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Complete List of Authors:	Sobreira, Tiago; Purdue University Avramova, Larisa; Purdue University, Chemistry Szilagyi, Botond; Purdue University College of Health and Human Sciences, Davidson School of Chemical Engineering Logsdon, David; Purdue University, Chemistry Loren, Bradley; Purdue University, Chemistry Jaman, Zinia; Purdue University, Chemistry Hilger, Ryan; Purdue University, Chemistry Hosler, Richard S; Purdue University Ferreira, Christina; Purdue University, Chemistry; Purdue University, Bindley Bioscience Koswara, Andy; Purdue University, Chemical Engineering Thompson, David; Purdue University, Department of Chemistry; Purdue University, Department of Biomedical Engineering Cooks, R. Graham; Purdue University, Chemistry Nagy, Zoltan; Purdue University, School of Chemical Engineering

HIGH-THROUGHPUT SCREENING OF ORGANIC REACTIONS IN MICRODROPLETS USING DESORPTION ELECTROSPRAY IONIZATION MASS SPECTROMETRY (DESI-MS): HARDWARE AND SOFTWARE IMPLEMENTATION

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Tiago Jose P. Sobreira [a] , Larisa Avramova[a,b] , Botond Szilagyi [c], David L. Logsdon[b], Bradley P. Loren[b], Zinia Jaman[b], Ryan T. Hilger[b], Richard S. Hosler[b], Christina R. Ferreira[a,b], Andy Koswara [c], David H. Thompson[b], R. Graham Cooks[b], and Zoltan K. Nagy*[c]

This study describes an automated system used for high throughput screening of reaction conditions based on accelerated reactions occurring in small volumes of reagents. Reaction mixtures are prepared in array format using a fluid handling robot and spotted on a flat polytetrafluoroethylene plate at densities up to 6,144/plate. The reaction and analysis steps are performed simultaneously using desorption electrospray ionization (DESI) to release microdroplets containing the reaction mixture from the plate for reaction prior to arrival at a mass spectrometer. Analysis rates are up to 1 reaction mixture per second and data is recorded in real time using an ion trap mass spectrometer. Beacon compounds are used to triangulate position on the plate and this allows tandem mass spectrometry (MS/MS) to be performed on confirm products of interest. Custom software allows the user to control the system. It is also used to receive data from the DESI mass spectrometer to screen the spectra for compounds of interest, to perform MS/MS and to save data. This custom software also communicates with the software controlling the fluid handling robot (Biomek i7) as well as the Beckman software used to prepare reaction mixtures and also the software that controls the solvent used as the DESI spray. Data were recorded for N-alkylation, N-acylation and N-sulfonylation reactions in three 8-hour experiments on successive days to establish the ruggedness and repeatability of the system. Repeatability is high (94 - 97%) over this period with false negative 6% (depending on noise threshold chosen). Plates containing 384 reaction mixtures are analyzed in 7 min by moving the DESI sprayer in steps under the sprayer instead of continuously.

Introduction

The applications of high-throughput screening (HTS) were first popularized in the late 1990s. They have since proliferated throughout the field of combinatorial (bio)chemistry[1-2] and have recently captured increased attention from the chemical and (bio)pharmaceutical industry, catalyzed by the recent increased interest in applications of artificial intelligence (AI) and machine learning (ML)[3]. Within the realm of organic synthesis, generating high-quality and contextual chemical data for the purpose of exploring uncharted chemical space has become a technical bottleneck as sophisticated chemical feature engineering and AI/ML techniques have pushed the boundaries of how much insight may be gained from hundreds of thousands to millions of data points. Conceptually, HTS refers to a method for acquiring and processing a large amount of data per unit time and cost, normally in the order of ~1K-10K data points/day. For instance, recent minireview by Isbrandt *et al* discussed various microflow and droplet reactor-based HTS system with throughputs in the range of ~100-1000 reaction conditions/day[4].

More generally, the nature of HTS experiment varies from the biological to the chemical, the objectives of HTS are universal: (1) to generate libraries of useful information (e.g. gene sequences[5], organic reactions[6], and microbial strains[7]), (2) to explore or discover new parameter value ranges within a desired (bio)chemical reaction space (e.g. protein-ligand interactions[8] and polymorph-specific crystallization[9-10]), and (3) to optimize a set of known (bio)chemical reaction conditions (e.g. temperature ranges and solvent choices in organic reactions[6] and catalyst compositions in heterogeneous reactions[4]). The latest progress in the implementation of AI/ML significantly elevates the impact an HTS system can have on the scientific community by enabling deeper comprehension of the accumulated, high-dimensional and complex dataset, and in turn, the screening problem at hand. To attain such feats, HTS has historically involved shrewd design of hardware and software for streamlining the process of rapidly preparing samples, performing assays, and acquiring and processing data in chemically and biologically insightful ways. Specifically, the ideal HTS system is one which allows for low sample volume, rapid assay, system integration and automation, and in the case of dense datasets, effective data compression. These four fundamental factors of HTS combine to lower the costs of material and labor as well as increase the rate of data generation. As a result, current state-of-the-art HTS technologies often make use of nanoliter-to-picoliter fluid handling robots to mix and deposit samples in parallel, such as in microarrays or microfluidics, coupled with rapid spectroscopic- and spectrometric-based analytical techniques, including UV/Vis, IR, Raman, NMR and MS[11-13].

[a] *Bindley Bioscience Center, Purdue University, West Lafayette, IN 47907-2100, USA.*
[b] *Department of Chemistry, Purdue University, West Lafayette, IN 47907-2100, USA.*
[c] *Davidson School of Chemical Engineering, Purdue University, West Lafayette, IN 47907-2100.*
*Corresponding author: zknagy@purdue.edu
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Recently, Wleklinski *et al.* developed a novel HTS method capable of screening up to ~3600 reactions/hour based on the novel phenomenon of accelerated reactions in microdroplets coupled with desorption electrospray ionization mass spectrometry (DESI-MS)[13]. DESI-MS is an ambient ionization technique whereby analytes from an inert surface are desorbed in the form of charged droplets and are directed to the inlet of a mass spectrometer such as an ion-trap[14]. The reagents of interest are first prepared in microwell arrays and then "spotted" onto an inert substrate, such as polytetrafluoroethylene (PTFE). This DESI substrate is subsequently sprayed with charged solvent droplets causing the spotted reagents to desorb, producing charged microdroplets in which the reaction occurs during droplet evaporation. The resulting ions are guided towards the MS via a metal ion-transfer line for MS analysis. Consequently, through mechanical movement of the DESI substrate underneath the sprayer full mass spectra of the entire reaction set can be generated (Figure 1 (Top)). While there are many subtle factors to be considered when comparing different HTS system's performance, including accessibility to different types of reagents and reaction conditions, amounts of sample requirement, possibility for scale-up, etc., strictly comparing in terms of throughput, the DESI-MS HTS system has at the time of writing the highest throughput reported.

To advance the implementation of the novel DESI-MS HTS method by the chemical and (bio)pharmaceutical community at large, an integrated approach to hardware and software design for creating a replicable HTS platform is essential. In this article, we describe our DESI-MS system implementation - referred to internally as the "Purdue Make-It" System - to accomplish this goal. The platform is comprised mainly of commercially available hardware augmented with a few custom-designed but readily 3D-printable parts. The different devices are actuated in an integrated manner using a combination of commercial software and the corresponding software development kits (SDKs) and in-house programs developed using open-source packages. To describe the system's build process, this paper is divided into four additional sections. Section 2, which is divided further into four in-depth subsections, describes the role of each hardware component, including the fluid handling robot, the selective compliance articulated robot arm (SCARA), the piezoelectric DESI spray solvent delivery system (PieSDS)[15], and the DESI-2D stage coupled to a mass spectrometer. Section 3 follows with details of (1) the integration of commercial software, the SDKs, and our in-house software, (2) the description of our in-house software, and (3) the workflow of all programs to control the integrated system during an HTS experiment. A case study of the HTS system is described in Section 4 where we demonstrate the system simultaneously performing and analyzing three classes of reactions, namely N-Alkylation, N-Acylation, and N-Sulfonylation over 8 hours per day for 3 consecutive days. The acquired MS spectra are then used to evaluate the data repeatability and validate the system's robustness. We also discuss important recent improvements that have been made to the system after the case studies. Finally, we conclude with summary of the current state of the system and its future directions and applications in Section 5.

System Hardware

The DESI-MS HTS platform consists of five centerpiece hardware components (Figure 1 (Bottom) and 3), namely a fluid handling workstation (Biomek i7, Beckman Coulter Inc.) which includes a pin tool and a plate holder, a SCARA robot (PF3400, Precise Automation Inc.), a DESI-2D imaging stage (DESI 2D, Prosolia Inc.), an LTQ XL mass spectrometer (Thermo Scientific), and a DESI spray solvent delivery system (ElveFlow). During an HTS experiment, the Biomek i7 mixes reagents in microwell plates and using the pin tool shown in Figure 2 B, deposits the resulting mixtures in arrays of spots onto an inert PTFE substrate. The PTFE substrate is hosted on top of a 3D-printed plate holder shown in (Figure 2 C), which is custom-designed to manage the different landing configurations of the different devices as it travels through the platform from preparation to analysis and finally to storage. Post-pinning, a magnetic-based servo shuttle, shown in (Figure 2 D), transfers the substrate to a location behind the Biomek i7. The SCARA then transports the substrate onto the DESI 2D stage which is attached to the LTQ XL. The DESI stage is connected to a piezoelectric solvent delivery system (or PieSDS for short) which delivers the DESI spray solvent during the analysis. More details of each hardware and its subcomponents are provided in the following subsections.

Fluid Handling Workstation

The Biomek i7 is a dual-arm liquid handling system with multichannel (384 format) and Span-8 (8 channels) heads for the disposable tips (CAD drawing provided in Figure S.1 in electronic supplementary Information (ESI)). The multichannel head is used for the pipetting of 384 samples simultaneously under the same conditions (volume, height, speed, mixing, layout, etc.), while Span-8 channels operate independently and can transfer the samples in unique patterns. In addition to pipetting, the reagents can be transferred using a pin tool (Double Float Plate Replicator, V&P Scientific). The pin tool consists of an array of 384 stainless steel slotted pins (Figure 2 B), which are designed to transfer 50 nL of liquid by a combination of surface tension and capillary action. The pin tool can be magnetically loaded to and unloaded from the multichannel head. The pins are re-usable and can be cleaned by sonication. First, pins are dipped into the source plate, then the hanging drop of solvent-solute mixture is transferred to the destination plate by touching the pins to the surface.

