Direct Analysis of Biotransformations with Mass Spectrometry – DiBT-MS

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Abstract: Ambient ionization coupled to mass spectrometry has the advantages of minimal requirements for sample preparation prior to analysis which renders it suitable for high throughput screening. We present a protocol that permits the application of this method in routine biotechnology and chemical biology laboratories which are using engineered enzymes to produce target compounds from substrates. We show how DESI-MS can be used to **di**rectly analyse the activity of **b**io**t**ransformations from crude cell lysate which we term DiBT-MS, this method is 10-1000 times faster than LC-MS and uses far less solvent. This protocol demonstrates the impact of solvent spray composition on ionization efficiency of the target analyte, the benefits of a nylon membrane slide and the reusability of sample slides in multiple experiments.

Introduction:

Desorption Electrospray Ionization (DESI) is a mass spectrometry technique performed under ambient conditions without complex separation and purification of samples. DESI-MS, developed in 2004[1], has simplified the imaging of tissue samples and has been adopted for the detection of a variety of analytes including protein complexes[2,3], bioassays[4] and lipids[5]. DESI-MS has recently evolved into an efficient semi quantitative screening tool for biocatalysis, termed direct infusion of biotransformation mass spectrometry (DiBT-MS) with the ability to screen hundreds of samples for the products of biotransformations concurrently using liquid handling robots in conjunction with the DESI stage[6].

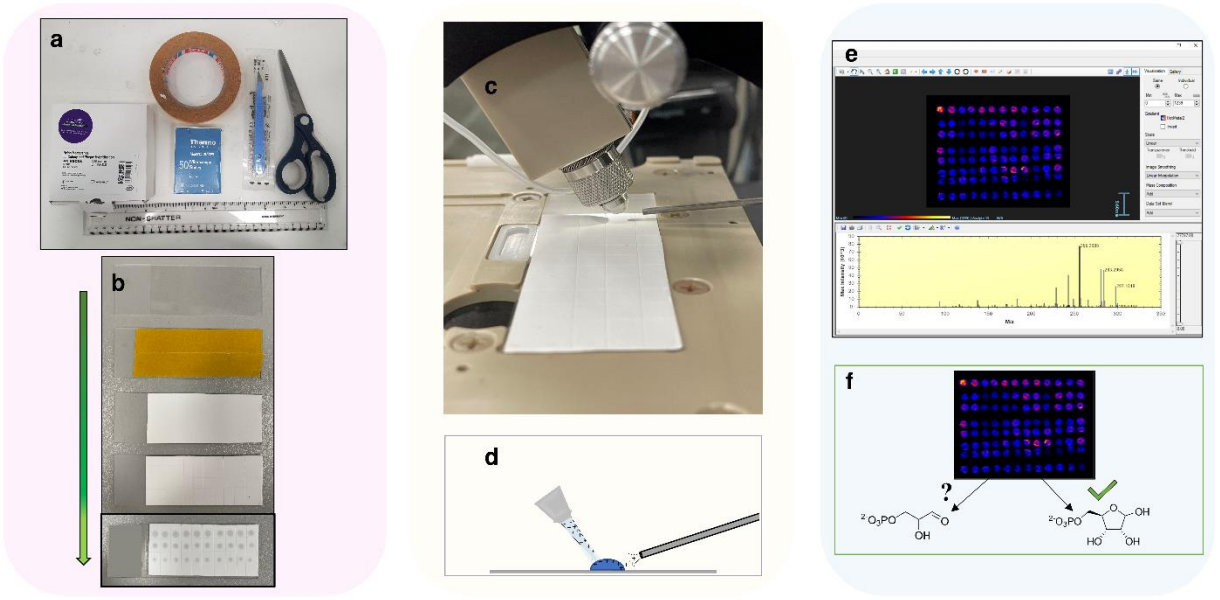

Figure 1: **Overview of the DiBT-MS workflow**. a) Materials required to prepare sample slides, including glass slides, nylon membrane, double-sided tape, a disposable scalpel, scissors and a ruler. b) Step-by-step visualization of sample slide preparation, from adding the double-sided tape to the glass slide (slide 2), affixing the nylon membrane (slide 3), scoring the nylon to make sample wells (slide 4) and spotting the nylon with sample (slide 5). c) Image of the Prosolia

sprayer head over the nylon membrane as an experiment was being recorded. Relative positioning of the sprayer head (70^{o} angle) and ion inlet (1mm from slide surface) can be used as a starting point for stage parameters when a new user is establishing their setup. d) Schematic of the protonation event that occurs on the sample slide, in positive ion mode. A microdroplet is temporarily formed on the surface of the slide, from which the sample is dissolved and then sucked into the metal ion inlet. e) Example of a molecular ion "hit" on a 96-well plate, visualized in HD Imaging, v1.4. f) Discovery of molecular ion associated with desired product formation and confirmation of successful biotransformation.

Successful transfer of target compounds in their ionised form to the mass spectrometer is dependent on physical (sample ablation) and chemical (protonation) processes, both of which must be optimised. The DESI stage has a solvent sprayer head that emits a charged solution onto the affixed DESI sample slide. This physical ablation of the sample by the charged solution (due to pressurized inert gas coming through the nozzle) allows the analyte of interest to be transferred into the gas phase through a thin film of solvated sample on the surface of the DESI slide and sucked into the ion source through a large extended metal source inlet arm (Figure 1, c and d).

Previous reports on optimisation of DESI include: a spotting-dependent robotic arm for more efficient data acquisition[7], manipulation of the droplet formation during sample ablation[8,9], and modulation of the electric field strength[4,10]. These iterations in general have resulted in increased ionization efficiency and lower limits of detection; however, these may not always be compatible with biotechnological screening assays[11]. Here we demonstrate an optimised methodology for

the screening of crude biotransformations using a commercially available DESI mass spectrometer.

Development of Protocol:

Collaboration and the need for objective reproducibility in medical research is well-known and vital for the production of non-biased and widely utilized clinical methdologies[12].

Direct infusion mass spectrometry is a particularly useful analytical technique for biocatalysis due to the simplicity of the sample prep; there are a number of reports that demonstrate well-resolved protein and small molecule detection from unpurified cells without sample extraction or extra purification[13–15]. A recent protocol has demonstrated the viability of native mass spectrometry experiments done directly from crude cell lysates[16]. We have employed a very similar sample preparation related to this protocol in a variety of experiments including the analysis of biotransformations from crude cell lysates or live bacterial colonies[17], and more recently in the rapid screening of biotransformations of imine reductases (IREDs) to elicit structure-activity relationships[18].

Our protocol herein demonstrates the optimization of carboxylic acid reductases (CARs) by DiBT-MS for amide bond formation and subsequent production of active pharmaceutical ingredients (APIs). CARs, multi-domain enzymes consisting of an adenylation (A-), peptidyl carrier protein (PCP-) and reductase (R-) domain, catalyse selective reduction of carboxylic acids to aldehydes[19,20]. It was demonstrated recently that isolated CAR A-domains function as standalone catalytic entities to facilitate amide bond formation, while the CAR's native function is abolished[21]. A wide scope of carboxylic acid substrates can be amidated in presence of amine nucleophiles under mild aqueous conditions, merely requiring adenosine 5'-triphosphate (ATP)

as a cofactor.[21] Their high selectivity and efficiency make them ideal candidates for bioengineering analysis in a high-throughput method. Enzyme cascade optimization is relevant for its use in the product formation of therapeutics that act as enzyme inhibitors such as iminosugars for antivirals[22], or biocatalysts with high chemoselectivity in a one pot synthesis[23] and as such is an ideal candidate for DiBT-MS high-throughput screening.

Given the visual data readout and the broad applicability to a variety of biological platforms this method has the potential to be used as a routine screen for chemical biology research removing the need for liquid handling or HPLC. The protocol is presented to educate on the following components of the DiBT-MS workflow:

- Creating customized nylon membrane slides for analysis of live cell colonies or crude cell lysate
- The reusability of sample slides for effective visualization of low-concentration samples on multiple days
- Optimization of ion transmission through modulation of the binary solvent system in the DESI sprayer head

Comparison with other methods:

For the analysis of biotransformation products, the go to method would be separation and identification by liquid chromatography mass spectrometry (LC-MS)[21]. In this protocol, DiBT-MS was chosen because either the products formed were not visualized by LC-MS, or LC-MS could not give any information on the amount of reactant consumed during the biotransformation. In

a comparative study of similar compounds, a large bioassay screen (of 3840 reductive amination reactions) using both LC-MS and DESI-MS methods provided the following comparison of compound identification: 71% overlap in identification, 15% observed by DESI-MS alone, and 14% by only LC-MS[7]. Given the considerable number of compounds exclusively visualized by DESI-MS or LC-MS, it may be prudent to test out experiment samples with both instrument setups. However, DiBT-MS provides a faster workflow and uses fewer solvents and chemicals[18,24].

