Revised Preparation of a Mimetic Tissue Model for Quantitative Imaging Mass Spectrometry

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Abstract

A revised methodology is presented for the preparation of a mimetic tissue model for use in quantitative imaging mass spectrometry (IMS). Tissue homogenates spiked with known concentrations of a compound standard are serially added to a mold and are snap frozen. The resulting mimetic tissue plug is then cryosectioned and thaw-mounted on the same slide as the tissue(s) to be imaged and quantified. From here, the sample tissue(s) and mimetic tissue model undergo identical sample preparation conditions for IMS. Once IMS is performed, the known analyte concentrations can be correlated with the average intensity of each layer of the mimetic model to generate a calibration curve. This standard curve can then be used to estimate the quantity of analyte that is present in the tissue(s) of interest. The matrix-matched standards applied in this approach account for common IMS issues such as ion suppression and extraction efficiency.

Introduction

Imaging Mass Spectrometry (IMS) combines spatial information with the highly selective and sensitive nature of mass spectrometry detection to yield a powerful molecular histology technique. In brief, this is achieved by rastering a sample surface as a 2-dimensional pixel array where at each position a mass spectrum is generated. By relating the intensity of any ion in the mass spectra with its corresponding x-y coordinates, a false color map can be generated which represents that ion's distribution in the sample. Over the past decade, significant contributions have been made to develop quantitative IMS which is the topic of several reviews.¹⁻⁴ Quantitative IMS has demonstrated promise in drug discovery and development where it can be used to assess drug distribution in tissue which has implications towards drug safety and efficacy.^{5,6} The presentation of a quantified tissue distribution has distinct benefits over the common approach of quantifying the tissue homogenate especially if tissue distribution is heterogeneous (localized). Two of the major challenges in IMS which can impact the accuracy of quantification are the suppression of ionization primarily caused by the endogenous biological matrix as well as the efficiency of analyte extraction from the tissue. There are several common approaches to building a calibration curve for quantitative IMS proposed in the literature which are outlined nicely by Porta.⁷ The first involves spotting the calibration standards directly onto the sample substrate (typically glass slide) adjacent to the tissue that is to be quantified. While this is a straightforward process, it fails to account for the suppression and extraction efficiency associated with the analyte in the sample tissue. A secondary approach is to spot the calibration standards on a control or surrogate tissue section to account for tissue suppression, but it is unclear how this approach relates to the analyte extraction from the tissue. A third method that was previously proposed by our group utilizes tissue homogenates spiked with known concentrations of analyte to yield matrix-matched standards. This method is designed to more closely replicate the suppression and extraction conditions experienced by the analyte in the sample tissue yielding a more accurate quantification.⁸ One drawback with this approach had been the elaborate process involved to generate the mimetic tissue model. After several iterations, a more efficient method has emerged to produce the mimetic tissue model as is outlined in this protocol and is summarized in the

flow diagram below. Briefly, a range of known concentration standard solutions are spiked into a series of tissue homogenates. These spiked homogenates are then serially frozen into a mold, producing a plug of tissue with a stepped concentration gradient of the analyte of interest. This mimetic tissue model can then be cryosectioned and thaw-mounted onto the sample substrate (microscope slide) along with the tissue(s) to be imaged and quantified. Once IMS is performed, the average intensity of each layer of the mimetic tissue model can be correlated with the known spiked concentration to generate a calibration curve. This curve can then be used to quantify regions of interest (ROIs) on the target tissue. [See figure in Figures section.](#)

Reagents

50 mL conical centrifuge tube (Falcon 50 mL tube, 352070) 3 mL syringe (BD 3 mL Syringe, REF 309657) Homogenizing vials (Precellys MK28-R, P000917-LYSK0-A) Steel homogenizing beads (MP Metal Bead Lysing Matrix, 6925-000) Dry ice chilled ethanol Microscope slides (Bruker Daltonics MALDI imaging slides, 8237001) Analyte standard Appropriate dissolution solvent for standard (example: methanol, acetonitrile, water) Tissue (Rat liver tissue used herein as example) _All studies were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution where the work was performed._ The following were utilized for imaging mass spectrometry MALDI matrix (DHB) Methanol Water Trifluoroacetic acid (TFA)