The high capacity robotic deck contains forty passive plate holders for standard or deep-well plates. Each holder can be used for a single plate or a stack of a few plates if needed for long-term experiments. An orbital shaker is included for vigorous mixing of reagents in plates. Two ultrasonic baths for pin cleaning and two waste stations for used tips are also included. Two grippers can move

independently to transfer plates within the deck or to the server shuttle, where they can be reached by the SCARA.

To facilitate manipulation of the glass plates on the deck of the fluid handling robot, we fabricated custom plastic plate carriers (Figure 2 C). The carriers have the footprint of a standard well plate, and the top surfaces of the plate and carrier are flush with each other to facilitate pinning. Since each plate requires a dedicated carrier for the duration of the experiment and experiments potentially include large numbers of plates, 3D printing served as a convenient and cost-effective method for fabricating the carriers. We also fabricated a riser plate, which supports each carrier when it is on the deck of the Biomek i7 (yellow portion in Figure 2 C). The purpose of the riser plate is to prevent the SCARA grip points on the plate carrier from being obstructed by the framework of the servo shuttle carriage. Further design details on the plate carrier and riser plate is provided in Figure S.2 in the ESI.

SCARA

The SCARA is a model PF400 from Precise Automation (Fremont, CA) controlled by Beckman SAMI EX software using PreciseSCARAModule 5.0. The SCARA is used to transfer plate carriers between the fluid handling robot and the DESI stage. The servo shuttle transports plate carriers out of the fluid handling robot, at which point the SCARA grabs and transports them to the DESI stage for analysis. When analysis is completed, the SCARA transports the plate carriers to the storage station.

Piezoelectric Spray Solvent Delivery System (PieSDS)

The PieSDS system was first developed by Szilagyi *et al.* to enable fast switching between different spray solvents and precise control of the flowrate during the DESI-MS analysis[15]. For implementation with the DESI-MS HTS platform, the hardware and software features of the system were extended to allow reliable autonomous operation. The system is composed of a combination of commercially available hardware. A piezoelectric pressure controller (ElveFlow OB1 MK3), which has two independent pressure channels, with a range of 0 - 2 bar, is used to deliver solvents from a series of reservoirs. Given its piezoelectric nature, the OB1 MK3 has low response (9 ms) and settling times (40 ms), and the pressure fluctuations are as low as 0.005 %. Switching between solvents is enabled by the use of a piezoelectric valve array (ElveFlow MUX flow switch matrix). The MUX valve array has 16, two position, on-off valves having valve opening/closing time of 25 ms and a hold-up volume of <10 nL. The flowrates are measured with a thermoelectric flowrate sensor (ElveFlow MFS2). The MFS2 has a dead volume in the range of µLs, which is not negligible when purging is needed in-between solvent switching. To the best knowledge of the authors, the chosen devices had the best performance available in the market in their category. The engineering diagram and CAD model of the PieSDS, enabling accurate, independent control of two solvent streams and quick solvent switching, is illustrated in Figure 3 (Top) and (Bottom), respectively.

During screening, one OB1 channel symmetrically pressurizes up to 8 solvent reservoirs (Figure 2 F). To enable solvent composition control, only one MUX valve is open per pressure channel. The solvents are connected to the same pressure channel thus cannot be combined with one another. Each flowrate sensor is calibrated per solvent, and identical solvents coming from the two pressure channels are merged by a T-junction. Solvent switching is then facilitated by changing the opening configuration of different MUX valves. The previous solvent needs to be cleaned out from the mixer and the silica capillary between the mixer and the DESI sprayer. In the developed configuration the considerable dead volume of the MFS2s is removed from the common rail, which improves the solvent switching time.

DESI-2D Stage

We modified the surface of the DESI stage to include a custom receiver for the plate carriers (Figure 2 E). The receiver includes grooves that interface with rails on the sides of the plate carriers. The grooves are tapered in three dimensions in order to guide the plate carrier into exactly the right position as the SCARA pushes the plate carrier into place. The guide grooves also ensure that the plate carrier does not come into contact with either the DESI sprayer or the mass spectrometer sampling capillary during loading and unloading. Once loaded in the receiver, the plate carrier is held in place by friction between the bottom of the plate carrier and floor of the receiver.

Software and Communication Protocol

The DESI-MS HTS system is operated using a series of software packages communicating with each other beginning with a prompt for user input (Figure 4 1A and 1B). A user is first prompted in terminal 1 with a simple-to-use, in-house data entry GUI where essential parameters, such as the plate layout, reaction conditions, and DESI-MS settings, are entered (Figure 5). The GUI is a gateway to CHRIS, which is built on top of two other applications - SAMI EX and the Integrated Flowrate Controller and Solvent Switching Software (IFC3S) (in-house). Meanwhile, in terminal 2, a user accesses the Biomek software, which controls the Biomek i7 with a set of pre-programmed methods for preparing the reaction mixtures. It interacts with CHRIS via SAMI EX, and together they enable networking between all the devices and for the entirety of the experiment. The IF3CS controls the flow of multiple spray solvents during DESI analysis while CHRIS obtains and analyzes the MS data corresponding to each reaction spot on the DESI substrate. More details of each of this application is discussed in the subsections below.

Biomek Software

The Biomek software controls the Biomek i7 fluid handling robot to create methods for preparing the reaction mixtures. Specifically, it has the ability to define new liquid types and labware

that develops precise and customized pipetting techniques. It also has the capacity to integrate with LIMS systems to import work orders and export data. New methods can be easily created through a user-friendly interface. The user can control various aspects of the pipetting process including the aspiration/dispensing height and speed, mixing within the plate, tip touching, tip pre-wetting, air gaps, blowout volumes, movement speed within the well and between plates, etc. The software analyzes and verifies the validity of the created steps and prevents the method from running until any errors are fixed. Also, it gives the estimated time to finish the method, so the user may optimize this time by re-arranging the steps or pipetting conditions. The Biomek software is capable of programming simultaneous actions if it involves different parts of the robotic deck. For example, the pin-tool held by multichannel head may be cleaned in the sonication bath, while the Span-8 head is pipetting reagents on the other side of the deck.

SAMI EX

SAMI EX is an automation workflow scheduling software from Beckman Coulter. It is designed to have a graphical workflow interface that enables simple method creation at a high level without needing to describe the details of how every plate must move across the system. It handles inputs within the hardware constraints and enables feedback to select appropriate actions during the course of a run based on sample data generated in real time. SAMI EX is also equipped with "SILAS" software modules, which are the communication protocols for interacting and integrating with other devices connected to the whole platform. A script-based SILAS module is used whenever appropriate for integrating our in-house software (i.e. CHRIS and IF3CS) with the rest of the system's software via SAMI EX. The full hardware layout including fluid handling robot, SCARA, and mass spectrometer including DESI stage is therefore represented within SAMI EX.

Integrated Flowrate Controller and Solvent Switching System (IFC3S)

The IFC3S enables programmatic switching of DESI spray solvents and flowrate control of two independent solvent streams during an HTS experiment while communicating with CHRIS (Figure 4). It is written in LabView using the ElveSys LabView SDK, which consists of LabView drivers for all ElveFlow devices. The first version of the IFC3S has been discussed previously (Szilagyi et al. 2019)[15], however, using the SDK, a significantly more capable control software and GUI was recently developed for this platform. Specifically, beyond the precise flowrate controller tuning that was implemented in the initial incarnation of the device, there are several other features required for reliable, automated operation, which are described below. A flowchart delineating the control schemes is provided in the ESI (Figure S.6).

Flowrate Stability Monitoring

The flowrate stability is critical to the outcome of DESI. Hence, the control and monitoring of its stability prior to and during the experiment is essential. As a prerequisite for feedback control, the difference between the setpoint and actual flowrate is calculated, and the variance of the data series generated over a certain time interval is calculated. The flowrate is considered stable if the variance is under 0.03 µL/min. Some variance under the 0.03 µL/min threshold is always generated by the measurement noise and the flowrate feedback control loops.

Automatic problem identification and troubleshooting in IFC3S

Destabilization and failure of the DESI spray solvent flow clearly leads to the failure of the DESI analysis, which must be detected in early stages so that appropriate actions can be taken. A two-level problem identification and troubleshooting protocol is enabled in the IFC3S.

Level 1 troubleshooting: purge the tubing for non-critical system failure. Numerous events may lead to the destabilization of the flowrate, with the most common being bubble formation or clogging. The flowrate destabilization is detected by IFC3S and communicated to CHRIS to pause the DESI acquisition. The system is then flushed using the maximum safe operating pressure. Flushing the tubing can remove bubbles or contaminants from the lines. If this simple method fails, the Level 2 troubleshooting is activated.

Level 2 troubleshooting: alert the user of a critical system failure. Every failure that leads to zero flowrate (gas or liquid leakage, depletion of pressure source, etc.) or a failed Level 1 troubleshooting is considered a critical system failure. These events are communicated to CHRIS to pause the DESI acquisition, and IFC3S sends automatic text and e-mail messages to the user indicating that the system needs human intervention.

Chemical Reaction Integrated Screening Software (CHRIS)

CHemical Reaction Integrated Screening (CHRIS) is an in-house software suite developed to integrate all parts of the DESI-MS system and to acquire mass spectrometry data. CHRIS subsequently searches the acquired data for *m/z* values that correspond to the starting materials, intermediates, by-products, and products of the reactions. The main part of CHRIS is written in Python 3 but it utilizes Visual Basic to generate the Xcalibur sequence file and Visual C++ 5.0 to interact with the mass spectrometer.

During an experiment, CHRIS receives a signal from SAMI, which indicates that the plate is ready. It then creates an output folder to hold the data and an Xcalibur sequence file (Figure 4). CHRIS then waits for another SAMI signal, which indicates that the plate has been successfully transferred from the Biomek i7 to the DESI stage. CHRIS then sends a contact closure signal to the mass spectrometer start the acquisition. Data are acquired by scanning over the plate line by line with each new line being a separate data file (Figure 6 (Top)). At the end of the experiment, CHRIS sends a signal to SAMI to

notify that the experiment is completed, and SAMI moves the plate from the DESI stage to the storage space.

