Vacuum compatible sample positioning device for matrix assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry imaging

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(Received 3 January 2011; accepted 3 May 2011; published online 27 May 2011)

The high mass accuracy and resolving power of Fourier transform ion cyclotron resonance mass spectrometers (FT-ICR MS) make them ideal mass detectors for mass spectrometry imaging (MSI), promising to provide unmatched molecular resolution capabilities. The intrinsic low tolerance of FT-ICR MS to RF interference, however, along with typically vertical positioning of the sample, and MSI acquisition speed requirements present numerous engineering challenges in creating robotics capable of achieving the spatial resolution to match. This work discusses a two-dimensional positioning stage designed to address these issues. The stage is capable of operating in $\sim 1 \times 10^{-8}$ mbar vacuum. The range of motion is set to 100 mm $\times$ 100 mm to accommodate large samples, while the positioning accuracy is demonstrated to be less than 0.4 micron in both directions under vertical load over the entire range. This device was integrated into three different matrix assisted laser desorption/ionization (MALDI) FT-ICR instruments and showed no detectable RF noise. The “oversampling” MALDI-MSI experiments, under which the sample is completely ablated at each position, followed by the target movement of the distance smaller than the laser beam, conducted on the custom-built 7T FT-ICR MS demonstrate the stability and positional accuracy of the stage robotics which delivers high spatial resolution mass spectral images at a fraction of the laser spot diameter. © 2011 American Institute of Physics. [doi:10.1063/1.3594099]

I. INTRODUCTION

Major developments in experimental techniques, instrumentation, and software have facilitated the use of mass spectrometers for biological imaging^{1,2} applications. Mass spectrometry imaging (MSI) allows the investigation of the spatial distribution of the molecular compositions within and across selected tissues as well as on the whole organism scale.³ The data produced by application of this technique in the studies of such diseases as Alzheimer's,⁴ cancer,⁵ muscular dystrophy,⁶ and Parkinson's⁶ provide medical community with a qualitatively deeper understanding of the nature of these maladies, while the ability of *in situ* study of the distribution of pharmaceuticals⁷ promises a significant acceleration in developments of remedies.

Two frequently used desorption and ionization methods for imaging mass spectrometer (MS) are matrix assisted laser desorption/ionization (MALDI) and secondary ion mass spectrometry (SIMS), although desorption electrospray ionization has recently been employed for imaging experiments as well.⁸ MALDI^{9,10} mass spectrometry is predominantly utilized in biological applications. Compared to

electrospray¹¹ ionization mass spectrometry, this method has some advantages: specifically lower sensitivity to contaminants, lower detection limits, and the ability to store samples and analyze them minutes (or days) later. Compared to MALDI, SIMS is suitable for studying elements and small molecules usually not exceeding 500 Da, while providing spatial resolution on a sub-micron level.

While MALDI offers an unmatched molecular resolution and range (capable of ionizing whole macromolecules and their non-covalent complexes), its spatial resolution is limited by the laser spot diameter. Even though modification of the laser optics^{12–16} and such experimental techniques as sample exhaustion¹⁷ is capable of producing much smaller “pixels,” the typical limit is $> 50 \mu\text{m}$.

All of these methods generate hundreds of sample spots (whether it be a MALDI target spot or a “pixel” in an imaging experiment), which must be analyzed in the mass spectrometer. Such a sample load requires automation. A typical automated source involves a 2D translation stage which positions the MALDI target plates or tissue under ionization beam's focal points. The laser (for MALDI experiments) or a primary ion beam (for SIMS) ionizes the analytes, which are directed into the mass spectrometer's ion optics for analysis. In general, commercial 2D translation stages have a number of problems associated with operation in vacuum. First,

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most of the stages (even some sold as “vacuum compatible” at exorbitant prices), are not vacuum compatible in which they are designed with numerous virtual leaks, and use motors and surfaces that must be kept lubricated, albeit quite often with non-vacuum compatible lubricants. Second, the motors and other electrical components are not designed with sufficient heat sinking capability and often overheat and seize or burn out. Third, the feedthroughs required in these stages are often inadequate in terms of the RF interference shielding, and the motors and feedback electronics themselves often generate substantial RF noise. Lastly, many instruments are designed horizontally, which requires the sample positioning stage to be mounted vertically and places different stresses on the vertical versus the horizontal positioning motors, loads, and feedback systems.

This article describes and presents a two-dimensional, vacuum compatible sample positioning stage specifically designed to address these issues. The stage was designed as a vacuum compatible device to operate at pressure around 1×10^{-8} mbar. While it is not exactly designed for operation at lower pressures, minor modifications would allow it. Positioning accuracy, RF interference noise, and vacuum compatibility, as well as the results from MSI experiments will be discussed.

