Title

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A newly developed method for capturing small molecule communication between tissues and cells using imaging mass spectrometry.

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Abstract

Imaging mass spectrometry (IMS) has routinely been applied to three types of samples: tissue sections, spheroids, and microbial colonies. These sample types have been analyzed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) to visualize the distribution of proteins, lipids, and metabolites across the biological sample of interest. We have developed a novel sample preparation method that combines the strengths of the three previous applications to address an underexplored approach for identifying chemical communication in cancer, by seeding mammalian cell cultures into agarose in co-culture with healthy tissues followed by desiccation of the sample. Mammalian tissue and cells are co-cultured in close proximity allowing chemical communication via diffusion between the tissue and cells. At specific time points, the agarose-based sample is dried in the same manner as microbial colonies prepared for IMS analysis. Our method was developed to model the communication between high grade serous ovarian cancer derived from the fallopian tube as it interacts with the ovary during metastasis. Optimization of the sample preparation resulted in the identification of norepinephrine as a key chemical component in the ovarian microenvironment. This newly developed method can be applied to other biological systems that require an understanding of chemical communication between adjacent cells or tissues.

SUMMARY:

A novel method of sample preparation was developed to accommodate cell and tissue co-culture to detect small molecule exchange using imaging mass spectrometry.

Keywords

Imaging Mass Spectrometry; Ovarian cancer; 3D cell culture; co-culture; tissue; cells; metabolomics

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DISCLOSURES:
The authors have nothing to disclose

INTRODUCTION:

Imaging mass spectrometry (IMS) has been optimized to characterize the spatial distribution of molecular features in three widely used applications: tissue slices, spheroids, and microbial colonies.[1–3] Tissue slices can be used to evaluate the localization of metabolites in the context of biological conditions in a host, either targeted or untargeted within a specific mass range. However, differences between molecular features are the most significant and obvious when a healthy tissue is compared to a diseased condition, for example, a tumor. This IMS approach is particularly adapted to detection of disease biomarkers, however, acquiring tissue samples at discrete stages in disease progression (such as tumor grades) precludes the identification of signals that could be important for initiation of the disease. The exchange of information through space is a ubiquitous feature of many biological systems, and tissue slices cannot capture this dynamic chemical relay. One technique that is capable of visualizing chemical exchange and diffusion is IMS of microbial colonies grown on agar plates; small molecules are able to diffuse through and across the agar and can be captured via matrix-assisted laser desorption/ionization (MALDI-TOF).[4] This growth setup can be used to identify molecules exchanged between discrete biological entities (colonies), and can also determine directionality of metabolite production. The platform originally designed for microbial colony growth was adapted to explore the primary metabolism of tissue explants grown with mammalian cells, and IMS was used to evaluate the dynamic chemical exchange in an *in vitro* mammalian system.

In the past several years, it has become clear that high grade serous ovarian cancer (HGSOC) often originates in the fallopian tube epithelium (FTE) and then metastasizes to the ovary during early disease development.[5–8] The reason that tumorigenic FTE cells spread to the ovary, where large tumors eventually form and metastasize further, is currently unclear. Previous research has focused on the role of ovarian proteins in primary metastasis to the ovary; however, it has recently been demonstrated that the transition from a healthy to a tumorigenic tissue results in massive disruption of cellular metabolism, and altered production of small molecules.[9–11] Therefore, we hypothesized that small molecules exchanged between the FTE and the ovary may be partly responsible for primary metastasis of HGSOC.

Using our newly developed IMS procedure, we have determined that coculture of tumorigenic FTE and healthy ovarian tissue induces the production of norepinephrine from the ovary. However, other cell types or normal FTE cells did not elicit this effect. An extraordinary benefit of this method is that the molecular production and exchange of signals that represent real molecules can be visualized, so even in a coculture it is possible to determine the source of a signal. This is an advantage over analysis of homogenized samples, where all spatial information is lost. In our model system, we were able to clearly assign the production of norepinephrine to the ovary. Norepinephrine has been linked to the metastasis and chemoresistance of ovarian cancers, and our detection of this molecule has validated that the novel IMS method can uncover biologically relevant molecules.[12–14] This validation lets us propose that this new application of IMS can be particularly helpful to research groups that are attempting to identify small molecules in coculture environments and to understand early events that influence cell transformation and metastasis.

