from 56 to 230 reduced the diameter from 2.5 ± 0.1 μm to 760 ± 60 nm, respectively (Figure 1b). Increasing "Velocity" increases the length of the taper but does not produce a second tapered region. In general, emitters with shorter tapers are easier to load with sample although some difficulties have been reported for emitters with very short tapers.[11] Increasing the "Heat" parameter, which controls the current supplied to the heating filament, from 535 to 575 resulted in a small decrease in the diameter from 2.3 ± 0.2 μm to 1.9 ± 0.1 μm with no significant change in the taper (Figure 1c). However, the reproducibility improved significantly at intermediate "Heat" values to a minimum of 2.33 μm ± 20 nm at a value of 545. Adjusting the cooling gas pressure from 350 to 100 results in a longer taper and a reduction in the tip diameter from 2.4 ± 0.5 μm to 660 ± 30 nm (Figure 1d). The tip diameters are less reproducible at a pressure setting higher than 300. This may be due to over-cooling during the heating step, resulting in the capillary snapping instead of separating into a fine point. The "Time" parameter controls the length of time that cooling gas is on (in units of ½ ms) during the pulling process. Above a lower threshold value, the "Time" parameter has very little effect on the resulting tip diameter. Changing "Time" from 235 to 125 resulted in no significant difference in tip diameter. Below a value of ~100, the tip is inadequately cooled during pulling and is drawn into a thin glass fiber with no discernable tip opening. Values above this minimum result in similar tip sizes. Overall, higher "Velocity", "Heat" and "Pull" settings, and lower cooling gas pressures, will result in smaller tip diameters (Table 1).

It is important to note that if the "Heat" or "Velocity" parameters are near a threshold value, then changes in ambient laboratory conditions may cause the instrument to cycle through multiple program loops, which will change the final tip diameter.[17] The ambient temperature, humidity, and temperature of the filament mounting block can all influence the number of loops, so it is recommended to optimize parameters to be distant from the looping threshold.

The reproducibility in tip sizes that are produced can be high. The average standard deviation (s.d.) in the diameter of the 21 emitters used to obtain the above data was ~6.8%. After characterizing the trends for each of the pulling parameters, emitters with tips as small as 220 ± 8 nm (s.d. = 3.6%) were made (Table 1). A reliable spray was maintained for ~30 min in variable-temperature ESI measurements of cytochrome *c* in 20 mM 7:3 sodium chloride:Tris buffer with these emitters.

Tips for Making Submicron Diameter Emitters

The parameters required to make reproducible emitters with submicron tips will differ for each capillary pulling instrument depending on the heating mechanism (filament or laser), orientation of the heating element with respect to the capillary, composition of the capillary, and a myriad of other factors.[17] Thus, the optimal parameters must be determined for each puller. Emitter composition (borosilicate vs. quartz) has minimal effect on the performance of emitters with submicron tips.[20] However, Na^+ may leach into solution with borosilicate emitters, and lead to some salt adduction and reduced signals.[20] This does not occur with quartz due to lower sodium content (~0.2 ppm).[20] Emitter openings should be broken by the tip puller because diameters can be irreproducible when the tips are clipped manually.[20] The following protocol

may assist users in finding parameters that reproducibly generate emitters with submicron tip diameters:

1. Run a ramp test using the capillary of choice; thin-walled, filamented borosilicate capillaries were used here. Sutter Instrument recommends filamented glass for emitters pulled below 3 µm in diameter.[21] Subtract 20 units from the ramp test value and use this as the starting "Heat" parameter.[17] To start, set the "Pull" parameter to 0 and set the "Velocity" parameter to a value between 80 – 120 that results in the capillaries being pulled in <8 s.[17]
2. Identify the lowest "Time" parameter that generates usable tips. On our device, this value is ~125. Set Time value to be ~100 units higher than this to ensure reproducible tips. For ultra-fine tips (below 200 nm), Sutter Instrument recommends setting the "Time" parameter to only 5 units higher than the lowest value that generates a usable tip.[17]
3. Vary the "Heat" parameter to determine the optimum value that results in the most reproducible emitter diameters. The "Heat" parameter should not be more than ~100 units lower than "Ramp" to avoid uneven snapping of the tips.
4. Vary the "Velocity", "Pull", and "Pressure" parameters systematically until tip diameters of the desired size are obtained. Typically, large "Pull" or "Velocity" values (>150) are necessary to reach tip diameters smaller than 400 nm. Large "Pull" and "Velocity" values are necessary to achieve similar size tips with theta glass capillaries. Lower "Pressure" values may be used to obtain smaller diameters as well, but result in a longer taper.
5. Run the ramp test every six months and check a minimum of three emitter tip diameters on both sides of the pulled capillary once every three months to ensure even pulling. The