The spray solvent composition for DESI is often a methanol and water mixture[25,26], with some other solvent compositions used in limited runs. Research has been carried out to determine the effectiveness of several non-aqueous binary solvent systems[26] on hydrophobic compounds, demonstrating better ionization efficiency due to solubility of the sample in the solvent spray. This work has been followed by advancements to liquid-handling robots, some of which have been paired with DESI instruments for rapid solvent changing (but no mixing of solvents), prepared in a 1:1 (*v/v*) ratio[27]. As the purpose of this protocol is not to increase the level of complexity in the methodology, but rather make it more accessible, we perform experiments using the traditional methanol and water mixture, but with smaller adjustments in the methanol concentration to demonstrate that the increase in ionization efficiency can be observed with two solvents of similar polarity and under small composition changes of 5% *(v/v)* incrementally. This allows the user to maintain the use of less-hazardous solvents that are consistent with historical DESI literature while adding further sensitivity to experiments.

Method optimization is still required for a general user when beginning a workflow with a compound that has not yet been visualized. This amount of optimization will be unique to this specific analyte of interest and overall complexity of the experiment and sample composition,

therefore creating an experiment which may be more arduous to a general user than desired. However, we believe that an increased knowledge of this technique will greatly further the advancement of mass spectrometry as a complementary tool to other biotechnological techniques.

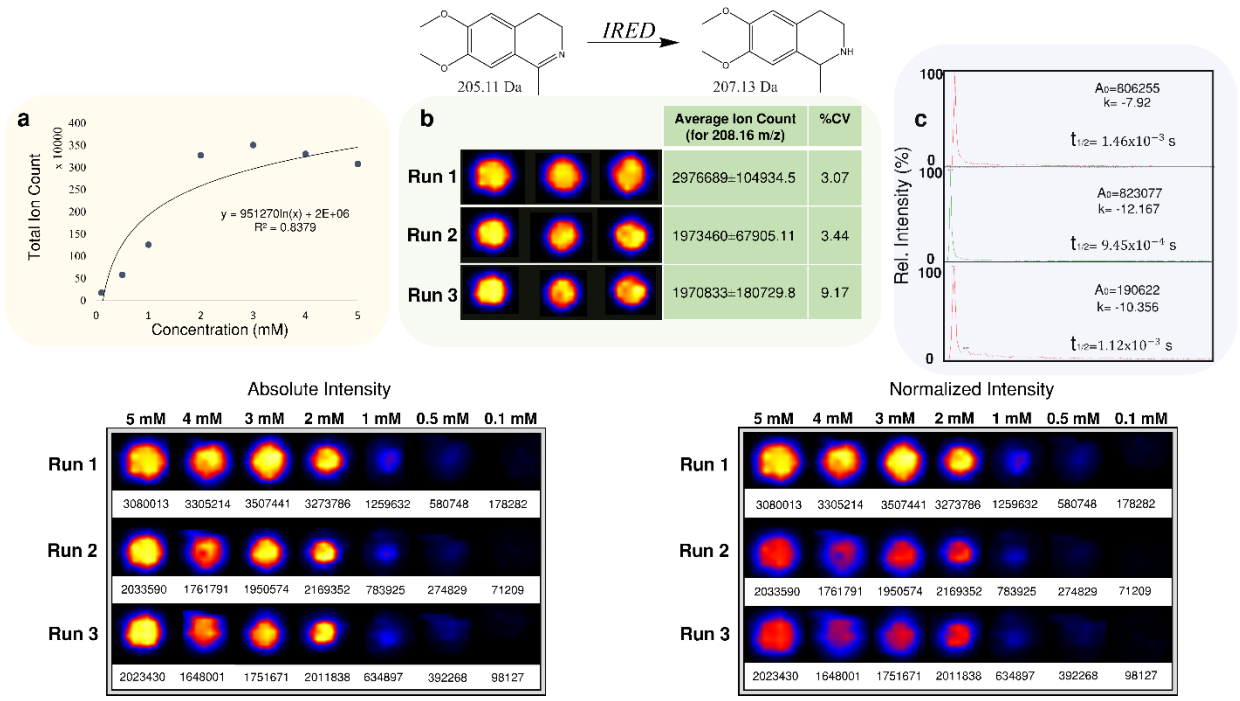

Figure 2: Experiments to demonstrate the reproducibility of the DiBT-MS workflow with nylon membrane slides. Two reference compounds (6,7-dimethoxymethyl-3,4-dihydroisoquinoline, 205.11 Da; 6,7-dimethoxymethyl-1,2,3,4-tetrahydroisoquinoline, 207.13 Da) are spotted in 500 nL volumes (ranging in concentration from 100 µM to 5 mM) onto nylon membrane and mass spectral images were acquired (500 µm x 500 µm pixels, rate of 1000 µm/s, 100% methanol as the sprayer head solvent). a) The total ion count (TIC) for protonated 6,7-dimethoxymethyl-1,2,3,4-tetrahydroisoquinoline (at 208.16 *m/z*) vs concentration spotted onto nylon membrane is plotted. TIC recorded increases non-linearly with concentration. b) Average TIC at 208.16 *m/z* for 5 mM spots of 6,7-dimethoxymethyl-1,2,3,4-tetrahydroisoquinoline in triplicate, with the percent coefficient of variation (%CV) to measure variability in DiBT-MS readout of the target

molecular ion across wells. The same spots were recorded in 3 consecutive experiments (acquiring over the same area) to demonstrate the presence of sample remaining on the nylon membrane after several ionization events. c) Spectra showing the TIC for the molecular ion at 208.16 *m/z* deplete over time while the sprayer head is passing over the 6,7-dimethoxymethyl-1,2,3,4-tetrahydroisoquinoline spotted well. Calculations of the half life decay (assuming first order decay reaction) are calculated for each run done consecutively, as in image B. d) Display of DESI-MS visualized spots on nylon membrane at 208.16 *m/z* across a concentration gradient, from 5 mM to 100 µM. Runs 1,2 and 3 were performed under the same acquisition parameters and sequentially. The TIC for 208.16 *m/z* ion in each droplet is displayed below the image of each droplet. e) Identical DESI imaging to the droplets shown in D, but with the heat map colouring normalized to the highest TIC. Run 1 shows consistently the largest TIC at each concentration, with relative intensity of TIC dropping to 66% of Run 1 in both Runs 2 and 3.

Experimental Design:

Consumables (nylon membrane, double sided tape) need to be purchased to generate sample slides for the DESI stage. Positive controls can be used throughout the experiments, including initial optimization with rhodamine 6G red pen ink (see below) and subsequent incorporation of a negative mutation in the biotransformation under study. Biotransformation lysates should be produced in mass spectrometry compatible buffers, with no or limited ionic detergents avoiding other excipient compounds that lead to ion suppression[28,29].

A syringe pump is required for the introduction of solvent through the sprayer head at a rate of 1-5 µL/min; traditional LC-MS fluidics with rates of 250-600 µL/min are not compatible with the DESI sprayer[30]. All steps in the protocol can all be carried out sequentially or on separate days, depending on the usage of the instrument and timing (see PAUSE POINTs below).

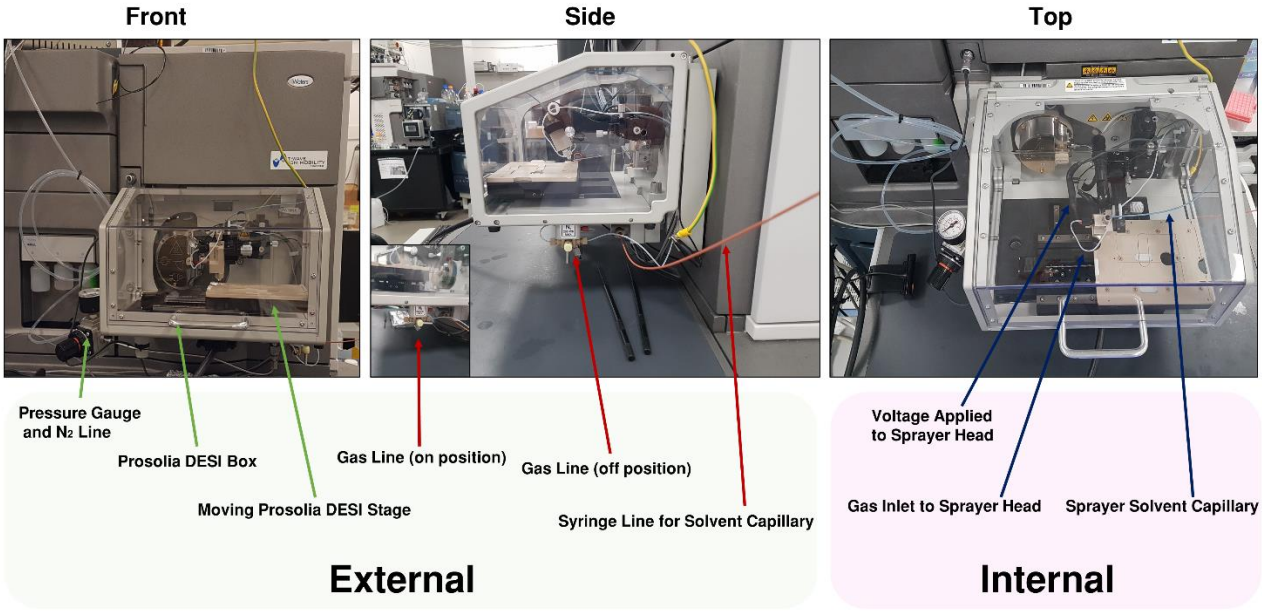

Figure 3: Identifying major physical components of the Prosolia DESI stage. This can be separated into external (outside the box) and internal stage (within the box). Three views of the Prosolia stage affixed to a Waters Synapt G2-Si instrument are shown, front (green arrows), side (red arrows) and top (blue arrows).