PROTOCOL:

1. Preparation of Reagents

1.1 MOE cells are maintained at 37°C with 5% CO_2 in a humidified incubator in αMEM media supplemented with 10% FBS, 2 mM L-glutamine, 10 mg/mL ITS, 1.8 ng/ml EGF, 100 U/mL penicillin-streptomycin, 1 mg/mL gentamycin, and 18.2 ng/mL estradiol-17β. It is important to pass the cells so that there are enough cells on the same day the mice will be sacrificed.

1.2 Prepare 2% agarose by mixing 1 g low-melting agarose with 50 mL distilled water. Autoclave agarose, allow to cool, and then aliquot (1 mL) before agarose solidifies. Agarose can be stored at −20 °C indefinitely.

1.3 1X DMEM media.

1.4 ITO-treated slide.

1.5 The 8-well divider is from a Millipore Millicell EZ-slide chamber slide. The dividers from other brands of chamber slides can be used, but for this experiment those from Millipore work the best, due to the adhesive rubber bottom, and because the parallel sides of the wells facilitate easy introduction and removal of a plastic divider for the divided wells.

1.6 Matrix for MALDI-TOF. Prepare 10 mL of 5 mg/mL 1:1 CHCA:DHB (Sigma) in 90:10 ACN:H_2O + 0.1% TFA. Sonicate until matrix is dissolved.

1.7 HTX TM Sprayer.

2. Mouse colony and ovary removal

2.1 Breeding pairs of CD-1 mice are maintained using standard housing procedures. Near the day of parturition mice should be checked every day so that the age of the pups is known. All animal procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Use and Care (IACUC) committee at the University of Illinois at Chicago.

2.2 When the pups are 16–18 days old, ovaries are excised and placed in pre-warmed Leibovitz's L-15 media with 1X penicillin-streptomycin using a sterile technique. The ovaries can be stored at 37°C for a few hours, if needed.

2.3 Under a dissecting microscope, carefully remove the ovaries from bursa and separate from the rest of the reproductive tract. As each ovary is removed from the reproductive tract, move it to a separate dish with Leibovitz's L-15 media, and cut in half axially. The ovarian pieces (now called explants) can be kept at 37 °C until plating of agarose.

3. Setting up and Incubating the ITO-Treated Slide for Cocultures.

3.1 Undivided Cocultures—3.1.1 Liquefy the agarose at 70°C on a hot plate.

3.1.2 Place the 8-well divider on top of the ITO-treated slide (Figure 1a). The rubber bottom on dividers from Millipore should aid in adhesion to the slide, but continuous gentle downward pressure during agarose plating ensures no leaking or mixing between wells.

3.1.3 Collect cells, centrifuge, and resuspend to 50k cells per 150 μL in DMEM media. If a different cell density is optimal, make sure that at this step the cell suspension is 2X the final density desired (eg, for a final concentration of 50k cells in 300 μL, cell density at this step should be 50k cells in 150 μL).

3.1.4 Before plating cell culture, add ovarian explant to center of well (Figure 1b).

3.1.5 Add agarose to each cell culture in individual eppendorf tubes just before plating. For each well, combine 200 μL of cell suspension and 200 μL of liquified agarose in an eppendorf tube. For example, for four wells, combine 800 μL cell suspension and 800 μL 2% agarose. Some mixture will be left over, but making slightly more than necessary avoids air bubbles during pipetting.

3.1.5.1 You will want to add agarose to individual cell cultures immediately before plating that cell culture. The agarose will cool in under a minute, so be prepared to plate quickly.

3.1.6 Immediately add 300 μL of the cell/agarose mixture to each well (Figure 1c). Depending on the experiment, it may be important to include controls such as (1) wells with no ovarian explants or cells, (2) wells with cells only, or (3) wells with ovarian explants only. Figure 1c shows three cell conditions and one media condition, each plated with and without an ovary.

3.1.7 Incubate slide at 37°C and 5% CO_2 in a humidified incubator.

3.2 Divided Cocultures.—3.2.1 Cut dividers from thin, smooth plastic. This experiment uses the sides of a sterile disposable media basin because they are flat and thin. Cut them just wide enough to fit snugly into the hypotenuse of the well (~13 mm).

3.2.2 Liquefy the agarose at 70°C on a hot plate.

3.2.3 Place the 8-well divider on top of the ITO-treated slide. Rubber bottom should aid in adhesion to the slide, but continuous gentle downward pressure during agarose plating ensures no leaking or mixing between wells.

