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Commentary

Expanding Native Mass Spectrometry to the Masses

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ABSTRACT: At the 33rd ASMS Sanibel Meeting, on Membrane Proteins and Their Complexes, a morning roundtable discussion was held discussing the current challenges facing the field of native mass spectrometry and approaches to expanding the field to nonexperts. This Commentary summarizes the discussion and current initiatives to address these challenges.

INTRODUCTION

Native mass spectrometry (nMS) has emerged as a powerful technique to study noncovalent complexes including protein·protein, protein·ligand, and protein·RNA/DNA complexes. The high precision of nMS enables rapid molecular-level assessment of binding stoichiometry, sample heterogeneity, and many other kinds of higher-order structural information especially when coupled with orthogonal separation and ion activation techniques. Recently, growing analytical needs for quality control of macromolecular biotherapeutics have propelled advances in nMS instrumentation and workflow development. The efficacy of biotherapeutics such as monoclonal antibodies, antibody-drug conjugates, and adeno-associated viruses relies on both chemical composition (e.g., primary amino acid sequence), and higherorder three-dimensional structure. nMS is rapidly moving from a technique only a handful of laboratories could perform, on in-house modified instrumentation, to a technique used across the globe on specialized, but commercially available, instrumentation. As more scientists from other disciplines adopt these methods, the nMS community is presented with a greater challenge of establishing standardized practices for the various steps of nMS analysis.

CURRENT CHALLENGES IN THE FIELD

When discussing the main challenges facing the field of nMS, and in particular the barriers to new users, the general consensus of the attendees of the discussion was that the biggest challenges are "*everything pre-MS*" namely sample preparation, cleanup, and introduction into the mass spectrometer, and in particular when trying to scale up these approaches to make nMS a higher throughput method. However, it is also important to consider the challenges that scientists will face during and after nMS data acquisition (see Table 1). Data acquisition requires simultaneously tuning several instrument parameters, such as pressures in various stages of the mass spectrometer and optimizing instrument transmission voltages (e.g., DC and RF settings) to maximize ion transmission, while preventing activation of fragile native ions. This process varies from instrument to instrument depending on the manufacturer, instrument geometry, and hardware, requiring instrument-specific standard operating procedures using standardized analytes. Lastly, nMS data processing is not as automated as bottom-up proteomics approaches. Spectra of complex mixtures can be challenging to accurately annotate. Like bottom-up data analysis, care must be taken when using software to ensure that appropriate settings are used, and annotations are correct. With the limited space here we did not discuss other exciting directions of nMS including integration with other techniques (e.g., ion mobility, fragmentation techniques, online separation methods, charge detection MS) that allow more complex samples to be characterized. The purpose of this commentary is to assess these challenges, and initiate a dialogue within the broader nMS community about:

- Starting an online forum for the community to engage on various topics
- establishing consensus protocols and standards for nMS, and contributing them to a central storage (e.g., in the community forum)

The online forum can serve as a medium for the discussion of the second point and to organize a community wide effort to populate the database with protocols and standard data. Other communities such as the Consortium for Top-Down Proteomics have recently taken similar steps to share optimized protocols, and interlaboratory top-down proteomics data for a small set of standard proteins acquired on different instruments to lower the bar of entry for users new to native top-down proteomics.[1]

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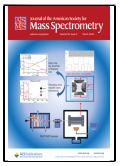

Table 1. Challenges Associated with Native Mass Spectrometry

Pre-MS Analysis	MS Analysis	Post-MS Analysis
• Sample preparation ○ Buffer compatibility ○ Clean-up procedure ○ Sample quantity ○ Sample purity • Sample Introduction ○ Choice of ESI source (nESI vs ESI etc.) ○ Electrospray ionization conditions (flow rates, source temperature, entrance voltages, emitter position)	• Signal optimization • Maintaining "native" conditions • Quality control of measurement ○ Ensuring ESI stability ○ Recognizing low quality signal	• ESI deconvolution • Assessing deconvolution accuracy/fit • Addressing differences in measured versus expected mass