Limitations:

With ambient ionization techniques, the most common method of compound ionization is through protonation/deprotonation or cationisation, depending on the solvent used for the charge transfer[31]. Ion suppressants in solution may limit the effectiveness of this technique.

Materials:

Reagents

The solvents used for the solvent spray can be customized to the user. In the examples discussed below, methanol and water were used as the solvent system. CRITICAL STEP: HPLC- grade materials must be used to ensure limited clogging in the fused silica capillaries that connect the syringe to the sprayer head. Additionally, no salts should be added into this solution. CAUTION

As the solutions used may be customized with more volatile solutions as desired, please take care to read the manufacturers instructions and take appropriate PPE precautions when necessary.

- HLPC-Grade Methanol (Sigma Aldrich, cat. no. 900688)
- HPLC-Grade Water (Sigma Aldrich, cat. no. 270733)

Reagent Setup

Solvent used for the sprayer head solution should be prepared on the same day of use and can be stored at room temperature. The minimum protein quantity or concentration of the specific compound will vary depending on the ionization efficiency. To begin, a minimum concentration of 1 mM is recommended for the compound of interest, at a volume dispensed of 500 nL per sample. Upon optimization, the working range for this protocol is generally 50 - 500 nL volume per spotted sample, at a concentration of 50 µM - 5 mM (a reference visualization is shown in Figure 2). Non-volatile salts and detergents are traditionally not compatible with mass spectrometry experiments, please try to avoid the use of EDTA, Tris, and other related detergents. When possible, a workflow that involves a transfer of your biotransformation lysate into a 50:50 methanol water solution (*v/v*) has been our standard workflow for biotransformations of crude mixtures.

Equipment

This protocol was developed on the Prosolia DESI 2D Omni Spray Ion Source, in conjunction with a Waters Synapt G2-Si mass spectrometer. The Prosolia stage and accompanying Waters software (HD Imaging software v1.4) are also compatible with the Waters Xevo G2-XS instrument. The mass spectrometer was operated using MassLynx v4.2 in either positive or negative ion mode. The recent introduction of the Waters Synapt XS with DESI stage has not been tested with

this protocol. The sprayer head that accompanies the Prosolia stage has since been updated in 2021 to a newer sprayer head that is now the default component on the Waters Synapt XS instrumentation.

Glass slides in the 76 mm x 26 mm or 75 mm x 25 mm format are required for fitting into the Prosolia stage. Double sided tape can be purchased from a general goods supplier and should not exceed 1mm in thickness, as this will impact the z-coordinate of the sprayer head and potentially lower the stage out of range for ion collection through the sampling tube. The nylon membrane can be cut to purpose as needed using a scalpel. Consumables listed below were used for the generation of this protocol, but substitutes may be made.

- Nylon Membrane (Roche, 82 mm diametre, cat. no. 11699075001)
- Scalpel
- Microscope glass slides (Thermo Scientific, 76mmx26mm, cat. no. 12352098)
- Double sided tape
- Ruler
- Red Pen (with Rhodamine 6G dye, available from Sigma Aldrich, cat. no. BAH378620152 or other retailers)
- Variable Volume Pipette (Thermo Scientific, 0.2-2 µL range, cat. no. 4642010)

Equipment Setup

HD Imaging Software and MassLynx Software must be up to date to ensure compatibility between the electronics of the mass spectrometer and the DESI stage.

Procedure

Prosolia DESI Stage Alignment by Red Pen Test

As per the Waters manual and general procedure for the Prosolia stage set up, there is already in place a positive control test meant to determine if the current DESI setup is acceptable for use. This section of the protocol is a brief summary of the required optimization.

Timing: 40 minutes (including solvent line flushing)

Although there is some minor variation in the positioning of the sampling cone to the DESI stage, general parameters for the positioning and placement on the XYZ coordinates are given below (Figure 4). The following is a brief set up described by Waters intended for alignment of the DESI stage with the corresponding DESI stage software, Omni Spray 2D.

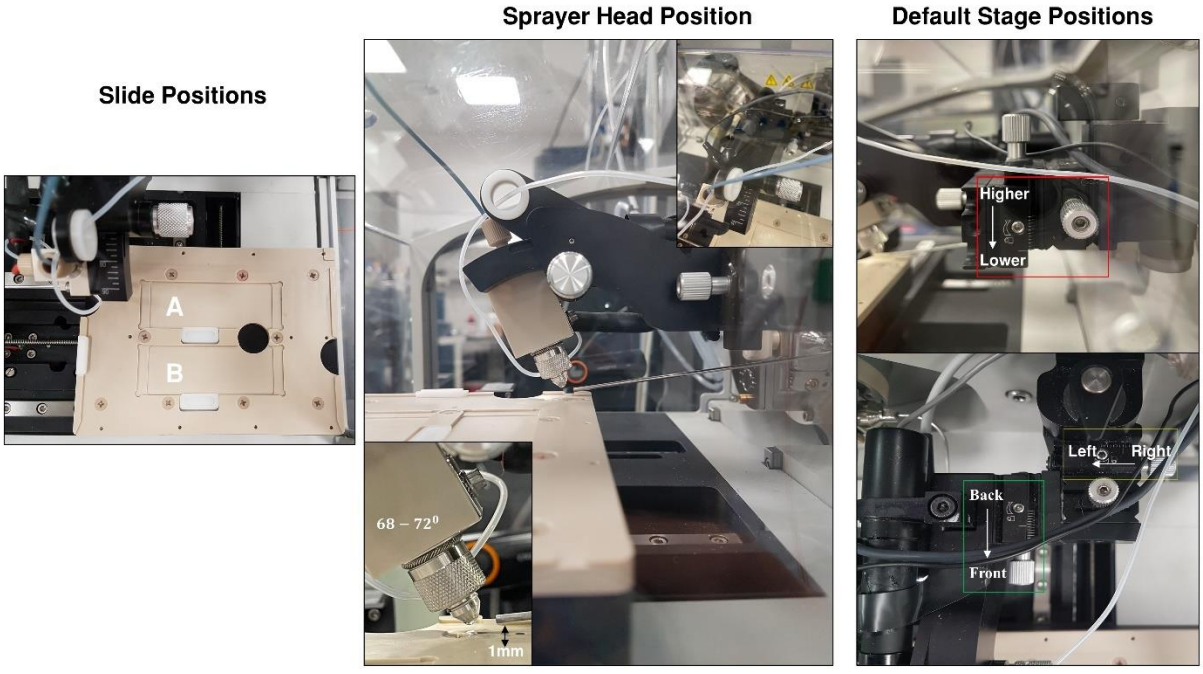

Figure 4: DESI Stage Default Starting Positions Slide Positions marked A and B correspond to HD Imaging software positions. Default sprayer head alignment with the DESI stage and metal ion inlet shown in the center image, with inset magnified sprayer head (with droplet of solvent formed) and the angle of incidence shown. On the right, default xyz stage positions (related to the movement of the sprayer head arm relative to the stage) for our setup are shown.

The sampling tray will move during operation. CAUTION Due to the amount of movement and fixed sample region of your plates, care must be taken to ensure that the HOME position of the tray is not altered between runs- this will affect the positioning and acquisition of your sample! CRITICAL STEP The home position and the x and y (mm) coordinates in the DESI Source Control window are not the same. HOME refers to the resting position of the solvent sprayer (at the top left of the sample tray, shown in Figure 4). X and Y origin coordinates refer to the top left position of the 76x26mm slide in position A (top slot of DESI stage, Figure 4).