condition of the cooling gas desiccant is also important for tip reproducibility. Frequent checking of the emitter tip diameters is recommended to ensure precise values.

This protocol should also be applicable to other filament-based capillary pulling instruments, such as the Sutter Instrument P-97 or P-1000. For laser-heated capillary pullers, such as the P-2000 from Sutter Instrument, the "Pull", "Velocity", and "Heat" parameters should follow similar trends to those shown here, albeit with different absolute values. However, the P-2000 also has two additional parameters: the "Filament" parameter, which adjusts the scanning pattern of the laser, and the "Delay" parameter, which adjusts the time between the laser turning off and the hard pull. Users of laser-heated capillary pulling instruments must also optimize these additional parameters.

Tips for Using Submicron Emitters for Native Protein MS

Care must be taken when loading the emitters and during electrospray in order to obtain native protein charge-state distributions. The following advice may help users maintain stable ion signal and achieve effective protein desalting.

1. Sample can be loaded into an emitter using a 10 µL syringe to deposit the sample near the beginning of the taper and avoid bubbles entering the tapered portion of the tip. Then, the emitter can be shaken or a centrifuge can be used to move the sample down to the tip and remove bubbles. Small bubbles can be visualized by holding the emitter up to a light. To avoid clogs from solution contaminants, we recommend filtering all buffers through a 0.22 µm filter prior to use.[20] Centrifuging the sample and pipetting from the bulk of the solution

(rather than near the surface or bottom of a sample container) may also reduce clogging of the emitters.

2. High spray voltages and short distances between the emitter and MS inlet can result in discharge due to the large electric field at the tip, breaking the emitter and interrupting spray. Spray may still occur but will typically be unstable and result in larger droplets, as evidenced by increased aggregation of analyte molecules resulting in higher abundance clusters in mass spectra.[8] We recommend positioning emitters ~3 – 5 mm from the instrument inlet and slowly increasing the spray voltage until there is stable ion current, typically at values of ~0.4 – 0.6 kV for emitters <500 nm in diameter (voltage difference referenced to instrument inlet). For emitters smaller than 1 μm, spray voltages in excess of 1.4 kV within 5 mm of the instrument inlet have a high risk of discharge between the emitter and instrument. A slight reduction in voltage (100 – 200 V) a few seconds after the spray stabilizes can improve the stability of the total ion signal.
3. In our experiments, spray voltage is provided via a platinum wire inserted into the back of the capillary that is in contact with the solution (Figure 2). This avoids the need to coat the emitter tips with an electrically conductive material to establish electrical contact. Metal-coated emitters with submicron diameter tips can also desalt ions effectively. They have the potential advantage of eliminating cross-contamination between samples,[14] although we have never observed contamination as a result of re-using the platinum wire. However, the metal coating can make visualizing bubbles in the tapered region more difficult.
4. Surface effects have been observed when using emitters with submicron tips as a result of protein-glass interactions. This can lead to time-dependent protein signals due to differences

in absorption and higher protein charging due to surface-induced unfolding.[22,23] It is important to keep these effects in mind when analyzing samples using these emitters.