3.2.4 After collecting cells, resuspend to 50k per 150 μL of media. If a different cell density is optimal, make sure that at this step the cell suspension is 2X the final density desired (eg, for a final concentration of 50k cells in 300 μL, cell density at this step should be 50k cells in 150 μL).

3.2.5 Insert plastic dividers diagonally into wells.

3.2.6 Add agarose to each cell suspension one at a time just before plating. For each well, combine 100 μL of cell suspension and 100 μL of liquified agarose in an eppendorf tube. For example, for four wells, combine 400 μL cell culture and 400 μL 2% agarose. Some cell/

agarose mixture will be left over, but making slightly more than necessary avoids air bubbles during pipetting.

3.2.6.1 You will want to add agarose to individual cell cultures immediately before plating that cell culture. The agarose will cool in under a minute, so be prepared to plate quickly.

3.2.7 On one side of the divider, plate 150 μL of cell/agarose mixture. Allow to cool and solidify (approximately one min) and then remove the divider.

3.2.8 Place ovary explant in center of empty half of well. Place 150 μL media/agarose mixture over top of ovary, and only in the correct side of the well.

3.2.9 Incubate slide at 37°C and 5% CO_2 in a humidified incubator.

4. Drying slide and preparing for MALDI-TOF.

4.1 After four days (or any preferred time point), remove the chamber divider from the agarose plugs and the slide (Figure 1d). Gently detach the sides of the agarose from the chamber with a flat spatula and gently pull the chamber upward, being careful not to move any agarose plugs. If they do move, gently reposition them so that they are not touching one another.

4.2 Place the slide in a 37°C oven for approximately four h, rotating 90° every h. The rotation of the slide is important to ensure even heat distribution throughout the sample.

4.3 Once dry, remove the slide from the oven (Figure 1e). The agarose plugs should be effectively flat and the slide should be removed before the agarose begins to wrinkle. The slide must be completely dried or this could lead to an explosion of the sample in the high vacuum environment of the MALDI-TOF mass spectrometer.

4.4 Apply matrix solution using the sprayer (Figure 1f). **Parameters: Temperature = 30°C, Flow rate = 0.2 mL/min, Number of passes = 8, Direction: CC, Distance =**.

4.4.1 In lieu of aTM Sprayer, an artistic airbrush can be used to apply liquid matrix. The same matrix solution can be used to spray, but approximately twice as much solution will be required. With the slide clamped so that it hang vertically, spray the slide from a 90° angle from approximately one foot away (see Hoffmann[15]) until matrix layer is visible.

4.5 Add 1 μL of calibrant (Phosphorus Red for targets <500 Da, PepMix for targets <5000 Da) to clear spot on slide. Phosphorus Red requires no mixing with matrix, but PepMix requires 1:1 mixture with matrix to aid ionization. Wait for calibrant to dry.

4.6 Draw an X using a permanent marker in each corner of your slide and take a optical image using a camera or a scanner. A 1200 dpi is required for high spatial resolution.

5. Imaging mass spectrometry in FlexImaging

5.1 Open a new sequence on flexImaging. Set the raster width to desired spatial resolution, at least 50 μm. With the TM Sprayer, it is possible to do 5 μm resolution, but this lengthens

the overall time to gather the mass spectrometry data while yielding comparable results and is also dependant on the ability to focus the laser in the MALDI-TOF mass spectrometer.

5.2 Upload the optical image of the slide to FlexImaging and set three teach points using the intersections in the X's drawn in each corner.

5.3 Designate regions of interest for imaging in flexImaging and name them accordingly.

5.4 Calibrate the instrument using FlexControl within 5 ppm error.

5.5 Save the optimized method and begin run. This experiment optimized the following parameters: Polarity = Positive, Detector = Reflectron, Laser size = 2, Laser power = 50%, Reflector gain = 3x.

6. Processing IMS data

6.1 To obtain statistically significant signals, import .mis file into the software of choice. In these experiments, we analyze IMS data using SCiLS Lab (Bruker).

REPRESENTATIVE RESULTS:

An optimal dried ITO slide will result in a flat desiccated sample with minimal to no wrinkles across the surface of the agarose and agarose pieces that maintain spatially separated on the slide. Other substrates aside from agarose may work, but often times do not maintain their integrity upon drying or removal of the well chamber which results in spreading across the ITO slide and loss of spatial information. For example, we have previously tested collagen as a substrate and this resulted in spreading and loss of spatial information when the well chamber was removed. The tissue used should be in the center of the well if it is undivided, or in the center of the half triangle if it is divided, so that acquisition of IMS data is not contaminated with edge effects.