Equipment

Bead homogenizer (critical) – FastPrep 24 Homogenizer (MP Biomedicals, Solon, OH, USA) Centrifuge Positive displacement pipette (critical) – Microman M250 positive displacement pipette (Gilson) The following were utilized for imaging mass spectrometry Mass spectrometer capable of IMS – 7T-Solarix FT-ICR mass spectrometer with MALDI source (Bruker Daltonics, Bremen, Germany) IMS software for data analysis – flexImaging (Bruker Daltonics, Bremen, Germany) Matrix application device (if using MALDI) – TM Sprayer (HTX Technologies, Chapel Hill, NC, USA) Digital Scanner – Aperio ScanScope (Leica Biosystems, Buffalo Grove, IL, USA) Cryo-microtome – CM1950 Cryostat (Leica Biosystems, Buffalo Grove, IL, USA)

Procedure

In accordance with the USFDA industry guidance for bioanalytical method validation,⁹ the ideal calibration curve should have at least seven points including a blank. The remaining six points should cover the intended concentration range and be evenly spaced along the x-axis (concentration) to avoid issues with leverage. The blank is included to more accurately assess the detection capability, limit of blank (LoB) and limit of detection (LoD), as this provides a means of determining background relating not only to electronic and chemical noise but also from potential isobaric species inherent to the tissue matrix.¹⁰ Therefore, the mimetic tissue model will consist of six levels of spiked homogenate and one

non-spiked homogenate for assessment of the detection capability. ****Homogenization**** (10 minutes)

By first intent, the homogenate of the mimetic tissue model should be made from the same tissue type as that which is being quantified. This, however, is not always going to be possible given that certain tissue types do not homogenize well (i.e. skin). Furthermore, appropriate control tissue does not always exist (i.e. tumor or xenograft tissue), and it may not always be possible to obtain control tissue from the same animal species. In these situations, we have found that rat liver tissue can be used as an effective surrogate. While the use of surrogate tissue can mimic the ion suppression and extraction from the tissue being quantified, the potential for unforeseen isobaric species in either the model or the target sample tissue should be noted. If an isobar exists in the sample tissue and not in the model tissue, the limit of detection can be underestimated and vice versa for the inverse situation. When preparing the tissue homogenate for the mimetic model, the use of a bead homogenizer without the addition of solvent is critical to yield a more morphologically relevant homogenate.^{8, 11}

1. Determine the minimum amount of homogenate that will be needed.
 - 1.1. The aim is to have about 300-400 mg of homogenate for each layer.
 - 1.1.1. Seven layers x 300 mg $\approx$ minimum 2 g of tissue per model.
2. Prepare the tissue for homogenization.
 - 2.1. If performing a large-scale homogenization (i.e. whole rat liver) choose an appropriate sized tube/vial.
 - 2.1.1. Whole rat liver can be aliquoted into two 50 mL conical tubes.
 - 2.2. Cut small enough portions of the tissue that will fit into your tube of choice.
 - 2.2.1. Portion enough tissue to at least satisfy the minimum amount required.
 - 2.3. Do not overfill the tube (only fill about $\frac{3}{4}$ to leave room for beads & homogenization).
 - 2.4. Add stainless steel homogenizing beads to the tube (MP Metal Bead Lysing Matrix).
 - 2.4.1. Roughly 15 beads for a $\frac{3}{4}$ full 50 mL tube will suffice.
 - 2.5. Wrap the top of the tube with parafilm to prevent loss of tissue.
3. Homogenize the tissue using the MP FastPrep 24 or similar ****bead homogenizer**** (critical).
 - 3.1. Homogenize at 4 m/s x 30 s for as many rounds as necessary (usually 2-3 rounds).
 - 3.2. Let the homogenate settle or spin down at low speed (1000 x g for $\sim$ 30 s) to collect as much as possible from the tube walls.
 - 3.2.1. The intention of spinning down is to collect the material off the tube walls but it may result in phase separation. If using the homogenate directly, gently stir to reincorporate the mixture.
 - 3.3. If the tissue was large enough to be spread over several tubes, it is suggested that these homogenates be combined to avoid potential bias in sampling from the tissue.
 - 3.4. Aliquot the homogenate into smaller single use containers ($\sim$ 2 g each) to avoid multiple freeze/thaw cycles. [See figure in Figures section.](#)

****Preparing the Mold**** (5 minutes)

The mold for the mimetic model is a modified 3 mL syringe (BD 3 mL Syringe REF 309657). Regardless of the exact type or brand of syringe, there are a few important attributes to the syringe choice. Given that the volume of the syringe ultimately dictates the amount of homogenate that is needed as well as the height of each layer of the model, the 3 mL syringe has a large enough inner diameter that can accommodate the tip for the positive displacement pipette and is small enough to limit the amount of homogenate required for each layer. Additionally, the plunger seal should be rubber-like material (avoid hard plastic) to limit the penetration of ethanol into the barrel during the freezing step.