1. Flush the syringe line with your preferred solvent for 30 minutes at 5 µL/min prior to starting your experiment. As a default solvent for these experiments, begin with 98% MeOH (2% H_2O, *v/v*).
2. Visually inspect the joints between capillary lines to spot leaks. After 30 minutes, there should be a solvent bubble formed at the tip of the sprayer head (as in Figure 4). TROUBLESHOOTING
3. CRITICAL STEP When the solvent bubble is observed, turn the nitrogen gas line on (refer to Figure 3). The spray should sputter and eject the excess solvent. Continuous sputtering and lack of solvent ejection may indicate a leak in the sprayer head or a blockage in your capillaries.

4. Open MassLynx. Open the Tune Page. Turn on the instrument by going to “Settings”, then “Voltages On”. The status bar in the bottom right corner turns green as the instrument becomes active.
5. Place a dot of red pen dye on the top left corner of a glass slide. Place this marked glass slide into slide holder position A (see Figure 4). Go to “DESI Source Control” of the MassLynx Tune Page. Disconnect the source. Open Omni Spray 2D.
6. Using the computer program Omni Spray 2D, position the sprayer head on the stage (by pressing the arrows) to the point where it reaches the red pen dye, and you see the characteristic ion of 443 *m/z* (representing protonated rhodamine 6G). Record x and y positions on Omni Spray 2D. CRITICAL STEP The TIC associated with the red pen dye should be at least $1x10^5$ counts, if not higher. The TIC should be 100x higher for rhodamine than the background ion intensity. This has some dependency on sprayer solvent concentration- with 15% H_2O (*v/v*) or higher, the TIC may decrease by at least 20% due to poor desolvation. Testing the red pen dye TIC is more consistent than looking at background ion intensity of the sample tray. TROUBLESHOOTING
7. Close OmniSpray. Return to the DESI source controls of MassLynx and enter in the new x and y coordinates for the origin of slide A. Press ENTER. Close OmniSpray and reconnect the DESI source control. The DESI controls within MassLynx should not be aligned with the DESI stage and slides.

Preparing Nylon Membrane Slides for DESI-MS

Timing: 10 minutes (to create 4 slides, up to 160 wells)

8. CRITICAL STEP The number of slides required to complete the visualizations of the samples will depend on the number of droplets that will be spotted onto the slide and the volume of each droplet. For a 500 nL droplet spot in 50:50 (*v/v*) H_2O: MeOH, the well spacings should be at least 5 mm apart (as spaced out in Figure 1b). Smaller droplet sizes will allow for smaller spacing between spots and therefore more spots per plate. Consider creating practice slides and doing multiple spotting attempts to determine the optimal number of spots per slide for your droplet size and solvent adhesion to the nylon membrane.
9. Cut the double-sided tape to cover the glass slide. Align the pieces of tape as closely as possible to ensure there are no gaps between them and the slide.
10. Cut the nylon membrane to overlap the area of the tape on the slide. CRITICAL STEP Ensure that there is no tape exposed, as this will diminish the ion sensitivity of the DESI.
11. Using a scalpel, remove the outside layer of the double-sided tape. Place the nylon membrane onto the tape, lightly pressing into it to ensure full adhesion.
12. Given the droplet size that will be used, the well size to be etched in with a scalpel will be unique to your experiment. Lightly score the nylon membrane with the scalpel. CRITICAL STEP Make sure not to cut too deeply or widely and expose the tape below, as this will cause the imaging to be distorted due to the high ion signal from the tape, alongside potentially creating another well in which your droplet may run into and deform the imaging.
13. Take a photo of the glass slide, with all 4 corners of the slide clearly visible. A coloured paper can be placed below the slide for contrast.

PAUSE POINT: Sample slides can be etched and photographed in advance and uploaded to HDImaging prior to experiment day. Photos of the slides can be transferred via email to the DESI computer or using a pen drive. Wells can be traced onto the slide and then spotted later, on the day of the experiment. If the slides are stored in a clean box, they should remain viable for a couple of weeks.

Slide Method Generation for DESI Acquisition

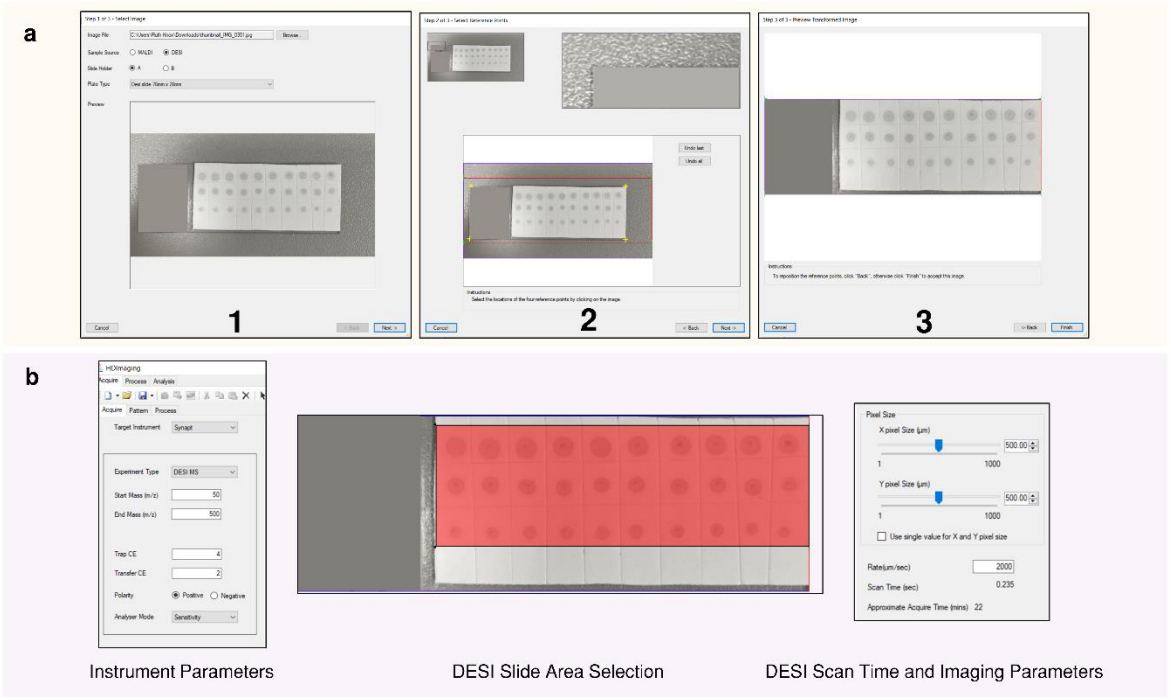

Figure 5: Generating Slide Area and Optimizing Visualization Parameters within HD Imaging. a) Screenshots of the import and slide selection process (described in protocol steps 14-17) for HD Imaging. Photo of the nylon slide can be imported to the acquisition computer by pen drive or email. 1. Select "DESI-MS" as the experiment type and choose slide position on the stage. 2. Align the DESI stage movements with the correct position of the slide. 3. Finalize the slide area and double check boundaries. b) Screenshots of protocol steps 18-22. The instrument parameters must be customized by mass range, analyzer mode and type of MS experiment (IMS vs MS vs MS/MS). The area selected for recording (red rectangle) should include a margin of nylon membrane outside the target area, to ensure complete recording of sample wells. Finally, default scanning parameters (and associated time for recording the experiment) are displayed.

Timing: 10 minutes

14. Import the image of the slide into the acquisition computer.

15. Open HDImaging. Select “New Plate”. The window that pops up is to find the uploaded image of the slide. Select position A or B for the sample tray (A is the top position, see Figure 4).
16. The slide dimensions are 76mm x 26mm if slides purchased match those in this protocol**.** Select the correct dimensions for the slides bought.
17. Using the magnified image of your slide, select the 4 corners of the slide (Figure 5a). After the 4 corners are selected, a corrected image of just your slide area is depicted. This is the area that you will now be able to run a DESI experiment on. Press “Create”. TROUBLESHOOTING
18. Under the “Acquisition” tab, fill out the required parameters for the mass spectrum wanted. Select the type of instrument you are using.
19. CRITICAL STEP Select the mass range appropriate for your samples, which must be within the mass range that the mas spectrometer was calibrated to. This cannot be changed in MassLynx or when in post processing on HDImaging, so it is best to select conservatively (a larger mass range than you predict for the product ions).
20. CRITICAL STEP Select the polarity that you would like to ionize the slide with. This will depend on if your compound of interest would be expected to protonate or deprotonated in the presence of your sprayer solvent.
21. Select “Sensitivity mode” for the most sensitive acquisition.
22. Under Acquire and Pattern: Draw a rectangle (using the blue rectangle square in the top tab) that outlines your sample region on the slide. Make sure to leave a few mm on each side away from your spots on the slide to ensure that you record the full droplet area (see

Figure 5b). A standard default pixel size is 500µmx500µm. The rate should be between 500-2000 µm/s. A slower rate will increase your total ion count and signal intensity but will take more time. Once these parameters are defined, the software will display the estimated running time.