II. METHODS

Savinase (synthetic serine protease subtilisin 309) was digested with trypsin and CNBr and 5 μ l was separated on a LC Packings nanoLC-system (Dionex, Amsterdam, NL) with a C₁₈ PepMap 100 pre-column (internal diameter 300 μ m, length 1 mm) and a C₁₈ PepMap 100 analytical column (internal diameter 75 μ m, length 15 cm). The eluents were 0.1% formic acid and 5% acetonitrile in water (A) and 0.1% formic acid and 5% water in acetonitrile (B). The LC method used a gradient of 20 min for 0%–70% B, followed by a 1 min gradient of 70%–95% B, 10 min of 95% B, and 12 min of 5% B for a total run time of 50 min. The capillary was coupled to a SymBiot I sample workstation (Applied Biosystems, Foster City, CA, USA) and the effluent was spotted on a premade MALDI foil (LabConnections, Northborough, MA, USA) in 30 s intervals at a spacing of 1 mm in a 10×10 matrix. A 20 min delay time between the start of the LC method and the start of the spot deposition was used. The foil was coated with a 10 mg/ml solution (60:40 ACN/H₂O (0.2% TFA)) of α -cyano-4-hydroxycinnamic acid with an ImagePrep matrix deposition device (Bruker Daltonics, Billerica, MA, USA) after spotting. Protein identifications were made by a Mascot database search (www.matrixscience.com).

Ten micrometer thick rat brain sections (Harlan Laboratories, Boxmeer, The Netherlands) were prepared at -20°C in a HM 525 cryomicrotome (Microm International, Walldorf, Germany) and thaw-mounted on indium-tin-oxide coated glass slides (ITO, 4–8 Ω resistance; Delta Technologies, Stillwater, MN). The sections were washed three times with cold 70% ethanol and coated with a 10 mg/mL solution (60:40 ACN/H₂O (0.2% TFA)) of α -cyano-4-hydroxycinnamic acid (Sigma-Aldrich, Zwijndrecht, The Netherlands) with an

ImagePrep matrix deposition device (Bruker Daltonics, Billerica, MA, USA).

MALDI FT-ICR MS experiments were performed on a custom built dual source FT-ICR mass spectrometer^{18,19} operating in a 7T unshielded superconducting magnet. The positioning device is mounted vertically in the MALDI source ~ 2 mm from the entrance to the mass spectrometer’s ion optics. A 355 nm Nd:YAG laser (BrightSolutions, Cura Carpignano, Italy) is used for desorption/ionization and ions are accumulated in an external octopole ion trap²⁰ before transfer to a capacitively coupled²¹ cylindrical open ICR cell.^{22,23} Ions were excited by broadband frequency chirp excitation and detected before a single zero-fill, Gaussian apodization, and Fourier transform. An AWG3 workflow-based data station is used for experiment control, data acquisition, and analysis.²⁴ Images were produced via an in-house developed software and visualized as “datacubes.”²⁵

III. DESIGN

The design parameters were not limited solely by the requirements of MS imaging experiments, but were also defined to handle the typical proteomics-type samples, for a versatile vacuum compatible positioning system. The full travel of the stage was required to be no less than $100 \text{ mm} \times 100 \text{ mm}$, as to accommodate the microtiter plates ($\sim 76 \text{ mm} \times 89 \text{ mm}$) commonly used in proteomics work. The speed was set to 2 mm/s, which would satisfy both imaging and MALDI FT-ICR MS time requirements, yet it is possible to further decrease the time of spot to spot travel for the shorter duty cycle analyzers. The positioning repeatability was set to 1 μ m, which exceeds the requirements for MALDI MSI experiments, yet further adjustments can be made to meet the spatial resolution requirements for SIMS imaging. Additionally, true vacuum compatibility, vertical load, and low (not detectable by FT-ICR) RF interference noise levels were imperative. Furthermore, to be practical, the device was to be as small as possible to minimize the volume which needs to be evacuated. Hence, conventional “stacked stage” arrangements having off hanging motors which sweep a significant volume of space as the stage moves were to be avoided in the new design.

The final stage design is shown in Fig. 1. The two-dimensional stage is a three piece single block design: the upper and lower plates and the central block which hosts two axis motors, linear encoders, position limit switches, and two temperature sensors. The system uses ballscrews and crossed roller bearings which require minimal lubrication; what little lubrication is needed is provided by low vapor pressure PTFE based grease.