Sample Preparation. Sample preparation for nMS can be a time-consuming process, often requiring multiple rounds of optimization. There is yet to be, and may never be, a single "one-size fits all" approach that works for all types of samples (or even all samples of the same type), and different approaches often have to be used for different types of samples. For example, membrane proteins may need additional steps of sample preparation namely exchange into a mass spectrometry compatible detergent or membrane mimetic,[2,3] while RNA samples typically need an ethanol precipitation step to remove nonvolatile salts. These steps are typically taken prior to the buffer exchange. Multiple methods exist for buffer exchange, including offline approaches such as spin columns, diafiltration, dialysis, and online desalting approaches using size exclusion chromatography or rapid size exclusion chromatography. The NIH-funded nMS Guided Structural Biology Center has begun to assemble such protocols on their web site (https://nativems.osu.edu/protocols) as a starting point for new users. Products that are commonly used for these sample preparation steps are listed:

- BioRad Bio-Spin P-6 Desalting Columns
- Thermo Fisher Scientific Zeba Spin Desalting Columns
- Millipore Amicon Ultra Centrifugal Filters
- Sartorius Vivaspin Centrifugal Concentrators
- Pierce Microdialysis plates
- Slide-A-Lyzer Dialysis Cassettes
- Thermo Fisher Scientific NativePac OBE-1

Online approaches to buffer exchange[4] can be useful for screening large sample sets for accurate mass or oligomeric state determination, however, the short elution times can limit the number of complementary experiments that can be performed (e.g., dissociation at multiple energies). A recent publication[5] demonstrates the capability of conducting high-throughput nMS-based workflows using an Agilent Technologies RapidFire 400 auto-sampler coupled to a Agilent Technologies 6560c drift tube ion mobility-mass spectrometer. The study demonstrates that the RapidFire can perform online buffer exchange, produce native-like ions comparable to nESI, and carry out multi-step nMS workflows on a sub-minute timescale. While the study does not demonstrate a high-throughput campaign, the results suggest it could be feasible.

A hurdle to online approaches, particularly within a core facility or to those just beginning in the field, can be the requirement for an LC, which can sometimes be cost prohibitive. More recently a simple infusion platform using a single isocratic pump has been presented for use with online buffer exchange and offers a lower cost alternative to an LC for such studies.[6] Online desalting approaches remain the highest throughput and would have the greatest appeal to those wishing to create a walk-up or open access facility.

In addition to the method of buffer exchange, consideration must be given to the concentration of both analyte and buffer. Nonspecific oligomers can form due to high concentrations of protein in a single droplet. For example, two co-exisiting monomers in one droplet can form a non-specific dimer to satisfy the protein-solvent interactions lost during droplet evaporation. This is especially a concern when the initial droplets are larger in diameter (e.g., from high flow electrospray), increasing the likelihood of a single droplet carrying two or more proteins. Previous studies have explored this aspect of nMS closely, and the reported outcomes are worth consideration while designing an experiment.[20] Such nonspecific interactions can adversely impact data interpretation when obtaining quantitative information such as binding affinities. Similarly, previous work has demonstrated that ionic strength of the electrolyte solution in which the sample is prepared can also affect the conformation(s) and oligomeric state of biomolecular complexes.[7,8] In addition, charge state has also been shown to play an important role in conformations adopted.[9] The most commonly used sample solution in native MS is ammonium acetate, with concentrations often in tens to a few hundreds of millimolars. Other volatile salts such as ammonium bicarbonate and ammonium formate have also been used. These salts do not buffer at pH 7. The sample solution pH can slowly change during electrospray, and can affect samples that are extremely sensitive to pH.[10,11] Finally for membrane proteins the detergent, membrane mimetic, pH, or cofactors are all important and can affect the oligomeric state observed.[12,13] There are multiple solution parameters, therefore, that may have to be optimized depending on the sample and the goal of the experiment. The introduction of variable-temperature ESI further illustrates the need for standard conditions for native MS experiments; conformations of proteins and complexes can be altered at both low and elevated temperatures. Additionally, variable-temperature ESI enables accurate thermodynamic analysis of solution phase biomolecular stabilities (folding and unfolding) as well as protein ligand binding.[14]