23. Save your method.
24. After the pattern file is saved (which tells the mass spec *where* it should be acquiring data), you must export the file to MassLynx (white and blue logo on the top banner). This will tell the mass spec what parameters it should be recording for this sample. Press Export. Ensure that the project file exporting to is your own.

PAUSE POINT: Methods can be generated on HDImaging prior to experiment day. They can also be exported to MassLynx at any point prior to experiment day.

DiBT Acquisition from Nylon Membrane Slide

Timing: 5 minutes-2 hours

25. Ensure that a steady flow rate from the syringe to the sprayer head has been established (same as step 2).
26. Open the MassLynx homepage. From the general page, go to File->Import Worksheet. Your sample file should be listed in your Project File.
27. Open the MassLynx Tune page. Ensure there is consistent signal and that the syringe is not leaking.

28. Adjust the syringe flow rate between 1-5 µL/min, depending on the signal intensity and ionization efficiency of your sample. Any higher flow rate will likely not be able to fully ablate the membrane surface and fully desorb your analyte of interest.
29. Insert your slide into slot A or B, depending on which was selected in the HD Imaging pattern file.
30. When you are ready to acquire, depress the play button at the top of the MassLynx method banner. Save your method file under the same name as the HDImaging file. The MassLynx software will now start its acquisition. The “Play” button in the middle of the MassLynx home page will be selected, and the status bar on the bottom of the MassLynx tune page will read “Acquiring”.
31. Once the DiBT acquisition is completed (demonstrated by the DESI head returning to the HOME position and the MassLynx Tune file displaying “Acquisition complete” in the lower left-hand corner), turn off the nitrogen gas line. Keep the solvent line flushing for another 15 minutes.
32. Put the instrument into standby mode by going to “Operate”, then “Instrument Standby”. The power notice in the bottom right corner of the Tune page will turn red.

PAUSE POINT: Raw file image processing can be completed at any point. There is no fixed timeline between acquisition and processing. Simply save the .raw files to your personal data repository.

Analysis of DiBT Acquisition and Imaging

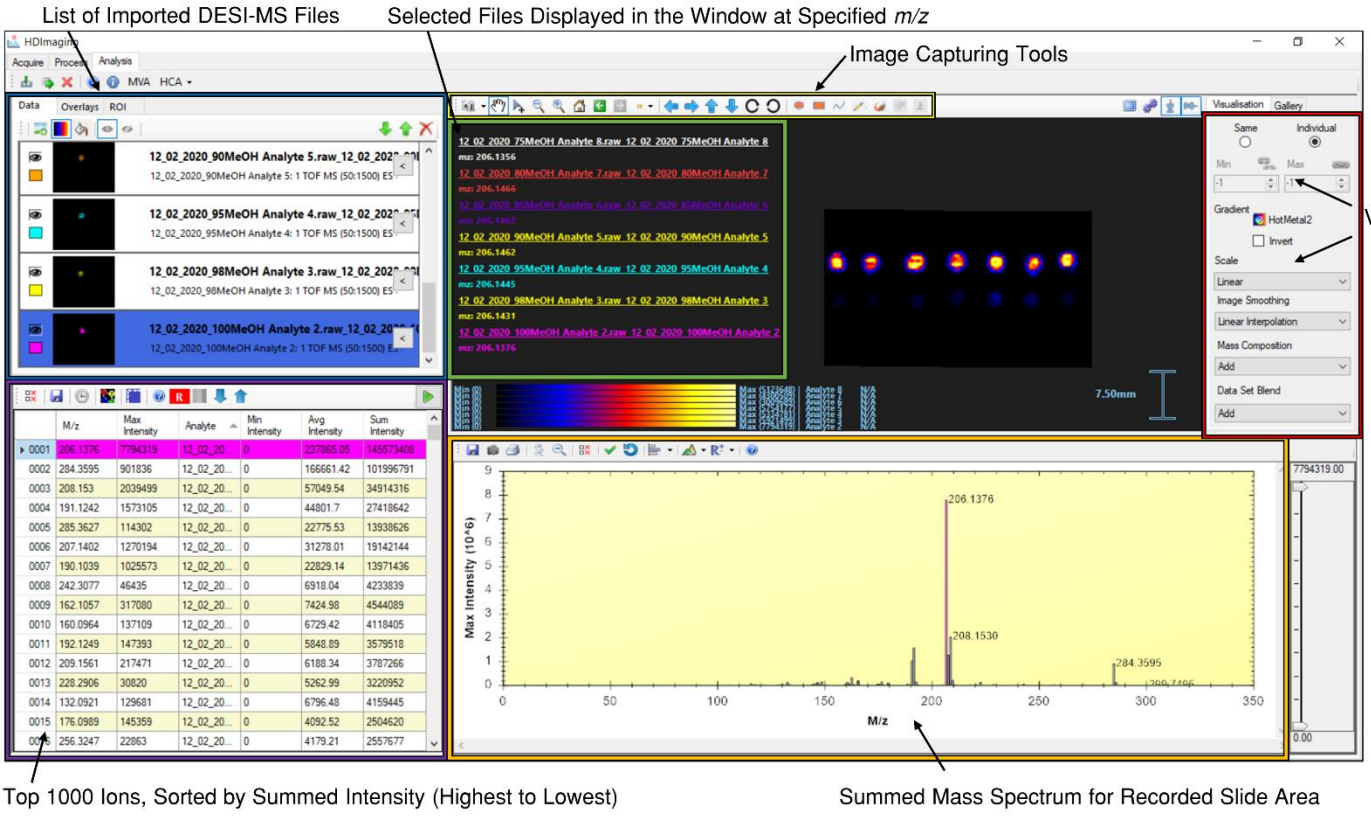

Figure 6: HD Imaging Data Analysis Window Overview for 6,7-dimethoxymethyl-3,4-dihydroisoquinoline Top left (blue square): List of imported acquired files (post protocol steps 33-37). A preview of each file is shown as a small black image, with a species highlighted. The total ion chromatogram over the complete slide and for the ions of interest is shown in Figure S2 (supplementary data). Current file that is visualized in the rest of the HD Imaging window is highlighted in blue (top left). Bottom left (purple square): molecular ions associated with the selected imported data file, categorized by summed ion intensity (high to low), with top 1000 ions displayed. Highest ion selected corresponds to the reactant depicted in Figure 2. Top right (red rectangle): Image selection tools to normalize visualization across image files. Additional options include blending results for multiple ions in the same image (data set blend) or producing a smoothed pixel image (image smoothing, linear interpolation). Bottom right (orange rectangle): mass spectra for the acquired file selected in top right. The selected ion (highlighted in purple) at 206.13 *m/z* corresponds to the protonated starting material for the IRED biotransformation

displayed in Figure 2 (and Figure S2, S3) and is also the most abundant ion recorded. The corresponding selected ion chromatogram is shown in Figure S4.

Timing: 15 minutes

33. Select “Process” Tab. The parameters for processing your raw DESI files are displayed. As default settings, good starting parameters would be: *m/z* window: 0.02; MS Resolution: 20000; Number of most intense peaks, 1000. CRITICAL STEP Ensure that the mass range selected for processing includes the expected *m/z* for your compound of interest and any potential side products. For example, for small molecules a *m/z* range of 50-500 is typical.
34. Select the green cross. A popup window will ask you to select the raw image files for processing. Select each raw file individually and press the central arrow to move these files over to the processing window. Press “add samples to the worksheet”.
35. There should now be files in the worksheet window, under “selected data”. Press the green arrow “play” to begin the processing.
36. A popup window will ask you to confirm your data processing options. Select which of the files in the open worksheet window you would like processed.
37. Processing will be displayed in the lower middle window. When processing is done, the command line “***Maldichrom.exe completed***” will be displayed. TROUBLESHOOTING
38. In the top left, select “Analysis” tab. In the top left, select “Import Data”. A popup window will now show all the previously processed files that can be imported.
39. Just like the worksheet window, select and drag over the files of interest into the working window. Press “Import”.

40. When the file is imported, it will appear in the top left window. Click on the file to have the mass spectra appear.
41. As a default, the mass spectral information is displayed in a table in order of decreasing summed ion intensity. By selecting any of the other column headers of the table (*m/z*, max intensity, analyte, min intensity, avg intensity), the data can be shown in a sequential decreasing or increasing order for that column. Alternatively, the mass spectrum shown in the right bottom can be magnified and the ion of interest can be selected from there.
42. Select an ion from the mass spectrum. The corresponding visualized area will appear in the top right window (Figure 6). The image displayed will be of the whole area selected in the method development, with the *m/z* of interest highlighted within that region. As a default colour scale, the presence of the ion of interest is displayed as low (blue) to high (yellow) on a sliding scale as a heat map. For that *m/z* value, the heat map will be normalized across the whole recorded area. TROUBLESHOOTING
43. Either through examining the top few summed intensity ions or by isolating the *m/z* range expected for your product, check the data for your expected product ion. Protonation or deprotonation is the most likely ionisation event for small molecules by DESI-MS. TROUBLESHOOTING
44. Examine the data for any other ions that appear as visualized droplets in your DESI acquisition and ensure they are independent of your result. Are there any ions related to the starting material? Does the *m/z* and corresponding mass relate to any possible byproducts of your reaction? CRITICAL STEP A negative control of the crude mixture that the compound of interest is solubilized in may be a useful shorthand way to determine

which ions are related to DESI sensitivity of background ions vs the reaction mixture components. Often, a crude reaction mixture will emit several highly ionisable compounds that may provide a false positive if of a similar mass to your desired product.