After the DESI-MS data is collected, CHRIS analyzes and processes the data using the following procedure. First, the Thermo RAW files are converted to a text format using msconvert from ProteoWizard[16]. These text files are then searched for the intensities corresponding to *m/z* 443, which identifies the locations of the 384 rhodamine B fiducial markers. A digital 2D matrix with the location of the rhodamine spots is subsequently generated. This matrix is then used to interpolate the coordinates and find the corresponding mass spectra for all spots on the DESI plate. A second digital 2D matrix is generated which contains the reaction details for each spot. This includes reaction conditions and all *m/z* values from starting materials, products, and any know by-products/intermediates. This second matrix is generated from a file which details the layout of each 384 well plate. The information in these two matrices is then combined, the first identifying where to look and the second what to look for, to calculate the intensities of all relevant *m/z* values for each spot. Output files are then generated which include lists of starting material intensities, product/by-product/intermediate intensities, and other ion intensities (unknown *m/z* values with ion counts above a defined threshold) as well as a graphical representation of the output via a web server (Figure 6 (Bottom)).

Case Study: N-Alkylation, N-Acylation, and N-Sulfonylation Reactions

Experimental

The robustness and repeatability of the system was evaluated by screening three classes of reactions: amine alkylation, amine acylation and amine sulfonylation. A variety of amines and electrophiles were used, and three different reaction solvents as well as three different DESI spray solvents were evaluated. The final concentration of all the reagents was 50 mM, and the ratio of amine to electrophile was 1:1. Acetonitrile (ACN), dimethyl sulfoxide (DMSO) and toluene were used as reaction solvents. The reagents used for these experiments were selected to provide structural diversity which would in turn impart various reactivity trends (Figure 7 (Top)).

Preparation of the reaction mixtures using the Biomek i7

Stock solutions (100 mM) were prepared for each reagent in each of the three reaction solvents in 3 mL glass vials. These vials where then placed into 24-position racks that fit onto the deck of the Biomek i7 (Figure 7 (Bottom)). The Span-8 head was then used to transfer the reagent stocks solutions into a 96-well deep-well plate (for the amines) and three separate 96-well standard plates (one for each electrophile). Transfer from these intermediate plates to 384-well final plates was also performed by the Span-8 head resulting in a total volume of 50 µL for each reaction mixture (25 µL of the amine and 25 µL of the electrophile). The intermediate transfer of the stock solutions to the 96-well plates allow for rapid 384-well plate preparation by the Span-8 head. The reaction mixtures were then carefully mixed in the 384-well final plates by pipetting up and down simultaneously using the multichannel head.

Each reaction class (alkylation, acylation, and sulfonylation) was placed in a separate 384-well final plate. The layout of each final plate was the same and contained three segments containing five rows each (120 wells per segment) with one empty row (row P). Each 120 well segment contained the 20 unique combinations of amine and electrophile in sextuplicate, and each segment represented a different reaction solvent (ACN, DMSO, or toluene). Each 120 well segment was then further divided into three 40 well segments, containing the 20 unique combinations of amine and electrophile in duplicate

Spotting of the reaction mixtures onto the DESI plates

Reaction mixtures from the 384-well final plates were transferred to the DESI plate using the 384-pin tool (50 nL/spot). Three different DESI plates were created each day (i.e. within 8 hours). The first DESI plate contained only acylation reactions. Eight replicates of the acylation 384-well final plate were spotted onto the DESI plate to create a density of 3,072 spots/plate. The second plate contained both alkylation and sulfonylation reactions. Four replicates of each final plate were transferred resulting in a final density of 3,072 spots/plate. The third plate contained all three reaction types. Seven replicates of acylation final plate, four replicates of alkylation final plate, and four replicates of the sulfonylation final plate were spotted onto the same DESI plate resulting in a density of 5,760 spots/plate. Thus, over three days (24 hours of experiment time) there were 90 replicates of each unique N-acylation reaction, 48 replicates of each unique N-sulfonylation reaction, and 48 replicates of each unique N-alkylation reactions. A unique reaction consists of a unique combination of amine, electrophile, reaction solvent, and DESI spray solvent.

DESI-MS conditions

The mass spectrometer (Thermo LTQ XL) was operated in positive ion mode with an *m/z* range of 50 - 500. The DESI spray angle was 55°, and the spray tip was placed around 1 mm from the surface of the DESI plate and 2 mm from the mass spectrometer inlet capillary. A voltage of 5kV was applied to the DESI solvent flow. The DESI-MS imaging lateral resolution was 350 µm, and with an instrument scan time of 80 ms, the resulting DESI stage speed was 4,376 µm/s.

Results and Discussion

During the HTS experiment, different components of the system operate in unison during different periods and for different duration. This is illustrated in Figure 13 (Top) for an 8-hour experiment. The results of the 3-by-8-hour experiments were used to study the robustness of the system as measured by the repeatability of the data generation. We analyze a total of 180 reactions, where each

reaction had between 24 to 42 replicates, for a total of up to 5,762 data points collected across 3 days (Figure 13 (bottom)). The repeatability of the system is discussed in the context of whether the mean and variance of the MS peak intensity distribution associated with the reaction products lead to variations in a "yes/no" decision of whether the reaction is successful. Specifically, we evaluated the acquired HTS data using two approaches, namely (1) statistical analysis of reaction product peak intensity across all the replicates and (2) principal component analysis (PCA) of the whole MS spectrum associated with a reaction spot on the DESI substrate.

Product MS Peak Intensity: The experimenter can choose to analyze a normalized peak intensity that corresponds to an expected (by)product of the reaction with a known *m/z*. This information reveals whether a reaction is a success and produces the expected product molecule – i.e. a "yes" or a "no" reaction - depending on whether or not the peak is above a certain pre-determined threshold. In particular, one can pre-determine an intensity cut-off corresponding to a yes/no decision based on heuristic knowledge of the sensitivity of the DESI-MS operation. We set a peak cut-off of >0.003 to represent a "yes" reaction and vice versa. It is noteworthy that the consistency of our decision-making regarding reactions whose product ionization is inherently around the 0.003 intensity cut-off would be less reliable (Figure 9). That is, reaction products whose ion intensities lie close to the boundary of the threshold will move in opposite directions as the threshold move up or down. Thus, in choosing an intensity cut-off, one can improve the consistency of the yes/no decisions by generally increasing the intensity cut-off, but at the expense of increasing the rate of false negatives (Figure 10 (Top)). At the cut-off where the rate of false negatives is lowest, we found that 94% of all reactions are reproducible at the 3000 and 6000 reaction spot density over the 3 days' worth of experimentation. The 6% inconsistency means that we may have a "yes" decision on one of the plates but not all of the plates across different days. Evidence suggests that 60% of the inconsistencies (4% of the reactions), are due to ion suppression while the rest (2% of the reactions) is due to the current DESI detection limit (Figure S.3 in ESI). A discussion regarding the actual results of the experiments, including which conditions were successful are included in the ESI (Figure S.7). Collectively, the source of non-repeatability may be due to several factors, including inconsistency in reaction mixture pinning and fluctuations in solvent delivery that may not have been captured by the flow sensor, and therefore, not corrected by the IF3CS control system. The DESI plates are also exposed to the surrounding, which contains elements that affect the ionization efficiencies of the target molecules and may change the profile of the spotted reaction mixtures over time. For instance, volatile reagents may evaporate unevenly across the plate causing discrepancies in (by)product signal intensity between the first and last spot on the same DESI plate due to differences in the time of analysis. There is also the possibility that reactions occur in a thin film rather than in droplets leading to variations in the time scale of formation of (by)products. These are inherent uncertainties within the current system which cannot be readily controlled at the moment. One way to overcome these variations is to have replicates of the same reactions in different spotting patterns across different plates. In this way, fluctuations in signal intensity of the same reaction can be meaningfully captured and success or failure of reactions can be confidently discussed.

Spot MS spectrum: The second approach for robustness analysis of the system concerns the use of the entire MS spectrum of a reaction spot and is intended to study the variability of the product and background peaks from one reaction spot to the next and from plate to plate across the few days of experimentation. In particular, given the high sensitivity of the DESI-MS method and that the method is performed in an open, ambient environment, a DESI-MS analysis of the same reaction but from different spots or plates yields somewhat different spectra. In order to capture this variability, we use principal component analysis (PCA) to compare the spectra of a standard reagent, namely rhodamine, replicated over multiple plates. There are 384 rhodamine spots per plate and 3 plates per day for a total of 1152 replicates and, in turn, spectra. The PCA analysis of these spectra showed that the first 3 PCs capture ~70% of the variance between spots from different plates and 9-22 spectral components capture ~90-99% (Figure S.4 (Top) in ESI). In other words, 22 different sets of peaks associated with each rhodamine spectra vary significantly from spot-to-spot. Figure 10 (Bottom) shows a plot of the coefficients of the 3 most significant principal components (PCs). They occupy the same space between substrates and suggest that the variability of the MS spectrum, while large, is fully characterized and reveals the consistency of each HTS experiment and those across plates. This observation is also consistent when considering the similarity between plots of mean MS spectra of rhodamine averaged over 3 days and 1152 replicates per plate (Figure S.4 (Bottom) in ESI).

Recent Advancements in the DESI-MS HTS Platform

Since we completed the case study described above, we have made significant improvements to our data collection procedures. The first of these improvements is that we now collect data only from the center of each spot. Our previous method of data collection (Figure 6 (Top)) scanned over the entire surface of the DESI plate. Whether a plate contained only 384 or the full 6,144 spots, the analysis time was always the same (approximately 3 hours). The new, spot-to-spot method of data collection acquires data only from locations that the user specifies, which significantly shortens the analysis time of plates containing fewer than 6,144 spots (Figure 11 (Top)). In addition, for a plate that is fully utilized and contains 6144 spots, the analysis time also shortens from 3 hours to 1.7 hours.

For the spot-to-spot method to work, the locations of all 6,144 spots must be known before the analysis begins. This is accomplished by first using the pin tool to place a dark dye solution just in the three corners of each DESI plate (top left, top right, and bottom left). The user then locates the center of the DESI spray using the camera on the DESI stage. The DESI stage is then moved

sequentially to each of the three pinned dye spots, the center of the DESI spray is aligned with the center of the dye spot using the camera, and the location of the DESI stage is recorded. Using the coordinates of these three spots, the locations of all 6,144 spots are then calculated. When the analysis begins, the DESI stage moves sequentially to each spot selected by the user and acquires data for approximately one second. A plate containing only 384 spots can be acquired in under seven minutes, whereas this same plate would take three hours using the previous method.