5. When solutions containing high concentrations of nonvolatile salts are used with emitters that have small tips diameters, protein ion desalting should occur spontaneously within the distances and voltages given above. The desalting phenomenon is characterized by a large drop in the total ion signal (primarily salt clusters), significant reduction in salt clusters in the mass spectrum, and resolved protein charge-state distributions. The onset of desalting occurs more readily with decreasing tip diameter and smaller emitter tip diameters leads to less salt adduction.[1] The effect of desalting and tip size is illustrated in Figure 3, which shows a spectrum of 10 μM yeast alcohol dehydrogenase obtained from a 150 mM NaCl/25 mM Tris solution using emitters with either a 9.2 μm (top) or 220 nm (bottom) tip. For the emitter with the larger tip diameter, the ion count is high (8.8×10^5) but any protein signal that may be present is obscured by abundant signal from large, unresolved salt clusters. In contrast, the ion count obtained with the emitter with the smaller tip diameter is significantly lower (1.8×10^4). Signal from salt clusters at higher m/z is dramatically reduced and charge-state distributions for the monomer, dimer and tetramer of the protein, from which their respective masses can be determined, are obtained.

This document encompasses advice gained over years of experience pulling and using submicron diameter emitters. We hope that this aids in fostering discussion about the issues encountered when using submicron emitters and encourages their more widespread use in the analysis of proteins and protein complexes by native MS.

Acknowledgements

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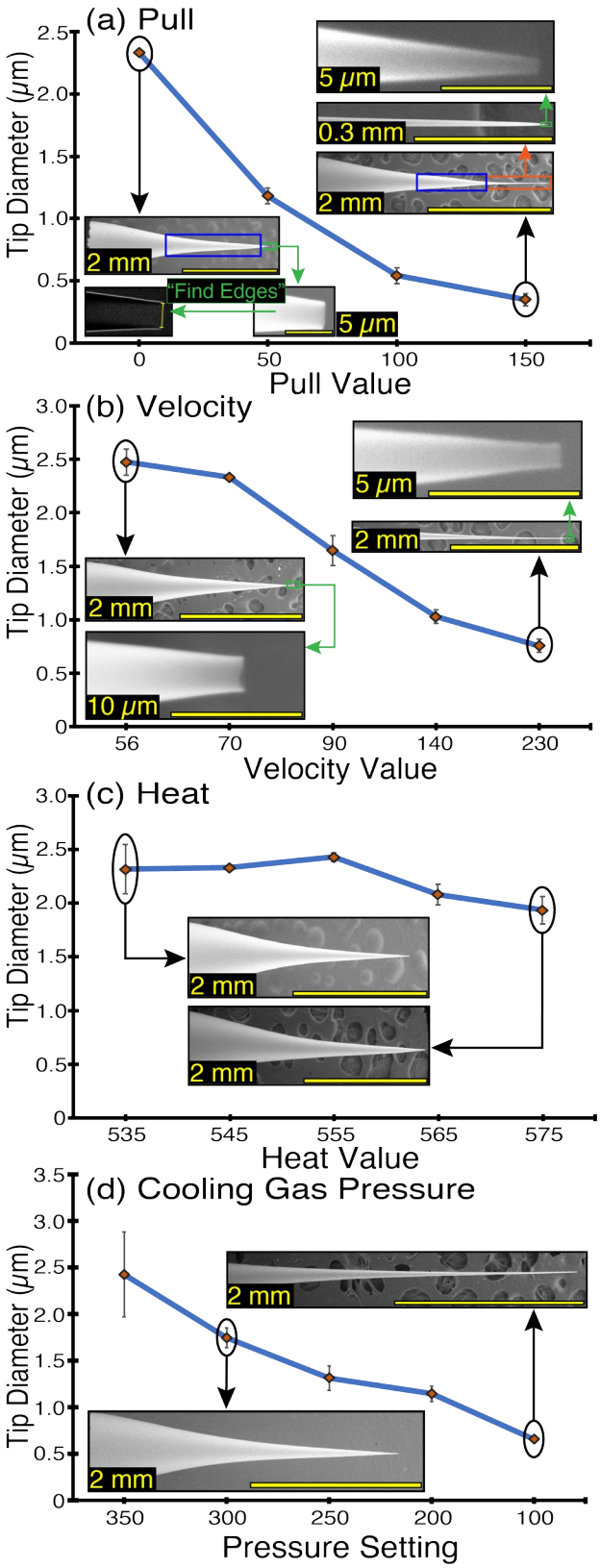