45. If multiple ions are to be visualized on the same area, by using “CTRL+ CLICK” on PCs or “CMD+CLICK” on Mac computers multiple ions from the data sheet can be displayed concurrently. CRITICAL STEP When ions are overlapped on the same image, the results will be normalized to the highest intensity peak, regardless of importance to the user.
46. When ions have been selected and the user would like to save the image, there are 3 options in the top left of the imaging window in a drop-down menu: camera (add image to gallery), save image and copy to clipboard (see Figure 6 for complete overview). Copying to clipboard allows for easy export to other programs. Whatever is within the visualized slide imaging will be saved when selected, not necessarily the complete area imaged. Press “Home” to reset navigation and centre your image.

Sprayer Solvent Optimisation for Increased Ionisation Efficiency

Timing: 2-4 hours (when necessary)

A solvent gradient may be necessary to improve the ion transmission of the compound of interest. In this case, several optimisation tests should be carried out to determine the ideal solution mixture for sample transfer from the slide into the gas phase. Ideally, the sprayer solution should be a combination of two or more solvents with differing hydrophilicity/polarity, as to provide a broadly soluble mixture for a variety of ionized compounds. During the desolvation process, the compounds are ablated from the slide surface and charge transfer

occurs with the spray solvent in a microdroplet on the slide surface. Secondarily, this charged analyte is desolvated as it travels from the surface of the microdroplet into the air, and then into the metal ion inlet before entering the mass spectrometer under vacuum. This protocol begins with the default solvent conditions of 98% MeOH, 2% H_2O (*v/v*). Although this solution contains two protic solvents, the sensitivity of the mass spectrometer can determine the effect of minor concentration changes between these two solvents.

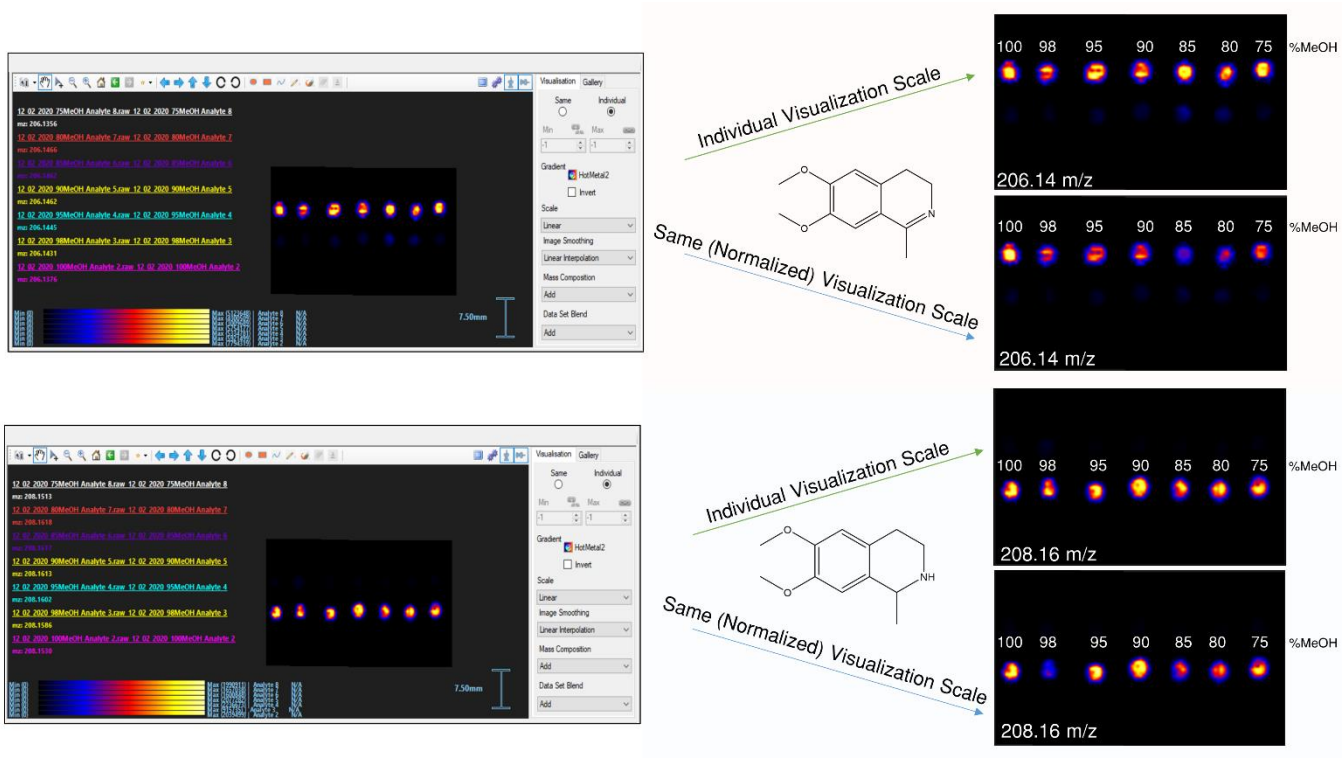

Figure 7: Solvent Optimization Visualization of 6,7-dimethoxymethyl-3,4-dihydroisoquinoline and 6,7-dimethoxymethyl-1,2,3,4-tetrahydroisoquinoline shown in HD Imaging. On the left, the processing windows of HD Imaging are displaying the nylon membrane highlighted at the molecular ions of 206.14 *m/z* and 208.16 *m/z*, which correspond to the protonated forms of 6,7-dimethoxymethyl-3,4-dihydroisoquinoline and 6,7-dimethoxymethyl-1,2,3,4-tetrahydroisoquinoline, respectively. The compounds were spotted onto nylon membranes in multiple wells, with each well being freshly spotted each time a different composition of the methanol and water sprayer head solvent was used, from 100% methanol down to 75% methanol: 25% water for the corresponding mass spectra and selected and total

ion chromatograms see supplementary data, Figures S1-15. Mass range recorded and HD Imaging processing parameters were kept identical. On the right, the MS image of each compound (absolute and normalized intensities) are displayed. For both compounds, using pure methanol as the sprayer head solvent produced the most molecular ions in the gas phase recorded.

For highly hydrophobic samples this may be ideal as the insolubility increases the efficiency of the desolvation process and therefore the total ion count within the mass spectrometer, providing a larger ion signal than a "wet" sample.

47. Make a nylon membrane slide with enough wells that you can run each solvent gradient mixture at least once (twice or in triplicate would be more robust).
48. Spot each well with your compound of interest, with the same volume and concentration in each well.
49. Generate a separate HD Imaging plate pattern for each area that represents a different solvent combination. Keep the polarity, mass range and pixilation identical for each method, just change the area recorded. Export each pattern method to MassLynx.
50. For each solvent change, rinse out the syringe three times with the new mixture before injecting the new solvent composition into the syringe line.
51. Turn the nitrogen gas off in between DESI acquisitions while the solvent is being changed.
52. To speed up the solvent change, remove the syringe line from the DESI stage. Flush (at minimum) 50 µL of the new solvent through this line. Reconnect the syringe line to the DESI stage.
53. If no connections are pre-flushed when switching solvents, wait 45 minutes with the syringe pumping at 5 µL/min until running the next DESI acquisition. If the syringe line has

been flushed with the new solvent, wait 30 minutes with the syringe pumping at 5 µL/min until running the next DESI acquisition.

54. Acquire a DESI imaging file for the same compound with different spray solutions.
55. Process each file in HD Imaging with the same *m/z* range, MS resolution, number of important peaks and *m/z* window.
56. Open the "Analysis" tab of HD Imaging and import all the data files associated with the change in solvent conditions.
57. For each imaging file, select the same *m/z* value (associated with the protonated/deprotonated target compound).
58. Expand the imaging window so that all the data files can be displayed concurrently.
59. Use the mouse button to individually move each image area so that the well for each is fully displayed. It may help to line the wells up in order of the concentration gradient employed (see Figure 7 for reference).
60. To the right of the imaging window, under "Visualisation", select "Same". This will normalize the ions selected and visualized to the same heat map.
61. Save the image of the normalized results. Repeat steps 57-60 for any pertinent ions.

Timing: Steps 1-7, 40 mins; Steps 8-13, 10 mins; Steps 14-24, 10 mins; Steps 25-32, 2 hours; Steps 33-46, 15 minutes; Steps 47-61, 2-4 hrs.