A series of experiments determined that an 8-well chamber was the best vehicle for incubation, as compared to a 6-well plate and a 24-well plate because the 8-well chamber results in minimal perturbations to the cells, whereas the other larger chambers requires agarose slabs to be transferred to an ITO or stainless steel slide. Additionally, the 8-well chamber allows for 8 conditions to be tested or controlled for in a single experiment. Because the sample preparation (precise media and agarose concentration, matrix amount, etc) differs slightly between runs, conditions can only be compared when they are acquired on the same slide. Further experiments determined that an ITO-coated slide was the best platform for incubation, when compared to a steel MALDI plate because the slide allows for visual verification of the cell state. For instance, using the ITO slide, we can verify that the cells have normal morphology and that they maintain even distribution throughout the agarose prior to desiccation. This will also allow for incorporation of fluorescent cell lines as needed. Additionally, removal of the chamber prior to desiccation is required to maintain the entirety of the agarose plug. If the slide is desiccated with the 8-well chamber adhered, the plastic pulls the agarose plug apart and results in major cracks in the plugs.

Another consideration prior to spraying is ensuring that the slide does not sit in the oven until the agarose begins to wrinkle. The wrinkling creates topographical differences in the surface of the agarose plugs and may affect the accuracy of the mass measurements.

Overall, we have optimized this slide setup to best detect exchanged small molecules in coculture. Therefore, during data analysis we are seeking molecular features that are only present or are significantly more abundant in an agarose plug that represents the biological condition of interest. Because there is the option to include seven other controls and conditions on the slide, this can be achieved visually and by the aid of statistical programs like SCiLS. Because this is the first pass of detection, the spatial positioning of the molecular features is extremely important, even though only the nominal mass can be detected on the MALDI-TOF instrument in our laboratory. Our dereplication process following the detection of spatially relevant masses at low-resolution is extensive so that we can obtain a high resolution mass and fragmentation data orthogonally. The first step that we take for dereplication is a search of the nominal mass through a database, such as the Human Metabolome Database (HMDB) for mammalian metabolites.

The first search through HMDB can be LC/MS, which detects molecules that have a matching nominal mass within the given error. Most of the molecules in the database have a significant number of spectra covering many techniques for comparison to experimental data. Once nominal masses have realistic candidates as their identity, it is often possible to compare physical data such as MS/MS fragmentation, UV profile, and retention time between the candidate structure and a standard. This dereplication workflow is how we detected norepinephrine in the coculture described.

DISCUSSION:

Identification of norepinephrine in the coculture of tumorigenic FTE cells incubated with ovarian tissue has validated that this IMS method is capable of detecting real and relevant small molecules from this system.[16] There is a growing body of evidence that implicates norepinephrine's role in HGSOC,[12,17,18] and this technique has contributed more mechanistic information. With at least eight biological conditions present on the same slide, the method can account for biological controls such as gene and cell specificity as well as media controls in a single IMS run. While the method was optimized to evaluate exchanged small molecules in primary metastasis in HGSOC, any cell or tissue type that can be placed in agarose can be substituted to analyze a wide variety of biological questions and disease states.

One of the most important steps in the protocol is ensuring that the tissue or cell types are in close proximity to one another and are present in the center of the agarose plug. Another important consideration is optimization of the drying time for the entire slide. This method was optimized using ovarian tissue, which dried easily and whose small size resulted in little drying complications. However, other tissues, with different composition such as fat, have very different drying profiles and therefore require more extensive desiccation optimization. After drying has been optimized for the biological sample, the remaining workflow should only minimal alteration.

During mass spectral acquisition, it is likely that many projects will require the analysis of compound groups other than small molecules. The IMS parameters for acquisition and the selection of matrix, therefore, should be adapted to generate the best data for the respective target, if it is known. Additionally, our screenings have only been done in positive mode with a 50:50 CHCA:DHB matrix, but negative mode experiments may prefer a different type of matrix, and larger molecules would be more easily detected in a different matrix as well. The method can be used with a wide mass range in an untargeted approach or with a more narrow mass range for a targeted search. In the case of the discussed method, small molecules were the target of interest because the focus was to detect molecules that were exchanged through the agarose between cell and tissue representatives. Although we cannot claim the limitations in terms of size and structure that inhibit movement through agarose, our aim was to detect small molecules, so our matrix decision and MALDI-TOF parameters were optimized for that goal. Future experiments are being developed to target lipids and proteins that may have been missed in our original acquisition method.