(a) Pull
Tip Diameter (μm)
Pull Value
5 μm
0.3 mm
2 mm
2 mm
"Find Edges"
5 μm
(b) Velocity
Tip Diameter (μm)
Velocity Value
5 μm
2 mm
2 mm
10 μm
(c) Heat
Tip Diameter (μm)
Heat Value
2 mm
2 mm
(d) Cooling Gas Pressure
Tip Diameter (μm)
Pressure Setting
2 mm
2 mm

Figure 1. The effect of tip puller parameters on the resulting inner diameter of the nESI tip: (a) Pull, (b) Velocity, (c) Heat, (d) Cooling Gas Pressure. Insets show the emitter morphology and close-up views of the tips. Numbers in yellow denote the size of the scale bars.

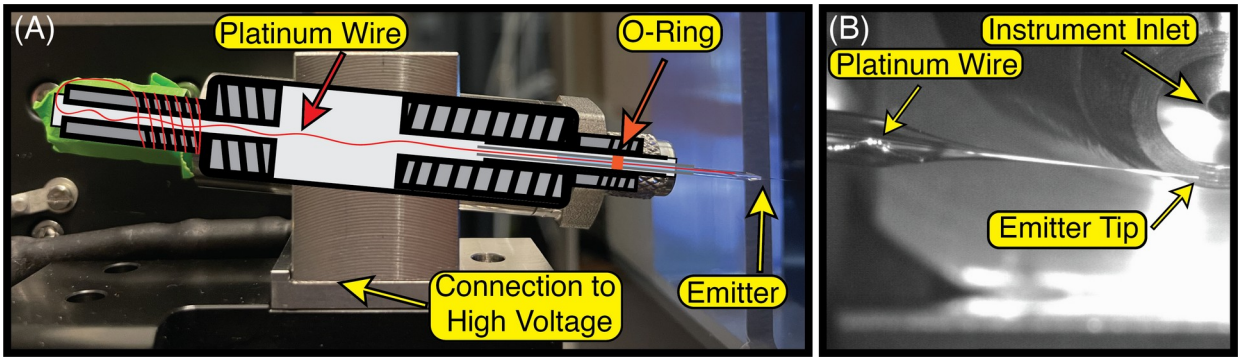


Figure 2. Photographs of (a) a Waters source block with a graphic cutaway illustrating the connection of the high voltage from the power supply to the solution by means of a platinum wire that is inserted into the solution and (b) a close-up view of an electrospray emitter with a 220 nm diameter tip and with a platinum wire inserted that is aligned to the inlet of a Waters Q-TOF Premier mass spectrometer used to obtain the data shown in Figure 3.