Brushless servomotors were used for a number of reasons. Neither linear nor ultrasonic piezomotors, which perform superbly in horizontal setup, are acceptable, given the vertical nature of the load. Stepper motors are not practical because they tend to generate a lot of heat, dissipation of which is difficult under the vacuum conditions. Brushless servomotors, on the other hand, are easy to tune both in horizontal as well as in vertical arrangements. Heat is produced only during motion and heatsinking can be accomplished by trivial

FIG. 1. (Color online) The positioning stage is a single block three tier design; (a) a blueprint of the fully assembled device with the top tier transparent; (b) a photograph of the middle tier housing all of the mechanical components and sensors.

thermal grounding, due to the placement of the coils on the outside of the case. Additionally, brushless servo motors are durable and do not arc in vacuum.

The motors are mounted to the central block via a copper bracket, which allows the block to act as a heat sink. It is only able to dissipate heat through conductive cooling at the bearings and radiative cooling through the environment. The conductive cooling is very inefficient since the bearings are of stainless steel (very low heat transfer capacity) and have low contact area. A copper band or braid was considered for improving conducting heat transfer, but a quick calculation of the heat transfer of such a band compared to the radia-

tive cooling rate (assuming black body) showed that the heat transfer through the band was negligible. The stage is black anodized to improve the radiative cooling (serendipitously, it also improves surface hardness, but it also increases the surface area which could cause problems in UHV operation).

The torque is transferred to the ballscrew via pulleys. The belts for these pulleys are actually small electro-welded stainless steel bands, and in order to keep them centered the pulley wheels are ground to be slightly convex. Tensioning set screws are mounted into the block to allow the pulleys to be tightened properly during testing. The ballscrew is mounted in two places: the pulley bearing assembly (double ball bearing) and the plate, to prevent over-determination of the position, which would result in “jamming.” The plates use cross roller bearings, which allows for a much more compact design by both low profile and elimination of the need for overhangs otherwise required by recirculating ball bearing.

The system uses 4 optical limit switches (two for each axis). The switch is basically a photodiode/phototransistor pair (see Fig. 1(b)) mounted on the central stage body, which is interrupted by an aluminum flag mounted on the upper and lower stage plates. This flag blocks the photodiode’s signal to the phototransistor when the stage moves to the end of its range of motion, and the electronics signal the stage to stop. Such a system, while similar to a rocker or a magnetic switch involves fewer moving parts and possesses greater positioning accuracy. It is important, however, to verify the cleanliness of the photodiode/phototransistor pair prior to final assembly in vacuum, as dust or grease can easily inactivate a limit switch resulting in motor burnout.

The stage control is implemented as a dual loop with the velocity feed-forward (Fig. 2). The feed-forward signal is generated by a trajectory generator, a functionality provided as a part of the Galil[®] controller. This feature allows for substantial increase in positioning speed especially during the initiation of motion. The outer loop control, which is also handled by the same controller, is a proportional (position) gain controller with the position information fed from the linear encoders. The inner loop is the proportional integral controller. The proportional (angular position) and

FIG. 2. (Color online) The control of the system consisting of the trajectory generator, inner velocity, and outer position loops. The trajectory generator generates both position and velocity commands, with the velocity gain K_{VFF} ; the main loop of the control scheme is a proportional only controller with K_{PP} proportional gain and K_{GP} position scaling factor; the inner loop is a proportional integral controller with K_{PV} proportional and K_{IV} integral gains, K_A amp and K_{GV} velocity scaling factors, K_J motor torque, and J inertia constants.

integral (velocity) information are received from the servo-motor tachometers and used by the controllers to adjust the current driving the motors.

The actual position of the load, as was mentioned above, is fed back into the controller via the linear encoder. Although it is possible to bypass linear encoders and use the positioning information directly from the motor rotary encoder (which is easily capable of submicron resolution), this will only provide the controller of the highly accurate angular position of the motor. Since there are multiple mechanical components with different rigidities involved in transferring motor rotation into the linear motion of the stage, the encoder's angular precision cannot be directly mapped to the plate's final positional accuracy. Instead, rotary encoder information is fed back into the motor's amplifier creating a velocity loop. This arrangement significantly simplifies tuning by reducing the number of interdependent parameters to adjust. In addition, the velocity loop allows not only precise point-to-point movement of the stage but also eases the tuning to follow the desired time trajectory, which makes positioning more accurate (especially given the unequal loads for different axes) while the stage is in motion. Overall, the system performs very similar to a proportional integral derivative (PID) controller with proportional and derivative terms provided explicitly in the form of position in the main and velocity in the inner loops, while the integral component can be thought of as a product of integration of the inner proportional term in the main loop.