Sample Introduction. The majority of nMS experiments in the literature employ nanoelectrospray ionization (nESI), which has low sample concentration and volume requirements (ul of sample, high nM to low uM concentrations). nESI also requires lower capillary voltage (~0.7 −1.5 kV) and source desolvation gas temperature, preserving the structure and noncovalent interactions of biomolecular ions. nESI typically involves the use of glass capillaries, which are prepared in-house using micropipette pullers. However, glass capillary nESI emitters are also available for purchase from instrument vendors and specialized companies. LC-compatible microflow multispray emitters are also available for use with low-flow LC sample introduction.[15−17] Despite these options, relying on purchased emitters

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long-term is often cost prohibitive to those beginning in the field or to core-facilities hoping to offer a new service. Automated high-throughput sample introduction instruments exist (e.g., Advion Triversa Nanomate, Agilent Technologies Rapidfire) which use liquid handling robotics to withdraw samples from a 96-well plate, desalt the sample online, and deliver to ESI emitters. While highly promising, these higher throughput solutions are often prohibitively expensive and usually rely on higher flow rate ESI probes, which consume larger quantities of sample. The success of these platforms, and other nMS studies using high-flow ESI probes[4,18] broadens the definition of "native electrospray ionization" to include both nanoelectrospray ionization (nESI) as well as high-flow electrospray ionization (ESI) probes. Recent ion mobility-mass spectrometry measurements confirm that higher flow rate ESI sources operated at lower source temperatures can still produce ions with native-like structures, as long as source conditions are tuned to balance ion desolvation and structure. Notably, even the lowest flow rate ESI sources still require ∼5 μL/min (300 μL/h) flow rates, but if sample quantity is not a factor, the robust dependability and ease-of-use of standard ESI sources is the best compromise for new users and/or core facilities.

Preparing Glass nESI Emitters. For those interested in pursuing nESI with glass emitters, pulling capillaries in-house is the most cost-effective approach to making emitters. Typically, laboratories use micropipet pullers, such as the Sutter P-97 or P-1000 to produce glass emitters, or the P-2000 to produce quartz emitters, giving users considerable control over the emitter material, and the emitter opening geometry and size. Initially this process can be challenging, especially for new users, and can be inconsistent if incorrectly tuned. To ease entry into this space, Evan Williams' group at UC Berkeley has led a proactive effort to assemble a thorough database of emitter pulling programs accompanied by pictures of the emitter geometry, measurements of the orifice, as well as details of the glass and puller filament used. They have also published an informative protocol on the optimization of various micropipette puller settings, and recipes for several types of emitters in a recent *J. Am. Soc. Mass Spectrom.* application note.[19] This collection of information from the Williams group is an excellent resource for new users and even experienced users who wish to reevaluate their protocols. Even with these resources in place, some obvious challenges must be highlighted. First, is the high initial cost for acquiring equipment necessary to produce emitters in-house. The micropipet pullers are typically on the order of $10,000−20,000, making them somewhat cost-prohibitive. Second, even though emitter pulling programs have been published, each program will require some tuning as every micropipet puller is slightly different and sensitive to the local environment in which it is operated, including the condition and age of the heating filament used for melting the glass and other components of the puller. Lastly, although not required, access to a scanning electron microscope is useful to measure the emitter orifices and confirm the reproducibility and quality of the nESI emitters.

Within the community, one will find many preferences for small nuances in the type of emitters that "work the best." First is the question of whether to use filamented glass, which wicks the sample to the front of the emitter by capillary action along the internal filament. The alternative, nonfilamented glass, can often require application of backing nitrogen gas to push sample to the front of the capillary to induce electrospray. Both types of emitters have inherent advantages and challenges, some of which tend to be based on anecdotal evidence. Next is the question of pulling emitters that are "pre-opened" or "pre-clipped" vs pulling emitters with a long taper which have to be manually clipped. The "pre-clipped" emitters are typically more consistent in the opening, while the manually clipped emitters can vary in opening size drastically. The advantage of manually clipped emitters is the ability to reopen emitters after a clog when working with low quantity samples. The last question revolves around the method of charging the emitter. Some use a platinum wire inserted into the emitter, while others sputter coat the emitter with a conductive material such as gold and/or palladium. Here, we present general recommendations for those starting out:

- For stable, soluble samples: preclipped emitter with a 2−5 μm opening, filamented glass
  - 2−5 μm produces a clean open orifice which is not prone to clogging by debris, filamented glass simplifies bringing sample to front of emitter. The loaded emitter might require slight backing gas pressure to push sample solution to the emitter orifice and initiate spray, but no backing gas required for acquisition. Sample loaded emitter can also be gently shaken, or centrifuged to ensure that sample solution is completely to the front of the emitter. The use of glass capillaries with an internal filament can also assist with this by wicking solution to the tip.
  - For larger diameter emitters, users will need to optimize the analyte concentration to prevent nonspecific oligomers as a result of producing larger droplets containing >1 analyte per droplet. This control must be performed when using nMS to investigate noncovalent biomolecular complexes.[20,21]
- For disordered/membrane proteins: unopened (manual clip) emitters, nonfilamented glass
  - "Disordered protein" refers to any proteins which are soluble, but have inherent structural disorder that may lead them to form insoluble aggregates over time, precluding analysis due to emitter clogging. Examples include amyloidogenic peptides/proteins such as amyloid β, α-synuclein, prion-like proteins, etc.
  - Membrane proteins are typically insoluble in aqueous solutions (e.g., ammonium acetate solutions). These samples are are generally solubilized using detergent or membrane mimetics,[22] with recent methods enabling direct analysis from native membrane bilayers.[23,24]. These additives can complicate electrospray due to changes in solvent conditions and can still allow for some sample aggregation resulting in emitter clogging.
  - Anecdotal evidence of filamented emitters being more prone to clogging with disordered or membrane protein samples, although the internal filament can help with wicking solution to the tip which can be beneficial in detergent containing samples.
  - Err on the size of clipping too small, since the emitter can be reclipped.
  - Backing gas will be required to push sample to front; however, it is not required for acquisition.

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- For charging the emitter, the easiest setup is to use a platinum wire inserted in the back of the emitter. Setting up the wire requires minimal additional hardware/instrumentation. The protocol can be found in the Protocols database on the web page for the Native MS-Guided Structure Biology Center (linked below).
    - Both methods of charging work will work well. Most users have a preferred method, however, it is due to familiarity and training experience. Some claims have been made regarding platinum wires leading to higher levels of protein aggregation in the emitter, however there are no data to confirm or refute this claim.
    - If the majority of samples will be disordered/aggregation prone proteins, and Pt wire charging is proving to be challenging, consider switching to Au sputter coated emitters.

An advantage of preparing glass emitters in-house is the ability to pull "submicron emitters" that are preclipped with openings <1 μm in diameter, which are shown to drastically reduce salt adduction of biomolecular ions enabling nMS out of solutions containing biological buffer components.[25−28] Capillary material can also be varied, with borosilicate glass typically being used in heated filament pullers and quartz often being used in laser pullers. Additional standard operating protocols related to nESI (such as pulling tips and setting up nESI sources for use with a platinum wire) can be found on the web site of the Native MS-Guided Structural Biology Center.

MASS SPECTROMETRY

After sufficient sample prep, careful operation of the mass spectrometer is required to make an optimal nMS measurement.[3,29,30] The most distinct requirement for nMS compared with other applications is the higher m/z range. For example, typical peaks for ∼100 kDa native protein complex are around 4000−5000, beyond the $m/z \sim 2000$ limit for most proteomics instruments. Even if the mass analyzer can detect at high m/z in theory (e.g., time-of-flight), the design of front-end ion optics can severely restrict the ion transmission of high m/z ions. Therefore, the hardware limitation must be considered first when instruments are selected for nMS applications. Next, while most analytical tuning is conducted in pursuit of maximum signal, signal-to-noise, and spectral resolution, the process of instrument tuning for an nMS measurement strikes a delicate balance between ion transmission, resolution, and preservation of native-like ion structure. Different tuning approaches are often required for individual proteins, protein complexes, membrane protein complexes, and nucleic acids (and their complexes). Membrane proteins for example require additional activation (typically in the source region) to release the protein or protein complex from the membrane mimetic.[3,31] Instrument settings must be optimized on a sample-by-sample basis, and often slightly readjusted between multiple runs of the same sample, to account for changes in electrospray quality/stability, as well as sample concentration and/or preparation. Standardizing this process would require the use of standard proteins in varying mass ranges, with published guidance of expected signal intensity (under well-defined instrument conditions) and quality in the optimized nMS conditions. Additionally, such a repository of standards would need to be curated with sufficient meta-data to separate data by sample identity, preparation, concentration, instrument being used for the measurement, and settings of the respective instruments at the time of measurement. With several commercial platforms now available for these measurements, this task would require a community-wide effort to compile this resource, and this is an effort currently being pursued by the nMS guided Structural Biology Center with a focus on soluble protein complexes.