A second improvement is that we can now analyze data in real time using CHRIS. Previously, data could only be analyzed after the entire data set had been collected. For large data sets, this meant that there were delays of an hour or more before any data could be reviewed. With the latest version of CHRIS, outputs like the one seen in Figure 6 (Bottom) are generated in real time. Spots appear in the output as they are analyzed, and the user can determine the success or failure of the reaction and view the associated mass spectrum in real time.

A final improvement is that MS/MS acquisition has been incorporated into CHRIS. Users first acquire full scan data using the procedures described previously. Once the data is analyzed by CHRIS, the user is provided a file which contains the data for each spot including reaction conditions, *m/z* values and corresponding intensities for starting materials, products, and other ions of interest, and importantly the XY coordinates of each spot. The user then creates a list of *m/z* values along with their corresponding XY coordinates for MS/MS analysis. The MS/MS analysis can then be performed on a duplicate DESI plate that was an exact copy of that used for the full scan analysis. The user first calculates the locations of all 6,144 spots using the coordinates of the three dye spots as described above, and the MS/MS analysis then proceeds in a spot-to-spot fashion, only acquiring data from the locations specified by the user (Figure 11 (Bottom)). The DESI spray rasters back and forth over each spot for approximately 18 seconds, during which MS/MS data is collected using three different relative collision energies (20%, 10%, and 30%). The MS/MS data is saved in the form of one RAW file per spot with an automatically generated name containing the XY coordinates and precursor ion *m/z* value. A supplementary video provided in the ESI demonstrates this significant capability.

Conclusions

In this work, we designed and built the Purdue Make-It System, which is a novel and replicable DESI-MS HTS platform comprised of commercial and custom-made hardware and software. We evaluated the robustness and repeatability of the system by performing a 24-hour HTS experiment spanned across three 8-hour days. The experiment involved determining the presence of expected products for three classes of organic reactions, namely amine alkylation, amine acylation and amine sulfonylation, where a variety of amines and electrophiles were used, and three different reaction solvents as well as three different DESI spray solvents were evaluated. The overall workflow of the HTS method is provided in Figure S.5 in the ESI. We noted that there is a trade-off between the repeatability of the yes/no decisions and the rate of false negatives for the data generated, which can be modulated by choosing the cut-off intensity. We found that 94% of all the reactions tested are consistently reproduced over 3 days at both 3000 and 6000 reaction spot density. We attribute the source of inconsistency to two major factors: inconsistency in reaction mixture pinning and fluctuations in solvent delivery. These issues are not fundamental and may be mitigated in the future. At the time of writing, the platform was also updated with several features including (1) a new, spot-to-spot method of data collection, which allows users to acquire data only from user-specified locations, (2) a capability for data analysis in real time, and (3) on-demand MS/MS analysis of a reaction (by-)product associated with a reaction spot. It is noteworthy that the system's throughput may be significantly improved if coupled with a reagent preparation system and that its impact can be readily extended towards biological applications whereby analytes from cells and tissue samples instead of reaction mixtures are of interest and can be deposited on the DESI substrate. As such, these are opportunities for immediate future work.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

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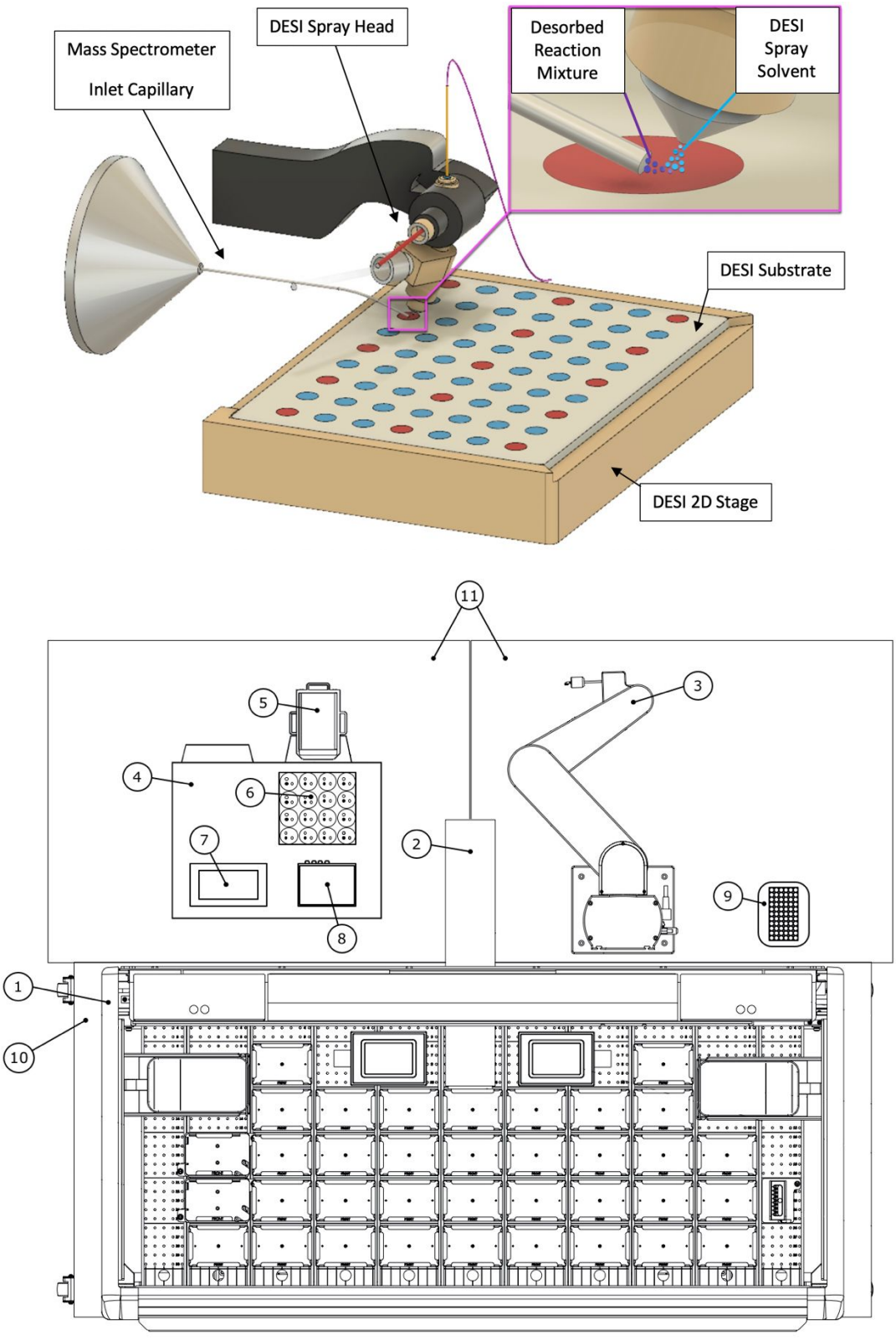


Figure 1 (Top) Schematic of the desorption electrospray ionization (DESI) source. The DESI substrate is sprayed with charged solvent droplets causing the spotted reagents to desorb, producing charged microdroplets in which the reaction occurs during droplet evaporation. The resulting ions are guided towards the MS via a metal ion-transfer line for MS analysis. (Bottom) Line drawn lay-out of the hardware components of the DESI-MS HTS system. 1) Biomek i7, 2) servo shuttle, 3) SCARA. 4) LTQ XL, 5) DESI stage, 6) solvent reservoirs, 7) ElveFlow pressure controller, 8) ElveFlow valve matrix, 9) DESI plate storage, 10 and 11) tables to support the equipment.