The sample translational stage requires 48 electrical feedthroughs to control the 2 motors, 4 encoders (2 linear and 2 rotary), 4 limit switches, and a thermocouple. Furthermore, it is critical that the feedthrough and cables are well shielded to reduce RF interference noise, due to the high sensitivity of FT-ICR MS to such noise. The feedthrough used is military standard with strain relief (55 pin) from Detronics Corporation (South El Monte, CA), but other alternatives are feasible as well, for instance, Caburn MDC (Harwell, UK) manufactures a number of UHV compatible feedthroughs, including two different 50 pin connectors (part numbers 1511007 and 1511008).

IV. RESULTS

The final assembly of the device is shown in Fig. 3(a). Its compact size allows for integration with the majority of existing MALDI sources. Figure 3(b) shows the stage (fully extended in one direction, exposing a motor, a pulley, and one optical limit switch) mounted on the back source plate of a custom built 7T MALDI FT-ICR MS with a high pressure source at Boston University.²⁶ Figure 3(c) is a photograph of the apparatus mounted in the source chamber, taken through a side window of the FT-ICR MS source.

The software interface is implemented as a lightweight dynamic linked library wrapper on top of the Galil[®] DMC software suite. This additional level of abstraction ensures flexibility and ease of access by a third party software by hiding the low level routines and significantly lowers the complexity of the interface. A custom built WIN32 application (Fig. 4(a)) controls the stage for testing, fine tuning, and manual operation. The application provides access to all function-

FIG. 3. (Color online) Final assembly of the positioning device; (a) lateral view of the stage in its fully compressed form; (b) mounted on the back plate of the MALDI source; (c) inside the MALDI source with a target next to accumulation hexapole (gas inlets for vibrational cooling are seen right next to the plate).

alities, diagnostic information, and statistics on the device. The stage has also been integrated into other available system control platforms, such as the workflow based AWG3 suite.^{24,27} A screenshot of the AWG3 GUI stage control for a MS imaging experiment is shown in Fig. 4(b).

The performance capabilities of the positioning stage were tested on the BU 7T FT-ICR MS with the device mounted vertically (Fig. 3(c)) in the MALDI source at a pressure of 1×10^{-8} mbar. The motion speed was set at 2 mm/sec, while acceleration and deceleration, as well as feed-forward gains were programmatically adjusted for the optimal positioning accuracy. All tests were conducted using proprietary Gallil[®] software tools.

Superimposed plots of the position and the corresponding positional error vs. time are shown in Fig. 5. The positional plot shows the initial rest at time zero followed by an acceleration event, steady motion, deceleration, and the final rest position at 1.5 mm roughly corresponding to ~ 0.4 s after the initiation. The error plot indicates that the positional accuracy of the device while moving is under $1 \mu\text{m}$ and the jitter that followed fluctuates between 0.1 and $0.2 \mu\text{m}$ and lasts for the duration of the movement resulting in a steady state error of $0.1 \mu\text{m}$.

The device was also integrated into the AMOLF FT-ICR MS system equipped with a 7T unshielded magnet,¹⁸ and was tested for mass spectrometry imaging applications. For proof of concept as a MALDI MSI positioning system, a high-performance liquid chromatography run of the savinase tryptic/CNBr digest was spotted on a MALDI target plate (Fig. 6(a)). The target was subjected to a FT-ICR MS imaging experiment (40×40 "pixels") which produced distinct and discrete spatial locations for a number of species at masses 961.0, 1100.7, 1200.8, 1205.9, 1440.6, 1457.0, 1821.5, 1933.2, 2368.1, and 2593.3 Da and are shown in Fig. 6(b)–6(l). A database search correctly identified the savinase enzyme with a significant Mascot score of 77 and an E-value of .031.

FIG. 4. (Color online) Software control interface; (a) a custom-built dedicated application for manual control of the device; (b) stage control GUI integrated into the AWG workflow based data acquisition and control system.

The laser spot size is the limiting factor affecting the resolution of microprobe mode MALDI MS imaging experiments (usually ~ 50 – 300 microns). One technique which allows circumvention of this shortcoming is “oversampling.”¹⁷ A typical oversampling MS imaging experiment consists of complete sample ablation at the initial position followed by moving the sample by a fraction of the laser spot size and collecting spectra (until sample exhaustion as well), hence acquiring sub-laser spot size information. To put it to a test,

a simple oversampling experiment with a pixel size of 50 microns (whereas the laser spot diameter is 300 microns) was conducted on a single MALDI spot containing a 50:50 bradykinin, angiotensin mixture (Fig. 7(a)) faithfully reproducing their spatial distribution (that of bradykinin on Fig. 7(b) and angiotensin on Fig. 7(c), respectively) even revealing some target surface imperfection, such as the scratch in the lower left part of the dried out droplet appearing throughout Fig. 7.