Troubleshooting:

Step	Problem	Possible Reason	Solution

2	Lack of solvent flowing into sprayer head	Leaks in capillary lines or clogging	To start, flush the lines with your solvent of choice at 5 µL/min for at least 30 minutes prior to running your experiment. Keep the gas turned off during this time. If problem persists, start by disconnecting the syringe line from the DESI stage and flushing it. Reconnect the line. Increase flow rate to 10 µL/min and disconnect the capillary line at the head of the sprayer solvent. Wait until consistent flow is established before reconnecting line to the sprayer head. Wait 5-10 minutes for the solvent to accumulate at the sprayer head. If the capillary line is made of fused silica and salt is observed in the line (white crystalline obstruction), either a portion of the capillary can be cut out using an etcher, or that part of the solvent line must be replaced.
6	Failure to establish red pen dye signal	Incorrect angle of alignment between the sprayer head and the sampling cone.	Prosolia default methods for the angle between the sprayer head and solvent vary depending on userand type of sample and slide material. The sprayer head angle can be adjusted as seen in Figure 4 if using a nylon membrane slide. For an easier task, coat a large area on a glass slide with red pen dye and begin by signal optimization in this larger area. The distance between the sampling cone and the slide should be 1 mm but consider adjusting up or down from there as necessary to see an improvement in the signal without touching the slide surface.
6	Low ion intensity	Lack of desolvation of sample	Sprayer solvent should be flow between 1-5 µL/min. Any higher flow rate will likely not be able to fully ablate the membrane surface and fully desorb the analyte of interest. Ideally, capillary voltage should be kept between 1-3 kV, but this will depend on the position of the capillary within the sprayer head and will need to be optimized every time the sprayer head is changed. Newer sprayer heads (designed for the Synapt XS instrument) operate optimally below 1kV capillary voltage.
17	Photo of slide does not appear in HD Imaging	Photo size is too large (>350 KB).	Consider reducing the photo quality or cropping the photo in the computer's photo program. Re-save the smaller image and re-import.
37	File will not load into Analysis	Corrupted imported .raw file	Delete the file from the worksheet window. Re-import the .raw file from your MassLynx folder.

	Window of HD Imaging		Check that the m/z range selected is within the range recorded on MassLynx. Re-run the processing.
42	File not visualized in HD Imaging	Corrupted imported .raw file	Delete the file from the worksheet window. Re-import the .raw file from your MassLynx folder. Check that the m/z range selected is within the range recorded on MassLynx. Re-run the processing.
42	File not visualized in HD Imaging	Window has moved out of sight.	Press “Home”. Open picture should be recentered in the top right window.
43	Ion not visualized in mass spectrum	Ion out of range of processed mass spectra	Re-process the DESI file in HD Imaging for a larger mass range.
43	Ion not visualized in mass spectrum	Ion too low intensity for processed file parameters	Reprocess the DESI file in HD Imaging to search for highest 10,000 ions instead of 1,000. Although this may help with detection of your peak, it will not be in high intensity. Try concentrating your sample and retrying the experiment with the same DESI parameters.
43	Ion not visualized in mass spectrum	Ion does not present in this sample, nor do any others associated with droplet	Try and find other molecular ions that can be visualized in the droplet. If there are no images that depict the droplet shape highlighted, there may be issue with the DESI stage or how the file was acquired. Attempt a red pen test to observe the activity of the DESI-MS stage setup. If signal is good with good sensitivity, re-run the sample and save under a new file name.

Results:

With this described protocol, the user should be able to effectively isolate and identify target compounds present in the wells spotted onto the nylon membrane, irrespective of the crudeness of the mixture (although the presence of detergents and ion suppressants may increase the number of molecular ions present and therefore make analysis more complex). As a continuation of the examination of IRED biotransformation standards in Figures 2, 6 and 7 for the reproducibility and day to day variation in DESI-MS performance, additional experiments are described on optimization of CAR A-domains. Initially, standards of reactants and products of the

CAR A-domain biotransformation were provided in a mixture of 1:1 water: methanol (*v/v*) to establish a baseline for the abundance of the protonated compound molecular ions and to further examine the effect of the sprayer solvent on ion transmission (and therefore sensitivity of the workflow).

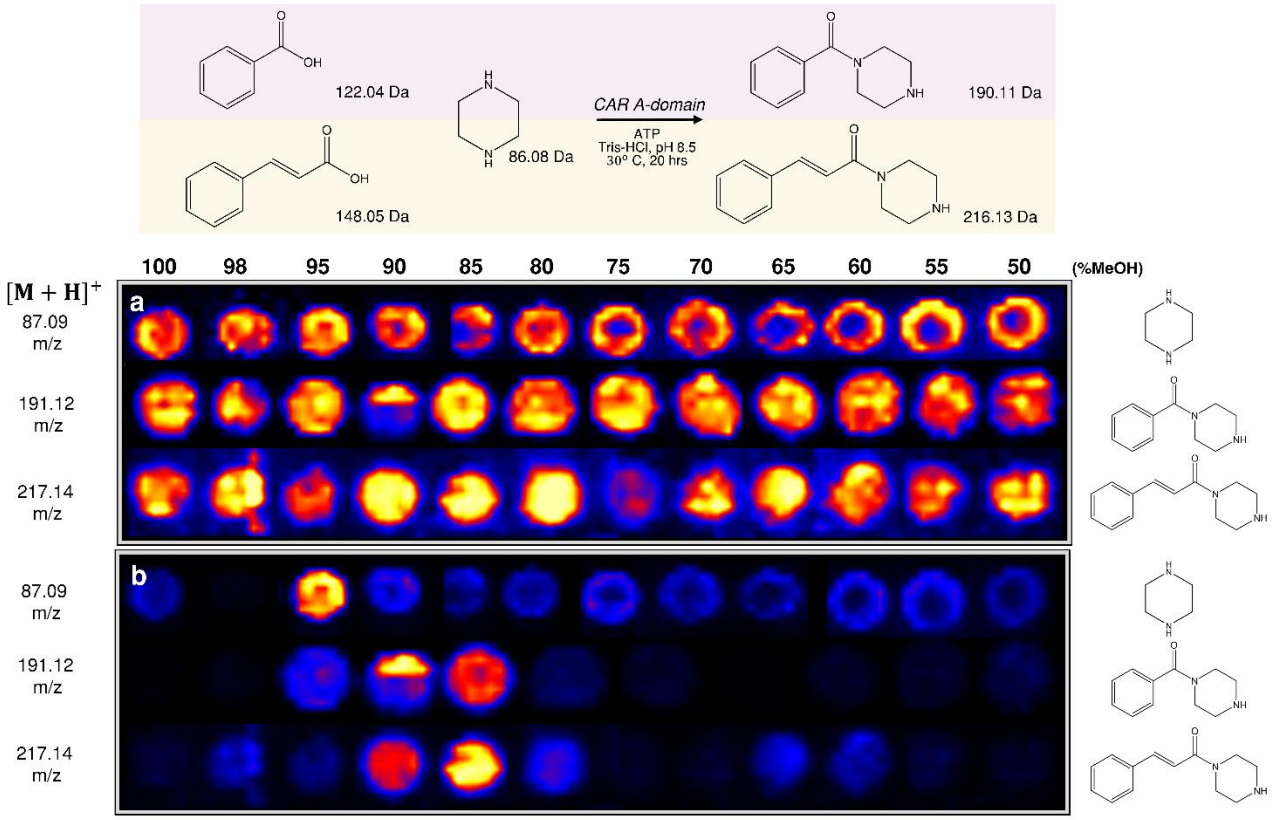

Figure 8: DiBT-MS of standards that are involved in CAR biotransformation reactions namely the reactants and expected products in the absence of enzyme. Top: Schematic of CAR amidation reactions, with monoisotopic mass listed. In purple, piperazine reacts with benzoic acid to produce phenyl(piperazinyl)methanone, and in orange, piperazine and 3-phenylacrylic acid react to produce 3-phenyl(piperazinyl)prop-2-en-1-one. All standards were provided at a concentration of 5 mM. a) DiBT-MS results for molecular ions 87.09 *m/z*, 191.12 *m/z* and 217.14 *m/z*, corresponding to protonated starting materials (associated structure on the right) shown in the absolute intensities recorded. Each droplet shown represents a unique well, recorded at 12 different compositions of water: methanol as the sprayer head solvent. b) The same results shown in A, but with the MS image normalized to the highest abundance droplets.