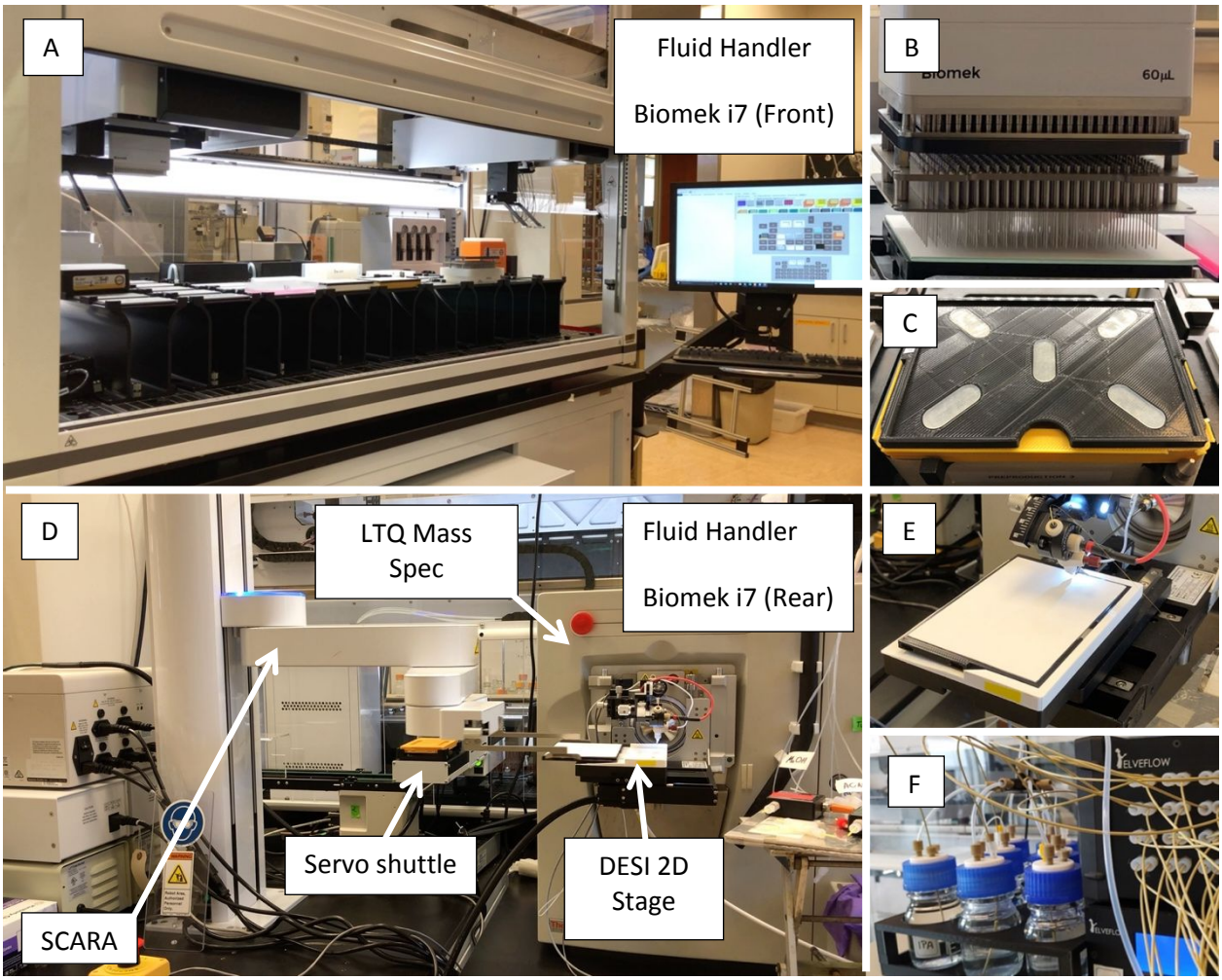


Figure 2 Photographs showing the key components of the DESI-MS HTS system, including (A) a fluid handling workstation (Biomek i7, Beckman Coulter Inc.) which includes (B) pin tool and (C) a plate holder (C), (D) a SCARA robot (PF3400, Precise Automation Inc.), (E) a DESI-2D imaging stage (DESI 2D, Prosolia Inc.) installed on an LTQ XL mass spectrometer (Thermo Scientific), and (F) a DESI spray solvent delivery system (ElveFlow).

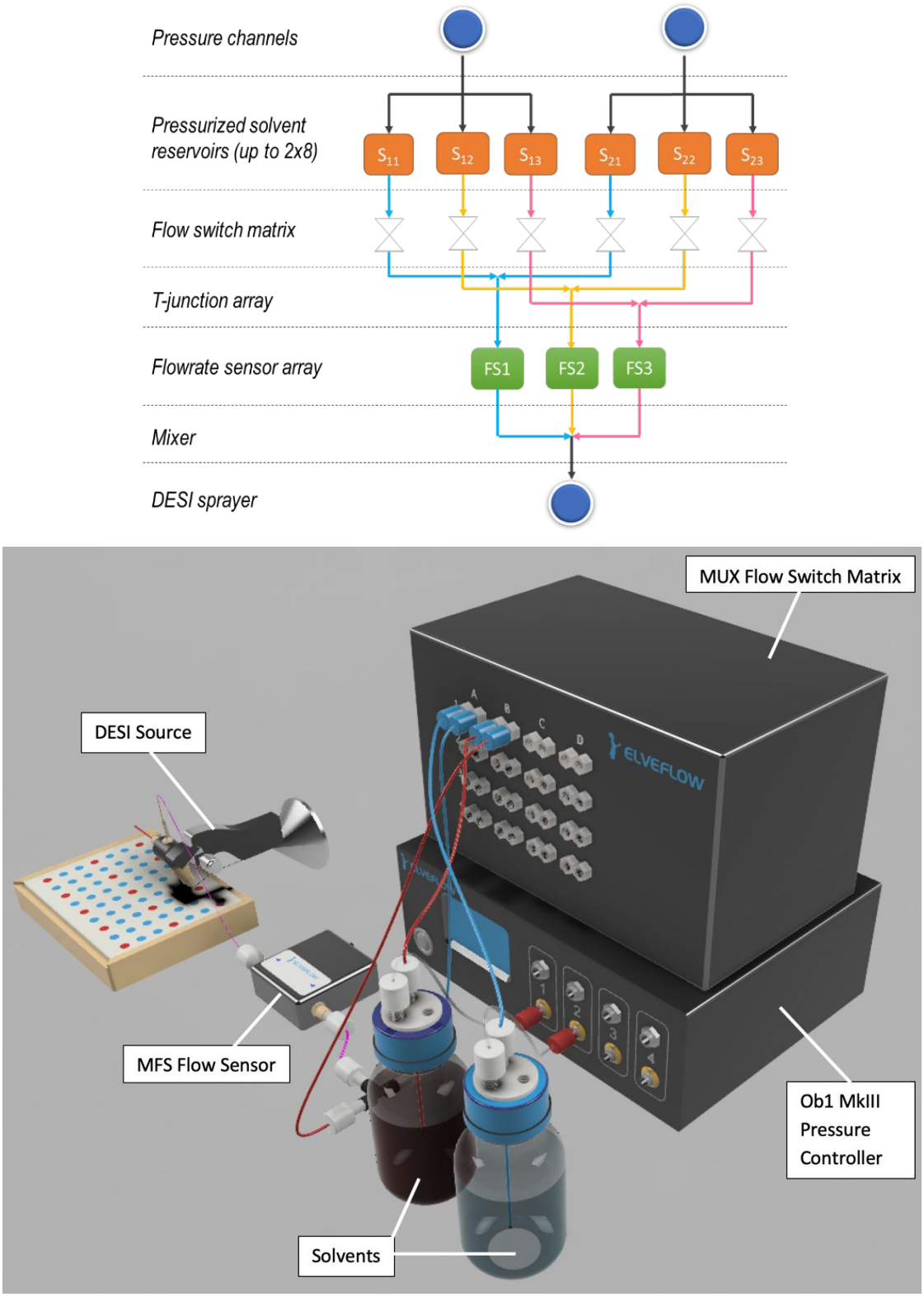


Figure 3 (Top) Simplified engineering diagram of the spray-solvent delivery system. The system is composed of a piezoelectric pressure controller, which has two independent pressure channels and is used to deliver solvents from a series of reservoirs. Switching between solvents is enabled by the use of a piezoelectric valve array while the flowrates are measured with a thermoelectric flowrate sensor prior to exposure to the DESI substrate via the sprayer. (Bottom) CAD drawing of piezoelectric-based spray-solvent delivery system (PieSDS), with two solvent control, including the ElveFlow devices and DESI source. The electrical cables are not shown for clarity. There is a total of 16 valve switches on the flow switch matrix (FSM) that can connect to 16 individual solvents and 4 independent pressure channels on the pressure controller (Ob1 MkIII). The pressure controller can connect to any combination of the 16 solvents for 4 independent flow rate settings.

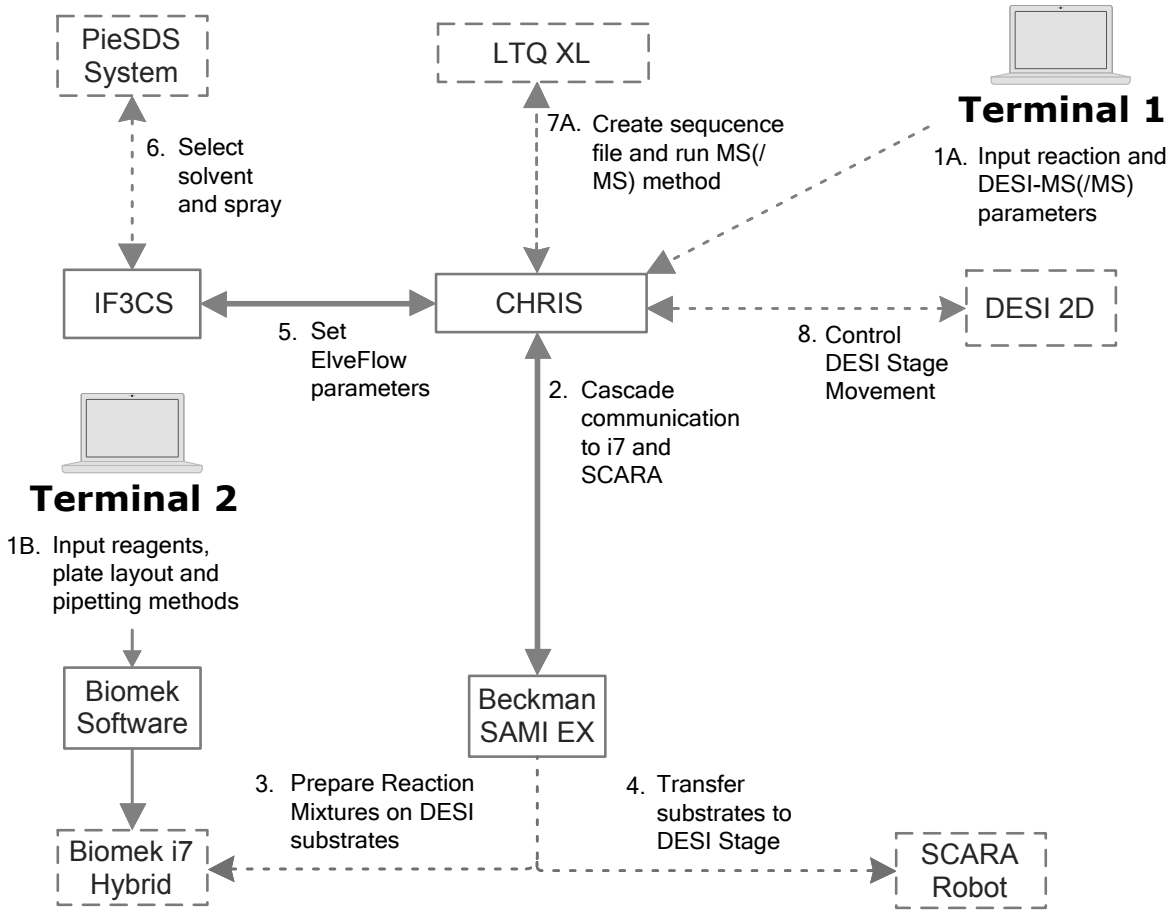


Figure 4 Communication protocol for the DESI-MS HTS system with hard line representing software and broken line hardware. A user is initially prompted with a simple-to-use, in-house data entry GUI in terminal 1 where essential parameters, such as the plate layout, reaction conditions, and DESI-MS settings, are entered. The GUI is a gateway to CHRIS, which is built on top of two other applications - SAMI EX and the Integrated Flowrate Controller and Solvent Switching Software (IFC3S) (in-house). Meanwhile, terminal 2 access the Biomek software, which controls the Biomek i7 with a set of pre-programmed methods for preparing the reaction mixtures. It interacts with CHRIS via SAMI EX, and together they enable networking between all the devices.