THROUGHPUT

An apparent hurdle associated with nMS is the low-throughput nature of the measurement. As discussed in this commentary, most nMS groups use static nanoelectrospray ionization, which requires manual sample cleanup followed by manual sample introduction (i.e., load into emitter, load to mass spectrometer, tune for signal etc.). This is, in part, one of the reasons why this method has yet to be embraced by the broader scientific community. Autosampling ESI hardware has been commercially available since 2003 (Advion TriVersa Nanomate),[32] and successfully deployed for high-throughput analysis of smaller analytes (e.g., small molecules, lipids, metabolites etc.), however fewer cases of high-throughput ESI of native biomolecular ions are available in the literature.[33,34] This is likely due to the complicated nature of samples analyzed in nMS studies and the common issues which hamper conventional manual nMS studies (e.g., inconsistent electrospray, emitter clogging etc.). Additionally, this platform relies on a well-plate format and does not incorporate a sample cleanup step, requiring manual sample cleanup prior to well-plate preparation. Recent advances in instrumentation have incorporated in-line sample clean up between the sample well-plate and the ion source (e.g., Agilent RapidFire); however, these instruments were designed for denaturing MS measurements. Some research groups have been working to develop in-line clean up cartridges for native sample clean up using size exclusion chromatography with promising results.[5,35] These results suggest that we are moving closer to a high-throughput solution for nMS analysis, but as with any high-throughput measurement, the efficacy of these measurements is highly dependent on the samples to be analyzed. A third and well-established method for automated sample introduction is online buffer exchange with an LC stack as demonstrated by VanAernum et al.[4] The primary advantage of this method is that it utilizes LC instruments that are often already present in most laboratories, eliminating the need to purchase expensive dedicated auto sampling instruments such as the ones discussed in this section. This method has been deployed for nMS analysis of various samples demonstrating its robustness to handle various proteins, and protein complexes.[36−38] Additionally there is software support for this method from instrument vendors, as well as software vendors enabling users to analyze raw data in a batch-analysis mode and generate reports summarizing the results of several consecutive runs (**Protein Metrics Byos** and **UniDec Processing Pipeline**[39]).

DATA ANALYSIS

Key steps in nMS spectral analysis include identifying ESI charge state distributions and deconvolving the distributions to obtain neutral analyte mass. Although many high-resolution MS systems are used for nMS, the high mass and heterogeneity of the samples make the m/z peaks broad. Even with the highest resolution instruments, isotopic resolution is challenging to achieve and not often pursued. Many data analysis software packages are designed for lower mass species with isotopic resolution and therefore are not suitable for nMS. Instead,

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Table 2. nMS Data Analysis Software Solutions

Program	Publisher/Vendor	Access URL
	Software Available for Free Use	
UniDec	Michael Marty (Univ. of Arizona)	https://github.com/michaelmarty/UniDec
MashNative	Ying Ge (Univ. of Wisconsin, Madison)	https://labs.wisc.edu/gelab/MASH_Explorer/MASHNativeSoftware.php
iFAMS	Jim Prell (University of Oregon)	https://github.com/prellgroup/iFAMS
	Software for Purchase	
MaxEnt	Waters	https://www.waters.com/waters/library.htm?locale=en_US&lid=1527409
BioPharma Finder	Thermo Fisher Scientific	https://www.thermofisher.com/us/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis.html
ProSight Native	Proteinaceous	https://www.proteinaceous.net/prosightnative
Protein Metrics Byos	Protein Metrics	https://proteinmetrics.com/byosphere/