In addition to the limitation of compound class detection, this method also requires that the cells be compatible with agarose, and that the tissue sample (if used) be adapted to fit into an eight-well chamber. Certain tissues can be adapted to larger wells if fewer biological conditions are required for comparison, but it is crucial that the selected tissue is capable of drying to ~10 μm height after desiccation. Multiple players could be evaluated such as more than one cell type in communication with a tissue. Because the sample preparation is able to spatially separate agarose-based cell cultures, it is possible to assign visualized *m/z*'s to the cell culture source based on observed diffusion patterns.

Using IMS to study tissue sections from human samples or transgenic mouse models recapitulates the complexity of tissues. However, human samples are difficult to obtain, which can dramatically limit the ability to study things like disease progression. Tissue from transgenic mouse models can be collected at well-defined time points. But, mouse models can be time consuming and expensive to develop. In addition, the full complexity of real tissues in both of these models can make it difficult to accurately evaluate communication between specific cells or tissues. In contrast, the method presented here is a highly adaptable. Different tissues or cell lines can be co-cultured on the same slide to examine differences in communication. For example, normal cells or tumor cells from the same tissue can be cultured on the same slide to understand differences due to transformation. Time course studies may uncover novel signaling cascades between cells, or small molecule inhibitors and/or RNAi could be incorporated to examine the role of specific signaling molecules or metabolites. We believe these possibilities will make this technique useful for studying cell-to-cell communication in a wide variety of contexts.

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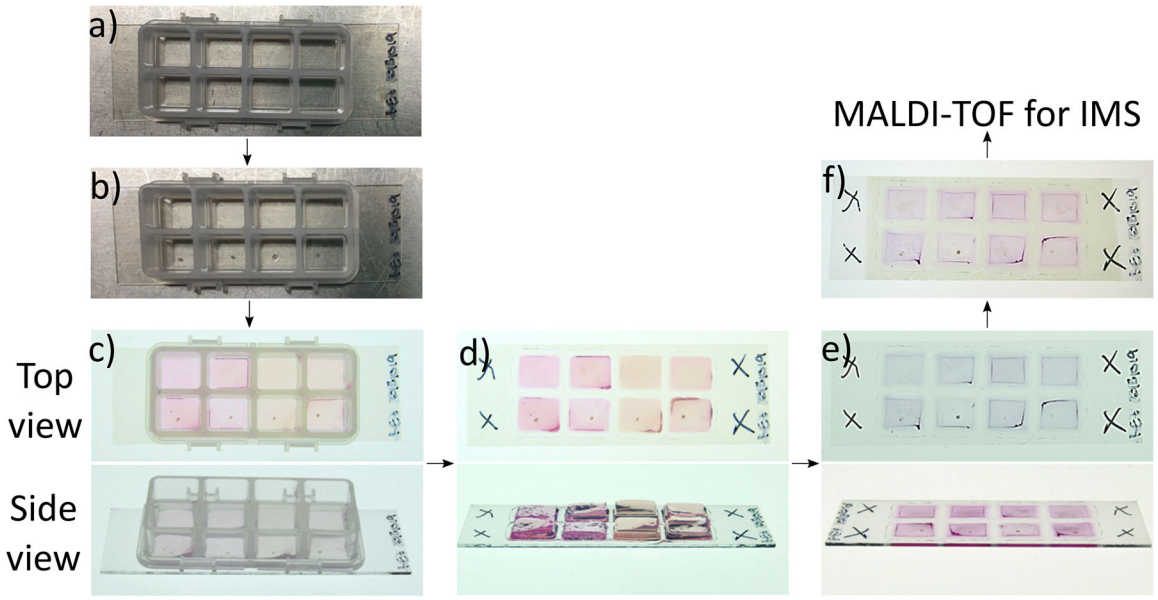

Figure 1.