1. Trim the syringe barrel.
 - 1.1. Pull back or remove the plunger.
 - 1.2. Cut the luered tip off the syringe (can use an HPLC tubing cutter). [See figure in Figures section.](#)
2. Trim the plunger seal.
 - 2.1. The plunger seal may have a conical shape which will cause a conical distortion in the first layer of the model.
 - 2.2. Cut the conical tip off the plunger.
 - 2.3. Re-insert the plunger into the syringe body to roughly the first volume demarcation.
 - 2.4.

Cut the extruding end of the plunger such that the bottom of the plunger is flush with the bottom of the syringe. —2.4.1. You can save this trimmed end of the plunger to later push the mimetic model out of the syringe. See figure in Figures section. _\((Optional)_

3. Create a handle for dipping the mold into the dry ice-cooled ethanol. —3.1. Drill or press a small hole through the bottom of the syringe barrel and plunger as shown in the figure below. —3.2. Press a small diameter L-shaped hexagonal \((allen)\) key through the hole. —3.3. This serves to lock the plunger in place as well provides a handle to dip the mold into the chilled ethanol. See figure in Figures section. ****Standard preparation and homogenate spiking**** \((20 minutes)\) To maintain the relevant morphology of the tissue, it is imperative that as little solvent as possible be added when spiking the homogenates. Ideally, the total volume of the spiked standard\((s)\) should be kept below $2-3\% \text{ (v/v)}$ compared to the homogenate volume. This may, however, be limited by the solubility of the standard. The solvent used to dissolve the standard should be water miscible to ensure efficient mixing with the homogenized tissue. The volume of standard added to each homogenate \((mimetic\ model\ layer)\) should be consistent. There is an attached "macro-enabled spreadsheet":http://www.nature.com/protocolexchange/system/uploads/7111/original/Mimetic_Tissue_Model_Calculations.xlsm?1534966473 which can be used to help determine the appropriate volumes and concentrations of standard to add given the weight of the tissue homogenate and the target tissue concentration. It is also possible to add multiple standards to the same mimetic model to allow for the quantification of multiple species. If the model is to contain multiple standards, their concentration gradients should be inverted such that the highest concentration of standard A should be spiked into the same homogenate as the lowest concentration of standard B. When spiking multiple standards, the total spiked volume should be kept below the $2-3\% \text{ (v/v)}$ cutoff, if possible. Ion suppression resulting from the spiked standard\((s)\) should also be assessed for the impact on the other standard\((s)\). It is not required to use a stable isotopically labeled \((SIL)\) standard unless quantification is to be performed on an endogenous species that is inherently present in the tissue homogenate used for the mimetic tissue model construction. The use of an SIL in this case will allow for deconvolution of the spiked standard level from the endogenous level in the tissue homogenate.

1. Determine and assign the intended range and intervals for the final tissue concentration of each level of the mimetic model.
2. Determine weight of tissue homogenate for each layer \((using\ the\ attached\ spreadsheet)\).

- 2.1. Record the weights of seven $\sim 2\text{ mL}$ screw cap vials \((1\ vial\ for\ each\ layer\ of\ the\ mimetic\ model)\).
- 2.2. Using a positive displacement pipette, aliquot approximately 300 mg \((\sim 300\ \mu\text{L})\) of tissue homogenate into the pre-weighed vials.
- 2.3. Record the weight of each vial and homogenate to determine the weight of homogenate in each vial.

3. Spike each homogenate with standard\((s)\).

- 3.1. Determine and prepare an appropriate dilution of stock standard to spike into each homogenate. —3.1.1. Standard solution volume / tissue homogenate volume should be $\leq 2-3\% \text{ (v/v)}$.
- 3.1.2. Standard should be dissolved in a water miscible solvent to ensure mixing with homogenate. —3.1.3. The volume of standard spiked into each layer should be roughly equivalent.
- 3.2. Add the appropriate volume of standard to each homogenate and mix. —3.2.1. Once the standard has been added, mix by adding 1-2 metal homogenization beads to each vial and load into the FastPrep 24 for mixing. —3.2.1.1. Mix at 4 m/s for ~ 30 seconds. —3.2.1.2. This mixing step is critical to ensure that the standard is evenly distributed throughout the homogenate. —3.3. The presence of air bubbles in the homogenate will result in voids in the final tissue section which should be avoided where possible. —3.3.1. Spin down each spiked homogenate vial at a low speed \((1000 \times g\ for\ 30\ seconds)\) to remove air bubbles which may have arisen during the mixing. —3.3.1.1.