spectral deconvolution in nMS heavily relies on fitting of charge state distributions. When analyzing pure, isolated proteins, this task is fairly straightforward since the detected peaks can be assumed to be part of a single ESI distribution and one can quickly assess the deconvolved measured mass against expected theoretical mass (e.g., calculated sequence/formula mass). However, nMS spectral complexity can scale rapidly with oligomeric states or the inclusion of binding partners (e.g., ligands, nucleic acids, other proteins, etc.), resulting in several ESI charge state distributions in overlapping m/z channels. While such complex spectra can still be deconvolved manually, this can result in an erroneous charge assignment and mass measurement. To address these challenges, especially with respect to new users or in native MS core facilities, automated deconvolution software is recommended. These software solutions implement mathematically derived deconvolution algorithms to fit raw data, identify charge state distributions, and output deconvolved mass spectra. Importantly, most automated deconvolution software currently available incorporate a measure of "fit accuracy" with scoring models for the deconvolved data giving the users confidence in the analyzed data.[40] New users can be trained in data analysis using data acquired for protein standards, as well as training aids such as interactive figures published previously by the Barran lab.[41] Representative free and paid software solutions are listed in Table 2.

DATA REPOSITORY

One area where improvement is needed, which was highlighted during the roundtable discussion, was the need for an appropriate repository for native MS data and adoption by the community. Not only is open access data becoming a requirement for many journals and funding agencies, but also the development and/or adoption of current repositories will have massive benefits in terms of developing methods for both data acquisition and data analysis (including access to data sets for software developers). While many data repositories do exist for MS data, these were often developed for omics data and therefore do not meet the specific needs for nMS analysis. Several groups have opted to upload raw data to MassIVE, which has suitable flexibility for such data sets (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp). Discussion also touched upon what kind of metadata should be required for nMS experiments, such as instrument type, sample solution, and instrument activation settings (in-source, collision cells etc.). In the case of ion mobility experiments, further details regarding type of ion mobility separation, measured collision cross sections, and calibration standards (if applicable) should also be included within the data set.

INTRODUCTION OF A SHARED FORUM FOR nMS

A barrier to new researchers in a new field is often not knowing where to begin and where or who to turn to for advice. With this in mind, the attendees of the Sanibel roundtable discussion stated a desire for a shared virtual forum for discussions. This could refer users to useful protocols, SOPs, articles, conferences, and workshops and be a place new researchers could ask for help. While this exists formally in other communities (such as the top-down proteomics discussion group), we are not aware of any currently active nMS discussion groups like this. With this in mind, we have launched the "Native Mass Spectrometry Discussion Group" on LinkedIn and invited interested community members to join and share their experience and start discussions about steps necessary to address some of the challenges outlined in this commentary. As an initial step toward standardization, the community would benefit from reaching a consensus on a data repository for nMS data, protocols, and software.

LOW-COST INSTRUMENTS FOR THOSE WHO ARE ON A BUDGET OR WANT TO KEEP THINGS SIMPLE

Although time limitations prevented discussion of this topic, it was discussed at the 2022 IMSC and deserves attention from our community. Wider adoption of nMS would be possible through the development of simple, robust, and lower cost instruments. While considering the development of a more economical instrument, several key questions arise. A few (not all) of these considerations are presented below:

- Would a regular flow ESI source suffice?
- Or would a lower flow ESI source such as microflow or nanoflow be required?
- What is the necessary mass range?
- What are the dissociation requirements?
- How many dissociation regions are necessary?
- Would in-source dissociation, or a single collision cell suffice?
- Is mass selection necessary?
- What is the "lowest achievable cost to consumer" to obtain a mass spectrometer which combines these baseline nMS capabilities?
- What options are available to repurpose or upgrade existing, used instrumentation to meet the necessary requirements for nMS?

OUTLOOK

nMS is an expanding field that complements many traditional structural biology studies and answers critical structural biology questions that are otherwise difficult to probe. Through the

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development of commercial high mass instrumentation, nMS is moving beyond the niche academic lab and into the wider community. However, some areas remain unstandardized, and community effort is required to address these. We hope the roundtable discussion at Sanibel and this resulting commentary will serve as a starting point to address some of these questions and help guide new researchers to the appropriate resources.

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Notes

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