Imaging MS: Normalization Approaches to Imaging Mass Spectral Data
Workshop Report
Tuesday 11th June, 5:45 - 7:00, Room 200GF

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Introduction

Imaging mass spectrometry continues to grow rapidly owing to its impact in the biological, pharmaceutical and materials sciences. Last year, a successful workshop was held titled 'Developing Quantitative Imaging' with lively discussion. One of the key obstacles to quantitative imaging is the need to account for non-uniform ion suppression effects when analysing heterogeneous tissue samples or comparing the mass spectral signatures from different patients. This process is referred to as normalization. Several normalization methods have been proposed, ranging from global mass-spectral normalization based on single metrics (e.g. total ion count) to molecule specific normalization that utilize isotopically labelled reference standards. The different normalization methods differ in their assumptions and thus how they should be applied, as well as in the ease and expense of their application. The aim of this workshop was to explore and discuss the current normalization methods, principally because of a lack of understanding in the wider imaging MS community.

For this year's workshop we decided to change the format from that used for the last two years. Previously discussion leaders were distributed in the audience to stimulate debate. While this format did foster wide participation in the 'individual groups' it also led to a significant degree of repetition, which limited the scope for debate. Instead this year we formally separated the discussion into distinct subtopics, to ensure that the normalization methods important for the different applications of imaging MS were covered. Specifically, after a very short general introduction we dedicated 15 minutes discussion for each of the following subtopics, each of which was introduced by an expert in the respective fields with a short 5 minute presentation to spur discussion.

I) **Global intensity normalization** – e.g. TIC, RMS (or other single value) for spectrum-wide scaling. This is the most commonly used method because it is the easiest to apply. However there are many questions regarding its application. For example Deininger et al. have demonstrated that better results can be obtained by omitting intense peaks. Often people use global wide normalizations simply because 'it makes the images look nicer'. Such statements indicate that there is insufficient understanding of normalization and which algorithm may be most suited to the data. Fonville et al. have reported heuristics to assess the performance of different normalization methods yet such measures are not widely applied. Introduced by Dr. Soeren-Oliver Deininger (Bruker Daltonics, Bremen, Germany).

II) **Molecule specific ion suppression using reference standards** – it has now been demonstrated that this produces the best quantitation results. However, it requires the use of suitable reference standards and so is best suited to targeted analyses, such as pharmaceutical imaging. Questions remain concerning the choice of suitable (inexpensive) reference standards, and how the reference should be added. Introduced by David Pirman (Pfizer, Connecticut, US).

III) **Molecule specific ion suppression (tissue exctinction coefficients or relative response factors)** – has been reported as an alternative method for quantifying the relative amounts of pharmaceuticals in different organs. The advantage is that it is inexpensive to apply because it does not need the addition of a reference standard. While there is the view that the results are less accurate there is a genuine question of if the accuracy is sufficient. Introduced by Dr. Jonathan Stauber (ImaBiotech, Lille, France).

IV) **Clinical analyses / 3D imaging** – what is the best practice to normalize MS signals between different tissue sections, whether from the same specimen (e.g. 3D imaging MS) or different specimens (clinical patient series)? How does one ensure equal performance of the mass spectrometer, or biomolecule extraction? Should one vary laser power / detector gain between tissues to get a similar tissue response? What methods would be best achieved for comparing datasets run at two different institutions? Introduced by Dr. Raf van der Plas (Vanderbilt, TN).

At ASMS this year, we enjoyed 180 presentations dedicated to imaging mass spectrometry sessions (cf 157 in 2012 and 120 in 2011).

Oral (3 sessions)	**Poster (9 sessions)** Number of posters given in parentheses
Imaging MS: Increasing Speed and Information Content	Imaging MS: Disease Markers (24)
Imaging MS: Biological Applications	Imaging MS: Method Development I (22)
Imaging MS: Pharmaceutical Applications	Imaging MS: Pharmaceutical Applications (24)
	Imaging MS: Small Molecules (28)
	Imaging MS: Software (12)
	Imaging MS: Instrumentation (23)
	Imaging MS: Large Molecules (4)*
	Imaging MS: Method Development II (21)
	Imaging MS: Quantitative Analysis (5)*

* Imaging MS: Large Molecules should be discontinued as for the last two years it has only contained a small number of posters. 8 poster sessions is sufficient.