When the DESI-MS spectra are processed in HDImaging and normalized for all the CAR reaction standards in Figure 8B, it is apparent that there is a narrow window (85-95% methanol) of sprayer solvent composition for the highest ion transmission from the microdroplet of the nylon membrane slide into the gas phase (and what is subsequently reported by the mass spectrometer as the TIC). It is especially exaggerated in the protonated piperazine wells, which have very high detection of the compound with the 95% methanol sprayer solvent, with fewer molecular ions visualized in the 98% methanol solvent sample. Although the piperazine compound is well visualized with the 98% methanol solvent (as seen in Figure 8a), typically the normalized spectra are examined for a large 48-96+ well assay to determine the best "hits" for the enzyme when compared with the wildtype enzyme or other variants that have also been examined. For this reason, ensuring the highest ion transmission possible for each compound prior to analysis of a larger assay is recommended. It is worth noting that piperazine is in a large excess (150 mM) in the biotransformation sample, while the carboxylic acid substrate is in the lower millimolar range (1-5 mM). In this respect piperazine can serve as an internal standard to check the system's performance.

It is also important to note that the optimal window of solvent composition that leads to high ion transmission and therefore sensitivity of this workflow is unique to the compound of interest, perhaps related to the intrinsic solubility of the specific compounds in the sprayer head solvent. Another publication is in process discussing the relationship between intrinsic and DESI-MS output, alongside a predictive methodology to be used in future method development.

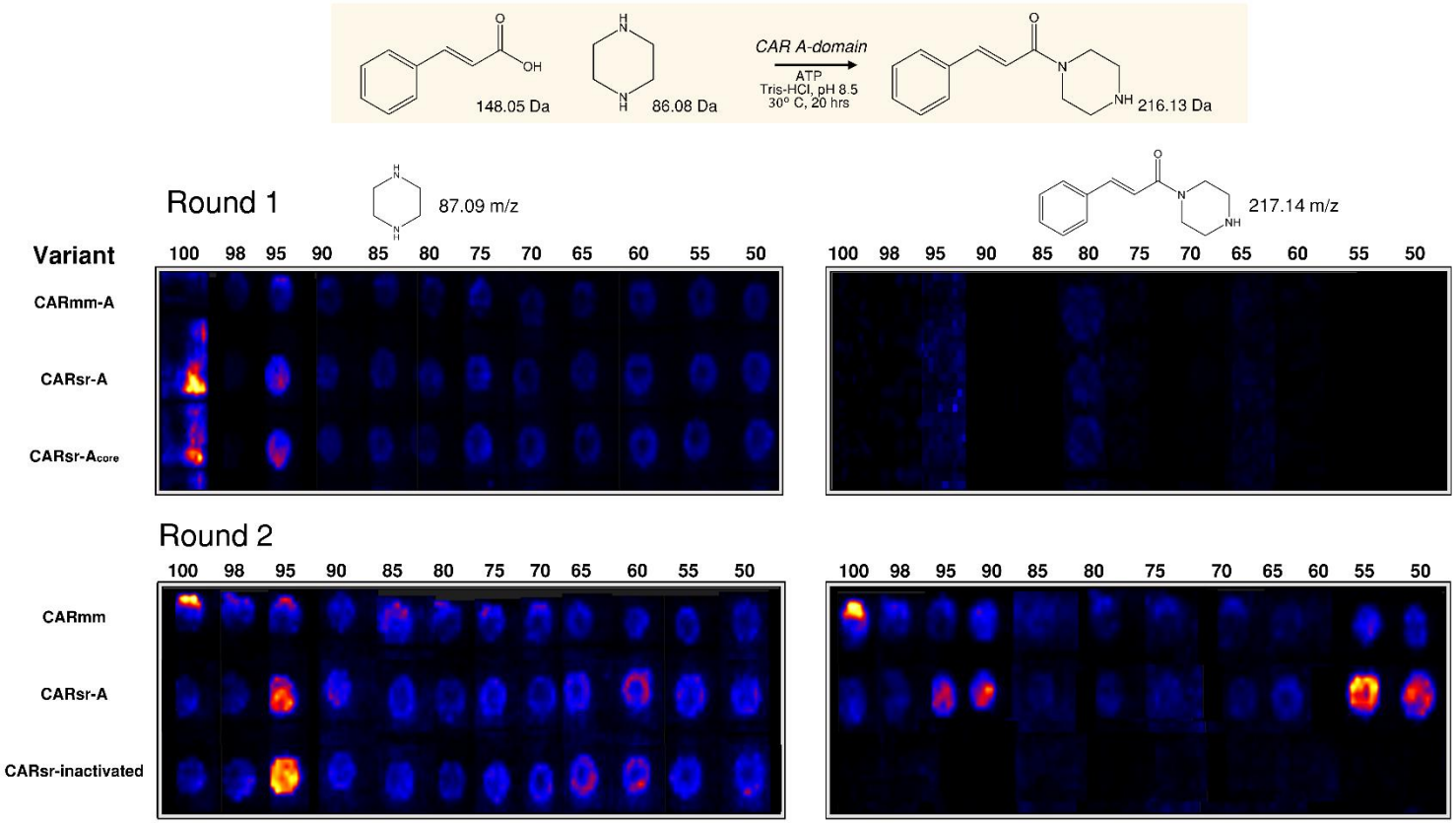

Figure 9: Optimization of CAR biotransformation through DiBT-MS collaboration with normalized intensity for visualization. Top: Schematic of 3-phenyl(piperazinyl)prop-2-en-1-one synthesis by family of CAR A-domains. On the left, CAR variants are listed to the side of DiBT-MS well visualization. The two rectangles on the left correspond to the molecular ion of the reactant piperazine (at 87.09 *m/z*), while the two on the right correspond to the product 3-phenyl(piperazinyl)prop-2-en-1-one (at 217.14 *m/z*). Every crude cell lysate was recorded with 12 combinations of water: methanol mixture as the sprayer head solvent. The reactant and (potential) product formations are shown for two different rounds of CAR enzyme optimization. Top, right: no evidence of product formation across any solvent composition attempted. Bottom, right: presence of product observed in two CAR variants. No presence of CAR product observed in the CARsr-inactivated wells.

After establishing that solvent optimization has an impact on the ion transmission of the CAR biotransformation substrates and products, the crude cell lysates of a variety of CAR

biotransformations were examined as well. The previously reported A-domain of CAR*mm* (*Mycobacterium marinum*, Uniprot ID: B2HN69) and variants of the A-domain of CARsr (*Segniliparus rugosus*, Uniprot ID: E5XP76) were selected as exemplary biocatalysts for this study: CARmm-A (truncation: CARmmΔ647-1175); CARsr-A_{full} (truncation: CARsrΔ655-1188); CARsr-A_{core} (truncation: CARsrΔ528-1188)[20,21]. An equal volume of methanol was added to the biotransformation sample to give 1:1 methanol:water (v/v) solution that was subsequently spun down to remove crude protein debris. As seen in Figure 9, DESI-MS results for presence of both starting material and final product are visualized in their protonated form for biotransformation lysates containing different CAR A-domains (CARmm-A, CARsr-A_{core}, CARsr-A, CARsr-A-inactivated). On the left-hand side of Figure 9, evidence of piperazine protonation is again visualized at 87.09 *m/z* with the largest ion intensity in wells that were sprayed with the 95% methanol solvent. In the second round of optimization, the final product at 217.14 *m/*z is visualized (Figure 9, bottom right corner) with all sprayer solvent modulations, in the two CAR variants (CARmm-A, CARsr-A) that maintained enzymatic activity within the active site. In the second round of optimization a heat-inactivated lysate sample of CARsr-A (CARsr-A-inactivated) served as a negative control in this experiment. Likewise, samples containing CARsr-A_{core} did not show any product formation due to the lack of the essential A_{sub} domain that contributes to formation of activated acyl species (acyladenylate) in the enzyme's active site[20]. Although not all of the variants are repeated between rounds of optimization in (due to selection by the biochemist), it is apparent for enzyme variants CARmm-A and CARsr-A (the top and middle rows for both rounds) that the modifications of overexpression, assay and sample conditions imposed

on the CAR biotransformations lead to new and improved production of the desired carboxamide.

With the biotransformations depicted in Figures 8 and 9, DESI-MS has demonstrated great use in the targeted development of APIs and other compounds through high-throughput screening of biocatalysts. Such experiments are reproducible day-to-day and can be easily interpreted due to the visual nature of the technique. With source and instrument conditions kept constant in the CAR experiments, sprayer solvent modulation had the same effects on ion transmission across multiple days, concentrations, and sample purity (standard vs crude cell lysate). We envisage that this protocol will serve as a starting point for biochemists, and chemical biologists to use a technique that facilitates rapid sensitive analysis and detection of target molecules, requires low sample volumes, low solvent use where in all sample handling is non-toxic to the user.

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Author Contributions:

RK developed the sprayer head solvent optimization method, acquired all spectra and drafted the manuscript. EK contributed to method development whilst studying at The University of Manchester and paper drafting whilst under employment by AstraZeneca plc. CS prepared CAR enzymes, and PEB, RS, CS, SF and NJT contributed to method development and paper revisions.

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