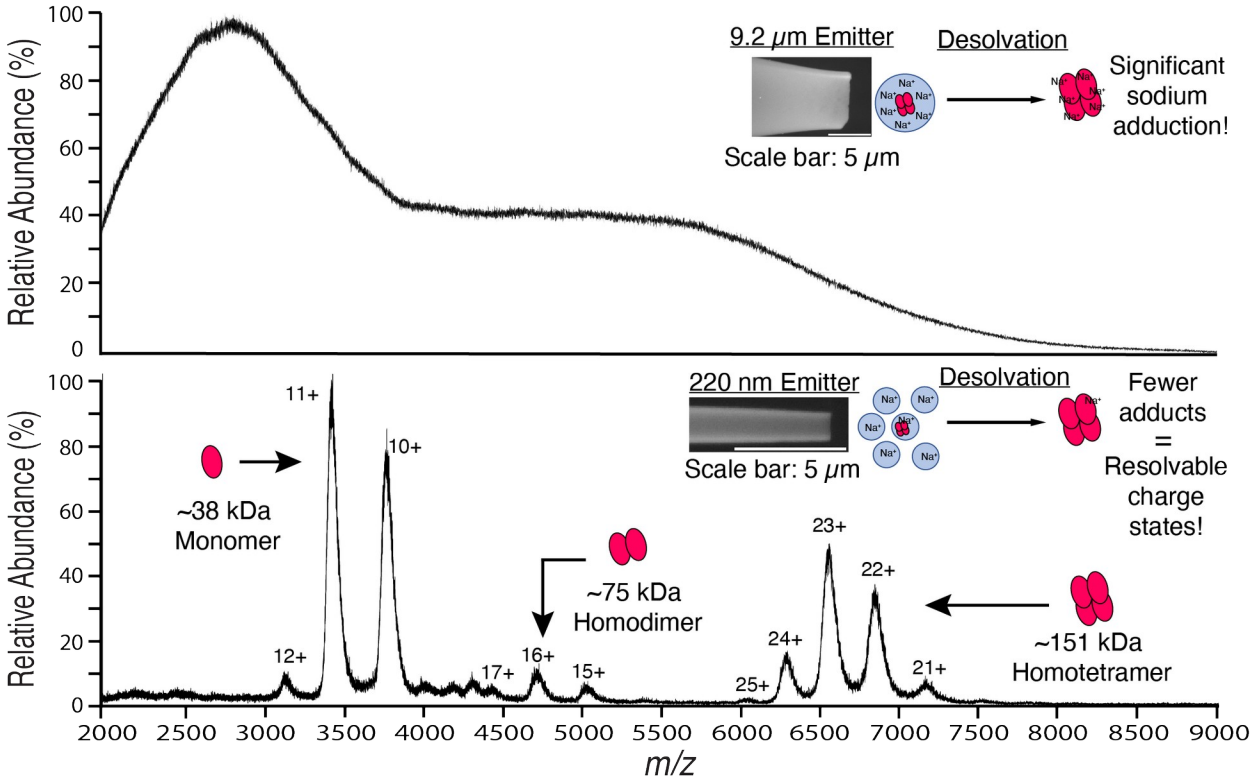


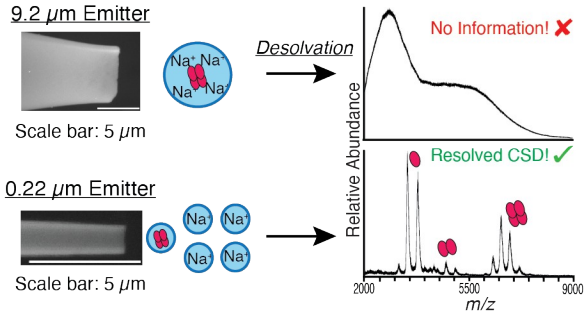
Figure 3. Electrospray mass spectra of 10 μM yeast alcohol dehydrogenase in 150 mM NaCl/25 mM Tris buffer solution (titrated to pH = 6.8) obtained using an emitter with a 9.2 μm (top) or 220 nm (bottom) diameter tip obtained with electrospray voltages of 1.1 kV and 0.5 kV, respectively. In both cases, spectra were acquired at the lowest voltage where consistent signal was obtained.

Table 1. Average Diameters and Standard Deviations (S.D.) of Electrospray Emitters Prepared Using Various Parameters in Order of Descending Emitter Diameter.

Heat	Pull	Velocity	Time	Pressure	Average Diameter (µm)	S.D. (µm)
545	0	56	235	300	2.48	0.12
555	0	70	235	300	2.43	0.040
545	0	70	235	350	2.43*	0.46
545	0	70	235	300	2.33	0.021
535	0	70	235	300	2.32	0.23
565	0	70	235	300	2.08	0.097
575	0	70	235	300	1.94	0.13
545	0	70	235	300	1.75*	0.10
545	0	90	235	300	1.65	0.14
545	0	70	235	250	1.31*	0.13
545	50	70	235	300	1.18	0.064
545	0	70	235	200	1.15*	0.083
580	0	230	235	300	1.06	0.037
545	0	140	235	300	1.03	0.064
545	0	230	235	300	0.756	0.059
545	0	70	235	100	0.655*	0.029
580	175	230	235	300	0.550	0.022
545	100	70	235	300	0.542	0.062
545	150	70	235	300	0.350	0.049
580	200	230	235	300	0.250	0.010
580	250	230	235	300	0.223	0.008

* Diameters marked with asterisk were measured approximately 6 months after other sizes.

TOC Graphic


9.2 μm Emitter
Scale bar: 5 μm
Desolvation
No Information!
Resolved CSD!
Relative Abundance
0.22 μm Emitter
Scale bar: 5 μm
2000
5500
9000
m/z

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