The first imaging MS oral session, Tuesday morning entitled Imaging MS: Increasing Speed and Information Content was very well attended, with an estimated audience of approximately 500-600. The second imaging MS oral session, Tuesday afternoon entitled "Imaging MS: Biological Applications" was less well attended, approximately 200 attendees. There were several high quality oral presentations in the session and so the low turnout was a surprise. One contributing factor was the 4 poster sessions between the two oral sessions. Many people were still present in the poster hall when the afternoon session began. The final imaging MS oral session, Thursday morning entitled Imaging MS: Pharmaceutical Applications, was well attended considering the usual lower turnout on Thursdays. The Imaging MS interest group meeting was well attended, with >90% seats in the room occupied.

The general consensus was that the imaging sessions were not distributed well throughout the 2013 conference, particularly the posters. The distribution in 2013 was

Monday: 0 Oral sessions. 1 Poster session.
Tuesday: 2 Oral sessions. 4 Poster sessions.
Wednesday: 0 Oral sessions. 0 Poster sessions.
Thursday: 1 Oral session. 4 Poster sessions.

Accordingly, on Tuesday it was difficult to visit all of the posters while attending all oral presentations. Almost 50% of the topic's posters were presented on the final day, which is normally less attended owing to people taking early flights home. For 2014, it would be appreciated to have 2 poster sessions per day.

One of the final topics discussed during the workshop was whether we should organize two separate imaging mass spectrometry workshops, one focused on biological applications and the other on pharmaceutical analysis. The principal reason is the very different considerations for the two application areas. The general consensus was that there should be only one interest group, but to organize two workshops during ASMS, one on each topic, and to take place on different days. If this is acceptable, then we feel the interest group should have 3 co-coordinators to spread the workload.

Finally it was announced by Jonathan Stauber of ImaBiotech that there will be an innovation in imaging mass spectrometry prize. Details of which can be found on the ImaBiotech website.

Discussion (for free discussion comments are not attributed)

Global intensity normalization:
It was demonstrated how, for MALDI ToF analysis, TIC normalization could adequately correct for small differences in matrix coating and/or laser power, but that the normalization method is prone to bias if the spectra contained high intensity, highly differential peaks. This was expanded upon further by stating that the issue for normalization bias is not whether they are intense peaks but whether the peaks changed significantly. It was also pointed out that the choice of normalization metric is dependent on which mass analyser was used owing to the different natures of the noise in MALDI-TOF versus MALDI-FTMS. In MALDI-FTMS the baseline is caused by electronic noise, which is approximately uniform between spectra, whereas MALDI-TOF instruments pick up a large amount of chemical noise that needs to be included in the normalization procedure.
It was also suggested that imaging mass spectrometry simply adopt the normalization procedures adopted in other imaging fields, which was then countered with the point that the nature of the data is different and so the normalization procedure will be different too (in agreement with the observation about the performance of different normalization metrics for different mass analysers).
It was also argued that normalization of ionization bias cannot account for factors such as tissue extraction efficiency. This was countered with the statement that one cannot begin to investigate extraction efficiency unless one can assess ionization bias. Ultimately however it was agreed that a single spectrum-wide metric cannot adequately address molecule specific ionization biases for all molecules present in a complex mixture.

Molecule specific ion suppression using reference standards
It was demonstrated how the addition of isotopically-labelled variants provides the most accurate quantitative data, by accurately mapping ionization bias throughout the tissue. A discussion began concerning the best way to apply the internal reference standards and its application in discovery based imaging mass spectrometry experiments. Specifically, it was postulated that it is difficult to apply on a proteome-wide or metabolome-wide basis and so imaging mass spectrometry should develop alternative methods for quantifying data. This was countered with the observation that SILAC and MIRACLE represent the two best methods for quantitative proteomics and metabolomics in the wider mass spectrometry community and that imaging mass spectrometry would be ill advised to plead a special case scenario because it is difficult to apply in an imaging context. This discussion did highlight the subject of what is considered an acceptable level of quantitation for the application at hand and if the added expense of isotopically-labelled variants was necessary.

Molecule specific ion suppression (tissue extinction coefficients or relative response factors
It was demonstrated how the relative response factors of a tissue could be determined by comparing the results for dilution series analysis on the MALDI target plate and on the tissue. It was then shown how the relative response factors were relatively insensitive to concentration and laser power. Accordingly a degree of quantitation could be achieved without the need for purchasing isotopically-labelled variants. In keeping with the previous discussion there were questions regarding the accuracy of the method, and its ability to map ionization bias in highly heterogeneous tissues. This was countered with the argument that the method is for determining average organ-concentrations of drugs.