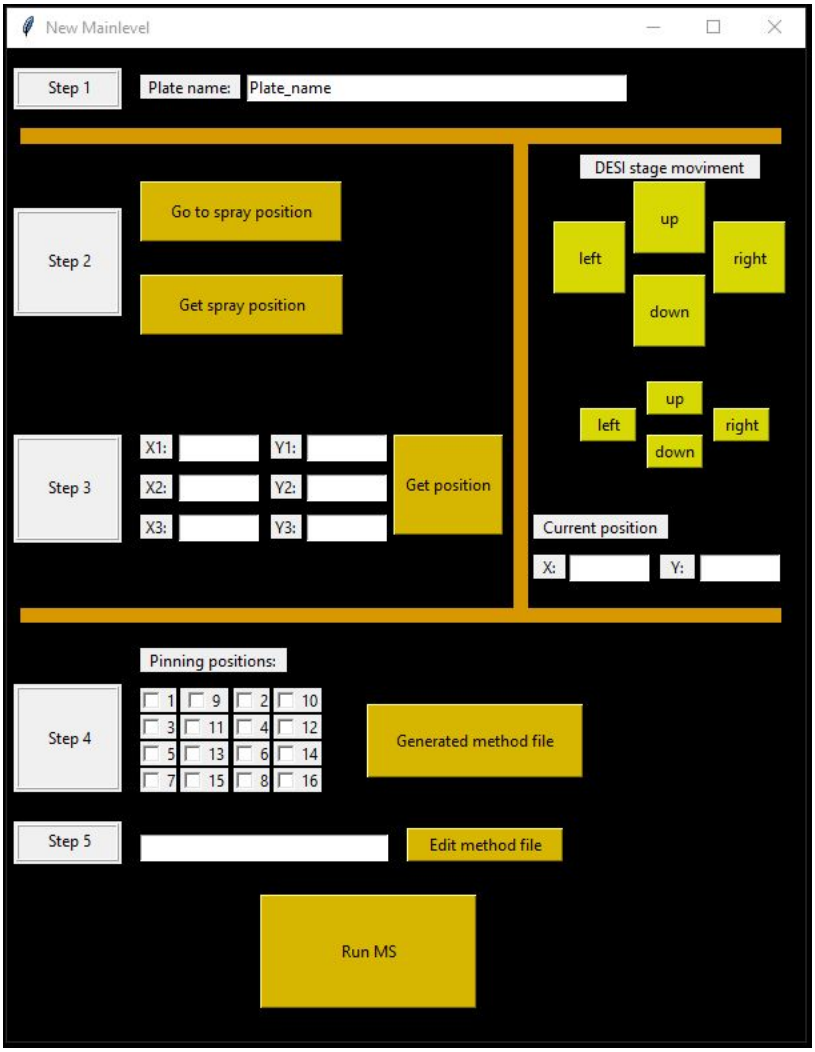


Figure 5 Screenshot of the input interface for the DESI-MS HTS System. The interface prompts a user for input parameters in multiple steps, namely the plate layout, reaction conditions, and DESI-MS settings, and allows simple DESI stage operations to be carried out, including manually moving the DESI stage and generating the method file.

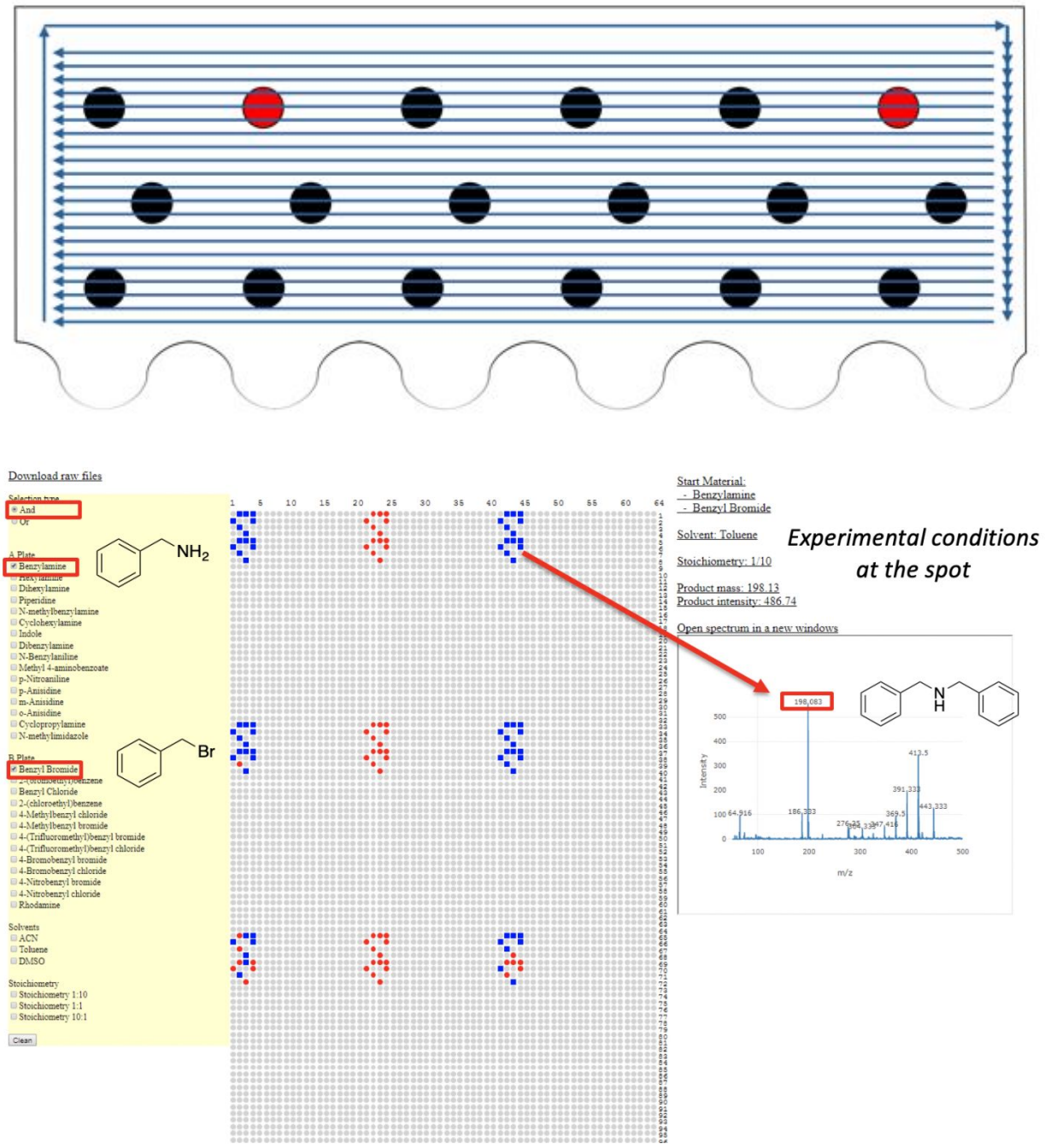
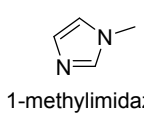


Figure 6 (Top) Scanning pattern used for the DESI-MS analysis. After each line, the current data file is closed, and a new data file is opened. The DESI stage then returns to the home position before moving to the beginning of the next line. When the contact closure signal is received, the acquisition of the next line begins. The red dots represent the rhodamine B fiducial markers. (Bottom) Screenshot of the web-based, graphical output of CHRIS. Reaction conditions such as reagents used, solvent, stoichiometry, etc. can be selected on the left to focus the view on the desired data points. Blue spots represent successful "yes" reactions, and red spots represent failed "no" reactions (i.e. product detected above/below a user-defined threshold). Each individual spot can be clicked on, which will display its experimental conditions and mass spectrum on the right.

Amines



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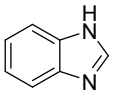

benzimidazole

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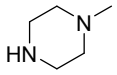
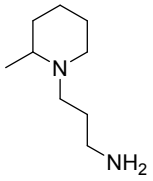


1-methylpiperazine

3

piperidine

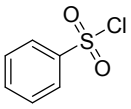
4



3-(2-methylpiperidin-1-yl)propan-1-amine

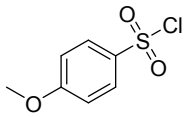
5

Sulfonyl Chlorides



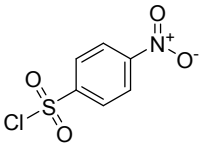
Benzenesulfonyl chloride

6



4-Methoxybenzenesulfonyl chloride

7



4-nitrobenzenesulfonyl chloride

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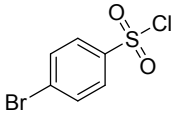

4-Bromobenzenesulfonyl chloride

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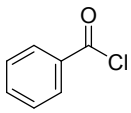
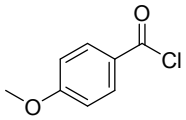
Acyl Chlorides



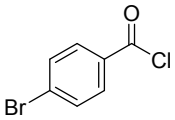
Benzoyl chloride

10



4-Methoxybenzoyl chloride

11



4-Bromobenzoyl chloride

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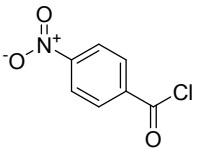

4-nitrobenzoyl chloride

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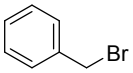
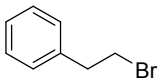
Alkyl Bromides