Higher speeds can result in a phase separation of the lipophilic components and should be limited or avoided if possible. —3.3.1.2. Some foaming may be evident on the top of the homogenate after centrifugation. [See figure in Figures section.](#) ****Preparing the Mimetic Tissue Model plug**** (15 minutes + > 3 hours to freeze, usually overnight) The next series of steps involves the construction of the mimetic tissue model. This process is the serial freezing of each of the individual spiked homogenates into a single tissue plug which can be sectioned along with the target sample tissue. To minimize the potential for carryover, the first layer added should be the blank homogenate followed by the lowest concentration spiked homogenate and working up to the last layer which is the highest concentration spiked homogenate.

1. Prepare the freezing medium. —1.1. Set up a small beaker with dry ice-cooled ethanol deep enough to nearly submerge the mold (syringe). —1.2. As is true with freezing other types of tissue, the speed of freezing can impact the quality of the tissue in terms of the formation of ice crystals and other artifacts. Typically, the faster the freezing the better the quality.
2. Add a layer of homogenate to the mold. —2.1. Using a positive displacement pipette, collect about 250 μ L of homogenate. —2.1.1. This is typically enough to evenly coat the plunger seal or previous layer. —2.1.2. The exact amount added is not critical but bubbles should be avoided where possible. —2.2. Wipe any excess homogenate off the pipette tip to avoid contaminating the edges of the mold. —2.3. Insert the pipette tip into the mold and expel enough of the homogenate to coat the plunger (or previous layer). —2.3.1. It is ideal to not expel all the homogenate as an air bubble is likely to form if you do. —2.4. If necessary, tap the barrel flange side of the mold down onto a hard surface to remove air bubbles and/or help the homogenate to settle to an even layer. —2.5. It is crucial to have the homogenate completely coat the previous layer.
3. Freeze the newly added layer of homogenate. —3.1. Dip the mold into the dry ice-chilled ethanol for at least 1 minute (or longer until the layer is frozen). —3.1.1. Dip at least far enough such that the newly added homogenate is below the ethanol level but do not completely submerge (i.e. the open end of the mold should always be above the ethanol level).
4. Repeat steps 2 and 3 for the additional spiked homogenate layers. —4.1. To avoid the potential for contamination, layers should be added starting with the blank then the lowest concentration spiked homogenate and ending with the highest concentration spiked homogenate. —4.2. Try to limit the amount of time between freezing sequential layers. —4.2.1. Once the fresh homogenate layer is added, it should be frozen as quickly as possible to prevent the previous layer from melting and mixing with the new layer.
5. Once the last layer has been added and frozen, remove the handle (allen key), wrap the mold in foil, and place in a -80°C freezer for at least 3 hours or overnight to ensure that it is thoroughly frozen. [See figure in Figures section.](#) ****Sectioning the Mimetic Tissue Model**** (10 minutes) Once the mimetic model is constructed for a given standard, applying the quantification of that standard to any tissue merely requires collecting a section of the mimetic model onto the same slide with the tissue to be quantified.
1. Equilibrate the model to the cryostat temperature as you would any other tissue.
2. Remove the model from the mold. —2.1. If necessary, use the palm of your hand to slightly warm the edges of the mold. —2.1.1. Be careful not to warm it too much or the tissue will smear as you are pushing the model out of the mold. —2.2. Start to slowly push the model out of the mold. —2.2.1. Use any tool to depress the plunger. —2.2.1.1. (i.e. a pair of forceps or the remainder of the plunger that you cut when preparing the mold). —2.3. As the model is pushed out of the mold it may stick to the plunger seal. —2.3.1. Use the plunger to orient the tissue model for mounting to the chuck. —2.3.2. Carefully separate

the plunger from the tissue model. [See figure in Figures section.](#) 3. Mount the mimetic model onto the cryostat chuck. –3.1. Apply a strip of mounting medium to the cryostat chuck. –3.2. Mount the mimetic model horizontally onto the chuck. [See figure in Figures section.](#) 4. Sectioning the model. –4.1. The model should be sectioned horizontally to avoid the potential for contamination between model layers. –4.2. Trim into the mimetic model tissue until there is enough surface area to collect a section. –4.3. Section the model at the same thickness as the tissue to be quantified. –4.4. Thaw-mount onto the slide with the tissue to be quantified. –4.5. Replace remainder of the mimetic model back into the -80°C freezer for storage. [See figure in Figures section.](#)