Workflow for sample preparation of undivided coculture. a) Adhere 8-well chamber to conductive side of ITO-coated slide. b) Place halved ovaries in center of wells for coculture conditions. c) Add 300 μL of agarose/cell suspension directly into wells. Make sure there are no air bubbles and that the ovary remains in the center of the well. If the pipetted agarose disturbed the ovary, gently use the pipet tip to recenter it before the agarose cools. d) After four days of incubation (or otherwise optimized time) remove 8-well chamber from slide. If agarose remains attached to chamber, gently detach agarose plug from chamber using a spatula and reposition it on the slide. The agarose will not adhere to the slide, so it will be easy to move. Draw an 'X' on each corner of the slide and take a photo. e) Dry the slide in a 37C oven for 4 hrs, rotating 90 each hour. Agarose should be fully desiccated and should lie flat on the slide. f) Apply matrix of choice via TM Sprayer or airbrush to slide. Matrix layer should be visible as yellow. Scan the slide on a scanner at 1200 dpi and and calibrants or standards for MALDI-TOF analysis.

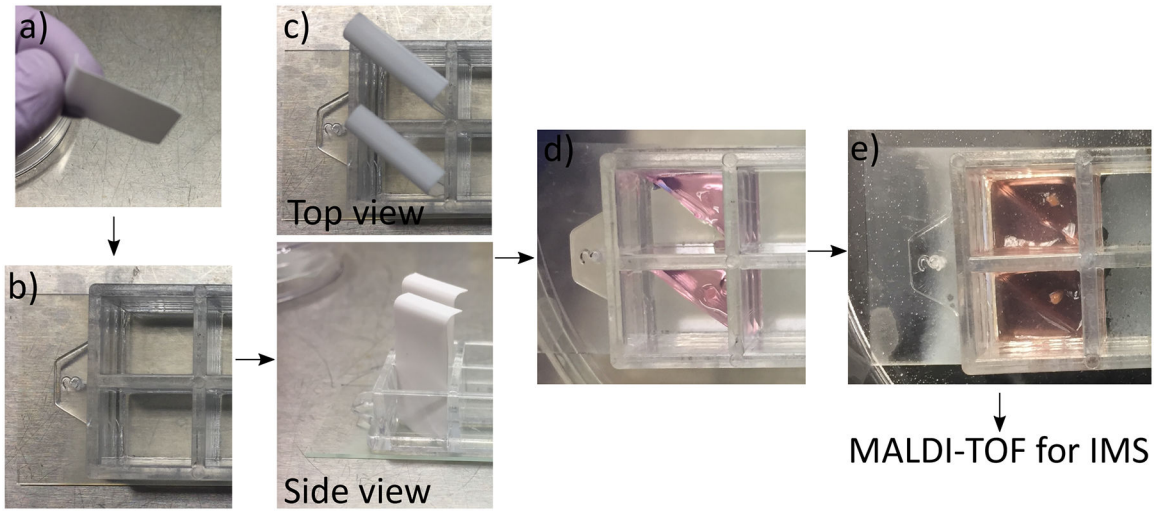

Figure 2.

Workflow for sample preparation of divided coculture. a) Cut tabs out of media basin (~13mm) and ensure that sides are straight. b) Attach 8-well chamber to conductive side of ITO-coated slide. c) Insert dividers diagonally into wells. d) Add 150 μL cell culture in agarose to one side of divider, allow agarose to cool, and remove divider. e) Add ovary to center of empty well half and cover with 150 μL of media and agarose suspension.

tabel 1.

XYZ

Name of Material/Equipment	Company	Catalog Number	Comments/Description
DMEM Media	Gibco	11995–065	Media mixed with agarose
Low-melting agarose	Sigma-Aldrich	A9414–10G	Mixed with media for plating
ITO-coated slide	Bruker	8237001	Platform for co-culture incubation
8-well chamber	Millipore	PEZGS0816	Repurposed from Millipore Millicell EZ-slide chamber slide
CHCA Matrix	Bruker Daltonic	8201344	Matrix sprayed onto dried slide
DHB Matrix	Bruker Daltonic	8201346	Matrix sprayed onto dried slide
Acetonitrile	Sigma-Aldrich	34998–4L	Solvent for sprayed matrix
TFA	Fisher Technologies	A116–50	Added to matrix solution
15 mL falcon tubes	Denville	C1017-O	
Eppendorf tubes	Genesee Scientific	22–282	To mix agarose and cells
Media basin	Corning	4870	Used to cut plastic dividers for divided chambers
Peptide Calibration Standard	Bruker Daltonic	8206195	Calibrant for medium mass range
Phophorus red	Sigma-Aldrich	343–242-5G	Calibrant for low mass range
TM Sprayer	HTX Technologies		For applying matrix
Centrifuge	Eppendorf	5810 R	To collect cells and remove supernatant
Autoflex speed MALDI-TOF	Bruker		For IMS