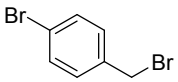
benzyl bromide

14



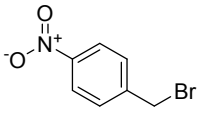
2-(bromoethyl)-benzene

15



4-bromobenzyl bromide

16



4-nitrobenzyl bromide

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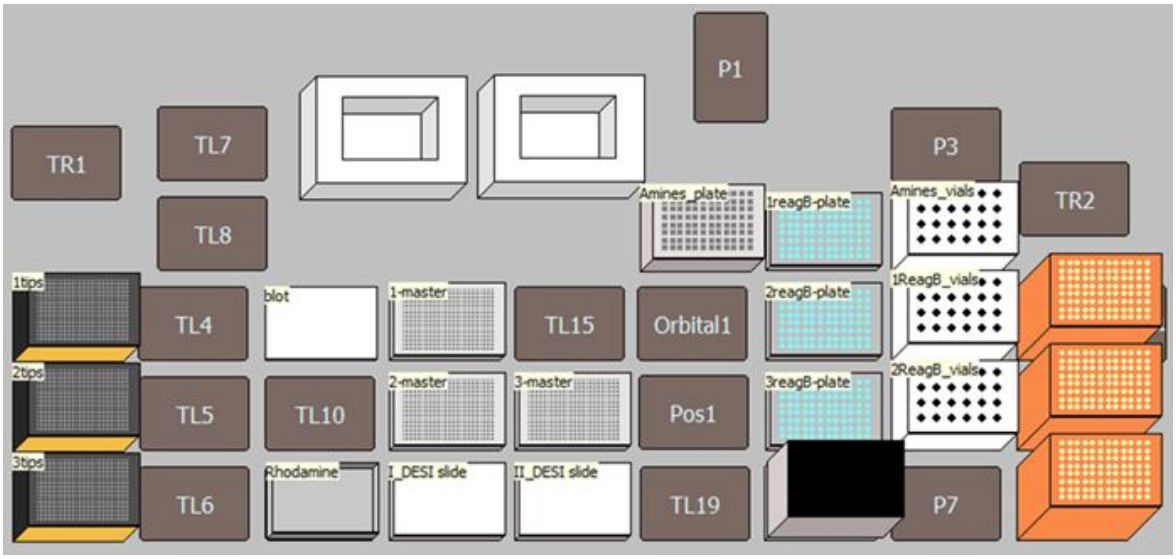


Figure 7 (Top) Molecular structure of the 17 reagents used for the 3-by-8-hour experiment by group (amines, sulfonyl chlorides, acyl chlorides, and alkyl bromides). (Bottom) Deck layout of Biomek i7 during the case study. The different deck's estate can stage different types of equipment suitable for reagent preparation and DESI substrate pinning. A detailed description of the component of the deck is provided in the ESI (Table S.1).

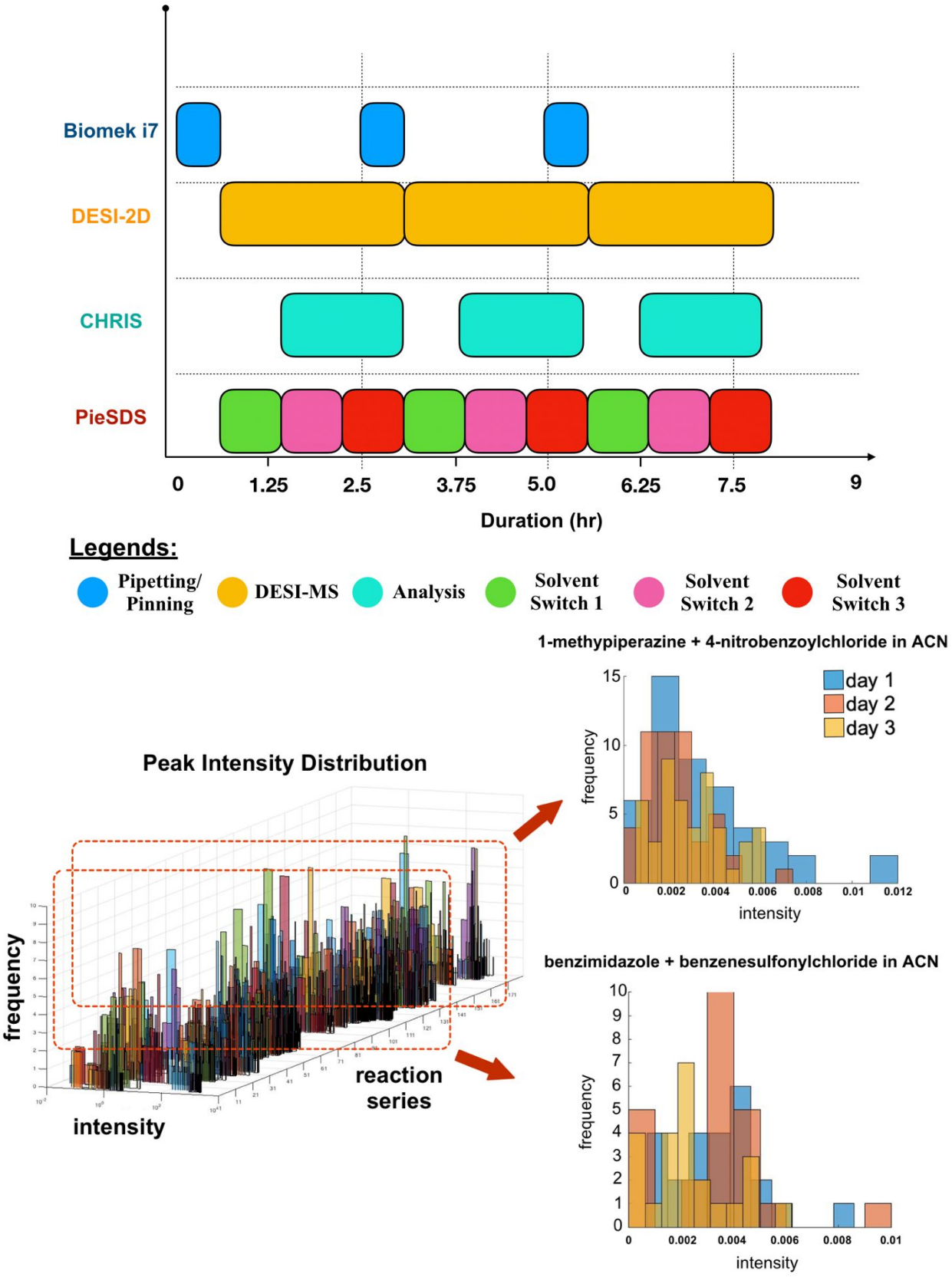


Figure 8 (Top) Duration of action for each component of the DESI-MS HTS system during the 3-by-8h experiment. The y-axis indicates the hardware responsible for each step of the experiment over an 8h duration. The legend shows the operation and the timing of execution by each hardware as shown in the x-axis. (Bottom) Bar graphs representing the peak intensity distribution of various reaction products in the reaction series. Examples of peak intensity distribution of product of two reactions are shown.

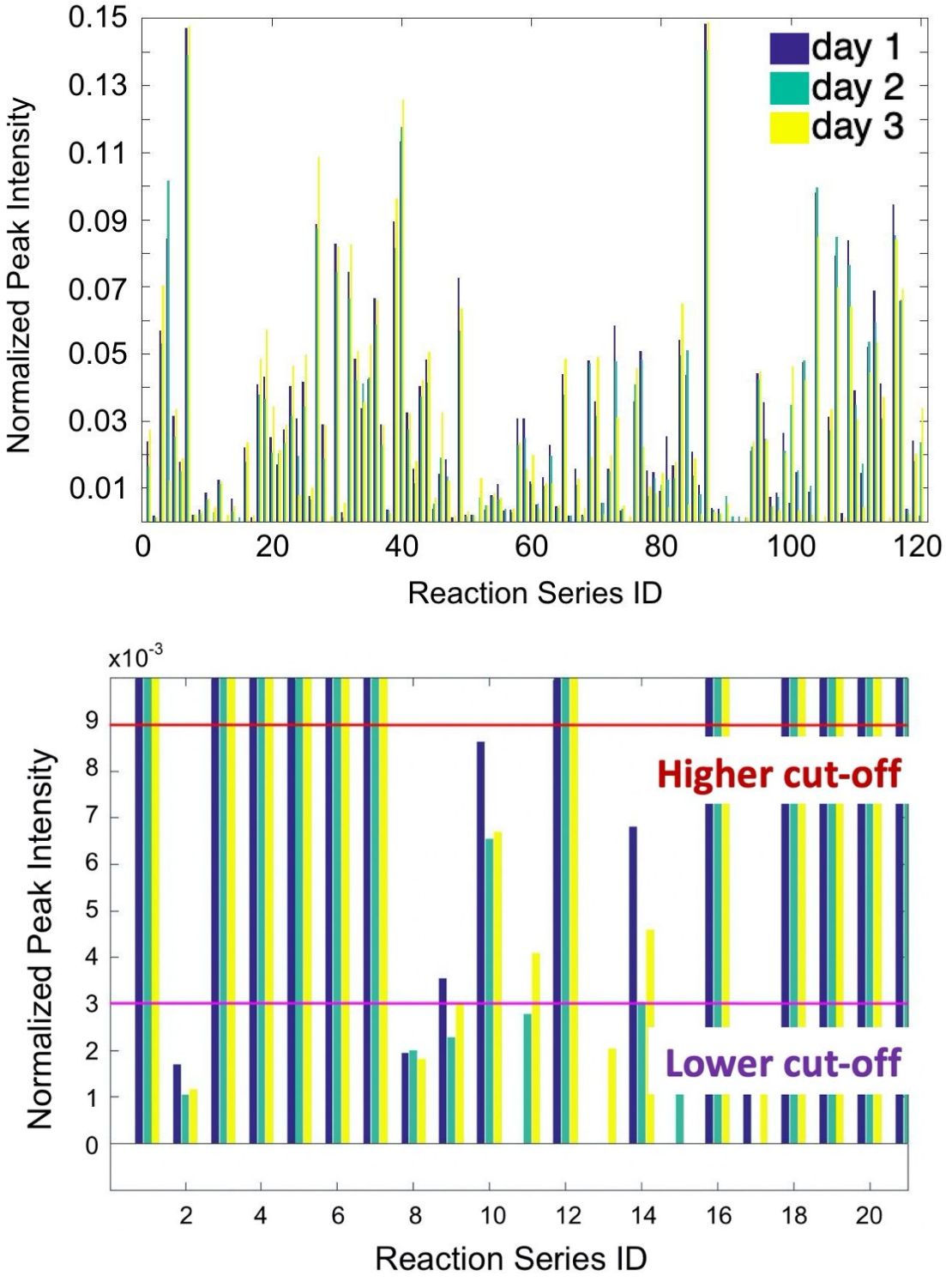


Figure 9 (Top) Normalized MS intensity of 120 reaction products performed in the 3-by-8-hour case study. (Bottom) Zoomed-in plot of the first 20 reactions with different "yes" threshold applied, including >0.003 (magenta) and >0.009 (red), which leads to different trade-off between repeatability and false positive rate of the system shown in Figure 10 (Top).