Clinical analyses / 3D imaging
The final topic concerned how best to normalize between different tissues, and the example given was for 3D imaging mass spectrometry. In 3D imaging the 3D datasets are reconstructed from the independent analysis of sequential tissue sections. Accordingly there is the need to normalize responses within the tissue, between different tissues (placed on the same target plate) and between target plates.

Discussion - Conclusions
Overall the discussion worked well and the majority of people felt that the workshop was successful. The essential points from the discussion are:

- Normalization is necessary to correct for ionization biases.
- Global, spectrum wide normalization can correct for moderate global ionization biases due to differences in matrix coverage or laser power.
- Different instruments may require different normalization procedures
- Labelled reference standards provided the best normalization and quantitation.
- Multiple normalization corrections may be necessary to compare samples run on different days and on different target plates.
- More tutorial / educational material is needed for normalization.
- It was also discussed that we need to ensure that any data refining is done with a scientific approach and not one in which the quality of the image is used to determine success.

Finally, it was noticed that while the introductory 5-minute lectures worked will and did indeed spur debate, many questions were addressed to the speaker. The previous year's group discussions did lead to more participation in the individual groups, but the subsequent questions were also addressed to the individual group leaders (who represented the group). The group format engendered more people to actively contribute, but at the expense of repetition. Accordingly in 2014 we may try a new format, in which individual groups are asked to discuss for 10 minutes *individual* topics, which will then be presented to the wider audience for active discussion.

Based on the discussion and from the survey responses the following oral and poster sessions are recommended for the 62nd ASMS (2014) sessions:

Oral	**Poster**
Biomedical applications	Pharmaceutical applications
Fundamentals, instrumentation and method development	Sample preparation
Pharmaceuticals and metabolomics	Small molecules
	Instrumentation
	Method development
	Disease markers
	Data processing and data analysis
	Liquid extraction based analysis*

* liquid extraction based technologies (Advion's LESA system or other liquid microjunction surface analysis based technique) are being rapidly taken up by the imaging MS community, especially for pharmaceutical applications, and were used in multiple oral and poster presentations. A dedicated poster session may be due.

The proposal by the Vice President for Programs, Professor Jennifer Brodbelt, to change session chairs every year is very welcome as it will better reflect the wide interest in imaging mass spectrometry. For 2014 the following chairs are suggested:-

Biomedical applications:	Prof. Axel Walch (Helmholtz Zentrum Munich), Prof. Richard Caprioli (Vanderbilt), Dr. Nathalie Agar (Harvard), Prof. Richard Drake (Medical Univeristy of South Carolina)
Fundamentals….:	Graham Cooks (Purdue), Liam McDonnell (Leiden University Medical Center)
Pharma & metabolomics:	Prof. Per Andrén (Uppsala University), Markus Stoeckli (Novartis, Basel), Tim Garret (Florida), Steve Castellino (GSK)

Acknowledgements

We thank ASMS for the opportunity to hold the workshop as part of the conference and all their help and support in doing so. The presenters are gratefully acknowledged for stimulating the discussion and getting everyone involved and excited about the discussion.

Results of the workshop survey are shown below (41 returns)

Workshop

Do you think this workshop has fulfilled the outline aims and objectives?

Not Sure 1	Poor 1	OK 17	Good 22

Have you found the format of the workshop successful?

Not Sure 0	Poor 2	OK 16	Good 23

Has the workshop been useful?

Not Sure	Poor 5	OK 18	Good 18

Do you have any suggestions or requests for next year's workshop?

- *Overview of computational tools. Alternatives to commercial software*
- *Emerging imaging techniques*
- *Clinical imaging mass spectrometry*
- *Tissue sample preparation and sample handling for peptide imaging*
- *Method discussion*
- *Multimodal Imaging and Imaging Mass Spectrometry*
- *Multivariate analysis*

Conference

Has the number and topics of Imaging MS oral sessions been adequate?

Yes 33	No 5

Has the number and topics of Imaging MS poster sessions been adequate?

Yes 33	No 4

Have the oral sessions been useful?

Yes 24	No 2

The following comments were mentioned several times:

1. Too application focussed.
2. Some talks lacked sufficient novelty.
3. More methodological detail in talks.

Suggestions received for oral and poster session topics next year (titles in bold were suggested more than once).

Oral	Poster
Data processing & analysis	**MSI Sample Preparation**
MSI for drugs & metabolites	Atmospheric pressure imaging
MSI on atypical samples	High throughput imaging
Quantitative MSI	High resolution imaging
Protein ID in MSI	Normalization
Pharma MSI of large molecule drugs	Unbiased biomarker discovery
Metabolomics MSI	Forensic applications
MSI in materials science	Inorganic imaging
	SIMS