Troubleshooting

1. Bubbles / Holes in cryosectioned tissue. If bubbles are apparent in the cryosectioned tissue, there are several steps where they may have been introduced. –1.1. The use of a non-volatile solvent, such as DMSO, has yielded a non-homogenous mixture once added to the tissue homogenate. –1.2. When mixing the standard with the tissue homogenate, there is potential to introduce air bubbles. A centrifuging step was introduced to minimize the effect of these air bubbles. –1.3. When collecting the homogenate with the positive displacement pipette, be sure not to collect air bubbles. –1.4. When expelling the homogenate into the mimetic model mold, be sure to not expel all of the material in the pipette as this may result in a large air bubble in the tissue plug. 2. Tissue melting during sectioning. There are several situations which can yield a mimetic model that does not section well. –2.1. High lipid content tissues are prone to poor sectioning (melting during sectioning). If a largescale homogenization was performed, be sure to combine the homogenates before aliquotting for the mimetic model to avoid bias in lipid content. –2.2. If ethanol enters the mold during the freezing step this can alter the density of the tissue homogenate. There are a couple of situations which can lead to this. —2.2.1. Submerging the mold too far where dry-ice cooled ethanol enters the top of the syringe. —2.2.2. If the syringe does not have a rubber seal, there is an increased chance that ethanol enters the syringe from the plunger side.

Anticipated Results

The figure below shows an example of a section of H&E stained liver tissue compared to a section of mimetic tissue model created from liver tissue homogenate. [See figure in Figures section.](#) Once the mimetic tissue model has been sectioned and thaw-mounted onto the microscope slide (ITO coated slide for MALDI) with the tissue(s) to be quantified, the slide can undergo the standard IMS workflow. For MALDI this would involve coating with MALDI matrix commonly using either sublimation or a commercial sprayer (TM Sprayer from HTX Technologies). For acquisition, a single ROI can be drawn which covers each of the layers of the mimetic model or individual ROIs can be drawn over each layer. Minimally, these ROIs should be drawn such that there are at least 200 pixels per layer. This will allow for a better assessment of the average intensity as well as the standard deviation of the intensity at each level which is used for the weighted regression. An example is shown below of the ion image from the mimetic tissue model and a section of dosed tissue along with the associated calibration curve. [See figure in Figures](#)

section. **Concluding Remarks** The mimetic tissue model represents an approach to IMS quantification which accounts for the major IMS challenges of ion suppression and extraction efficiency through the use of matrix-matched standards. The tissue homogenate used for the mimetic model represents the average ion suppression that would be expected from the corresponding target tissue. The protocol presented here is an improved methodology for a more efficient preparation of the mimetic tissue model. Because the model is placed on the same slide with the tissue(s) to be quantified, it undergoes the same sample preparation and acquisition conditions as the tissue(s) of interest. Due to the potential for variation in sample preparation, it is key that the mimetic tissue model be applied only within an acquisition. While the mimetic tissue model is seemingly a more involved preparation compared to the spotting approaches, there are a number of applications where the mimetic tissue model is more efficient. When replication is necessary or if the quantification is to be applied to multiple tissues, this merely requires a single preparation followed by the collection of another section of the tissue model rather than collecting a control tissue section and hand-spotting the calibration curve for each replicate. This is often the case in pharmaceutical analysis where tissues are available from multiple animals in different dosing groups or from various time points.

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Figures

Figure 1

Mimetic Tissue Model Workflow

Figure 2

Homogenization

Figure 3

Trimming the syringe barrel

Figure 4

Trimming the plunger seal

Figure 5

Creating a handle for the mold

Figure 6

Homogenate spiking and mixing

Figure 7

Preparing the Mimetic Tissue Model plug

Figure 8

Removing the model from the mold

Figure 9
 Mounting the Mimetic Tissue Model for cryosectioning

Figure 10
 Cryosectioning the Mimetic Tissue Model

Figure 11

Mimetic Tissue Model histology Comparison of a section of the Mimetic Tissue Model made from Rat liver homogenate with a section of in-tact Rat liver.

Figure 12

Ion image from Mimetic Tissue Model and resulting standard curve

Supplementary Files

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- [supplement0.xlsx](#)