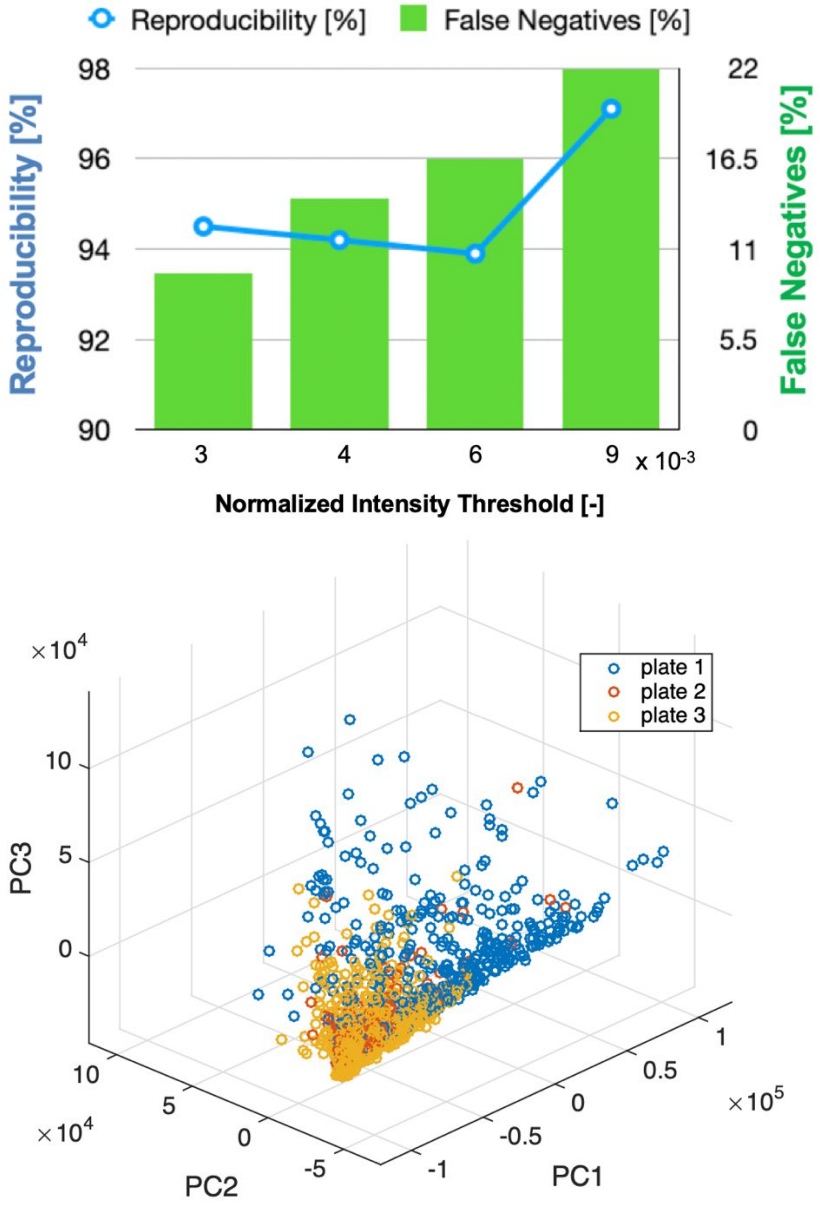


Figure 10 (Top) Trade-off between repeatability and the rate of false negatives at different normalized intensity threshold for determining yes/no reactions. (Bottom) Coefficients of the first 3 principal components (PC) of the spotted rhodamine spectra in 3 plates over 3 days. The PCs occupy the same space between substrates and suggest that the variability of the MS spectrum is well characterized and consistent in each HTS experiment.

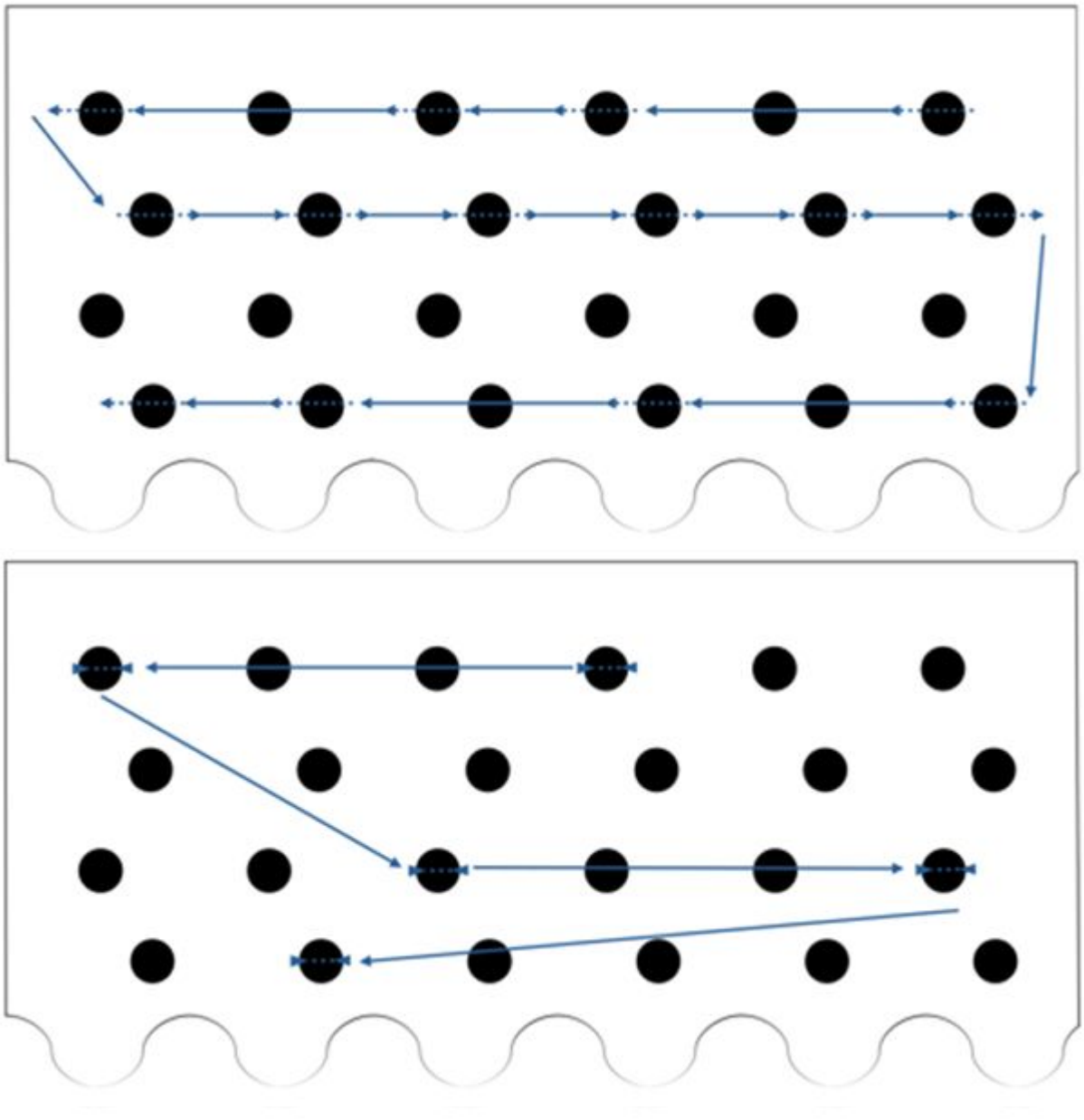
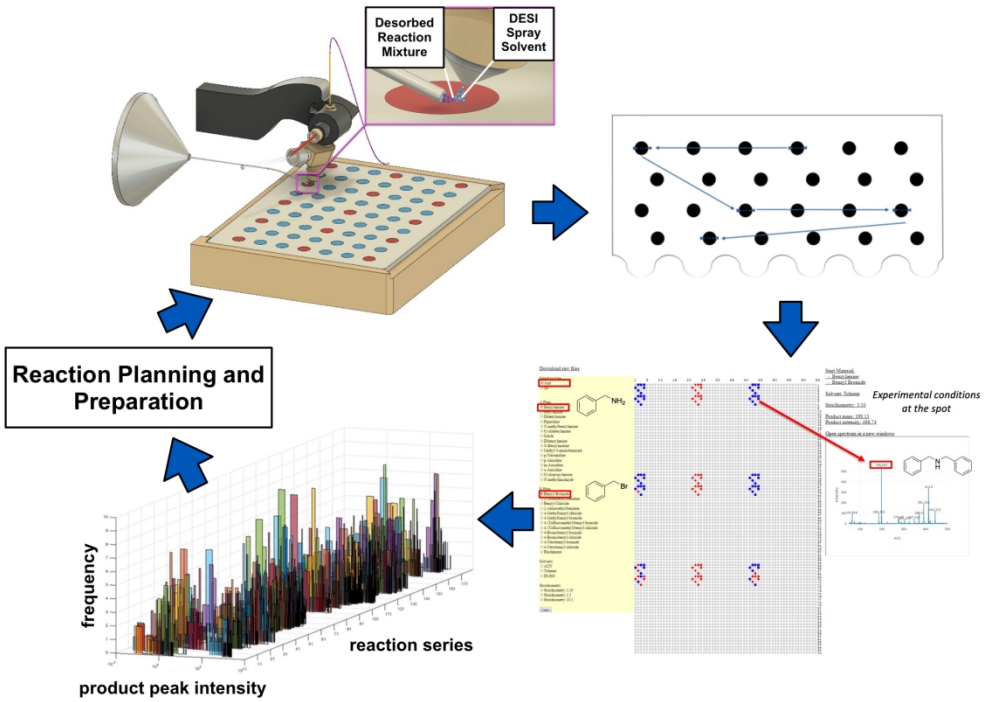


Figure 11 (Top) Spot-to-spot method of data acquisition. The spots that are skipped represent locations that were not selected for analysis by the user. (Bottom) Example of MS/MS spot-to-spot data acquisition. Data is acquired only from specific locations provided by the user. Since not all reactions produce product, MS/MS data is typically acquired from far fewer spots.

TOC Description:

Hardware and software Implementation of a novel high-throughput screening method based on desorption electrospray ionization mass spectrometry (DESI-MS HTS) capable of reaction analysis of up to 1 reaction mixture per second. The reaction and analysis steps are performed simultaneously using DESI to release microdroplets containing the reaction mixture from the plate for reaction prior to arrival at a mass spectrometer.



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