FIG. 5. (Color online) Performance evaluation analysis of the system. Blue is the position plot of the stage superimposed with the corresponding error (red).

FIG. 6. (Color online) An imaging experiment conducted on the simple tryptic/CNBr digest spotted on a MALDI target; (a) the plate after the sample deposition; (b) through (l) spatial distribution of the compounds with m/z 's of 961, 1101, 1201, 1206, 1440, 1457, 1800, 1821, 1933, 2368, and 2593 Da, respectively.

Figure 8(a) shows a MALDI FT-ICR MS imaging experiment of a rat brain section at a laser spot diameter of $\sim 300 \mu\text{m}$ (Figs. 8(b), 8(c) and 8(d)(I)) and an oversampling experiment at a raster size of $150 \mu\text{m}$ (Figs. 8(b), 8(c) 8(d)(II)). The results show distinct spatial localizations of putative lipids at masses corresponding to 827 Da (b), 768 Da (c), and 773 Da (d). Oversampling produces much more detailed and fine images (Fig. 8(II)) compared to the

FIG. 7. (Color online) Results of the "oversampling" experiment with the raster size of 50 microns conducted on the 50:50 mixture of bradykinin and angiotensin; (a) the target spot after the sample deposition; (b) spatial distribution of bradykinin at 904 Da; (c) spatial distribution of angiotensin at 1033 Da.

FIG. 8. (Color online) Results of the imaging experiments on the rat brain section (a) with raster sizes 300 (I) and 150 (II), monitoring spatial distribution of putative lipids at 827 Da (b), 768 Da (c), and 773 Da (d).

normal mode (Fig. 8(I)), but at the cost of decreased signal-to-noise ratio. This intrinsic trade-off is especially pronounced in Fig. 8(d)(II) where the compound has a broad distribution across the brain section, yet is not as sharply distinguishable as those in Figs. 8(b)(II) and 8(c)(II).

V. DISCUSSION

The primary design goal of this device is to maximize the positioning accuracy, however, minimizing the trajectory error of the device while performing MS imaging experiments is also imperative. “Pixels” must be close for better spatial resolution, but the greater deviation from the desired trajectory results in a longer settling time for the required positioning accuracy upon motion completion. This leads to an increase in the duty cycle of the experiment, which challenges large area MS imaging experiments.

Here, sub-micron trajectory accuracy was achieved with the compartmentalization of the control logic into the velocity and positional loops, as is described in the Design section.

Positioning accuracy and speed come with an intrinsic trade-off in the form of settling time. The demonstrated steady state error is $0.1\ \mu\text{m}$ and the amount of jitter does not exceed $0.2\ \mu\text{m}$, however, the time the stage requires to come to a complete rest upon arriving to its “final destination” is as much as the travel time. Hence, once the minimal amount of under/over-shoots is reached (by manipulating feed-forward, acceleration, and deceleration gains), the only way to decrease the settling time is to loosen the accuracy criteria.

The steady state error of $0.1\ \mu\text{m}$ is a very good achievement given the $.078\ \mu\text{m}$ resolution of the linear encoders. Reduction of the generally accepted margin of tenfold in respect to the sensor accuracy in PID controllers is possible mainly due to the design of the control logic. As was described previously, it is allowed for separate tuning of the proportional and derivative terms (as well as the implicit integral one) in different control loops.

Placing the motors in the central block of the stage (Fig. 1) makes the body of the device act as a heat sink as well as the shield for RF interference. This arrangement mod-

ified by use of a copper mounting bracket for the motors (Fig. 3(b)) proved to be effective against overheating during continuous operation of the motors (over several hours for imaging experiments). Additionally, the sub-detection level of RF interfering noise allows the stage to be moved even during the FT-ICR detection event. Thus, the only time the stage most definitely must be stationary is during the ionization event, leaving the rest of the FT-ICR MS duty cycle for movement to the next raster position; given the speed of $2\ \text{mm/s}$ and $.5\ \text{s}$ settling time, this does not add a significant overhead to any MALDI FT-ICR MS imaging experiment time. Moreover, the flexibility of scheduling the stage movement allows for introduction of internal calibrants,^{28,29} thus utilizing both high resolving power and mass accuracy of FT-ICR MS for MSI experiments.^{30,31}

Reported spatial resolution, although substantially improved, was by no means limited by the device positional accuracy. Reduction of the raster size in oversampling experiments (Figs. 7 and 8) inevitably leads to decreased signal-to-noise levels, since the decrease of a pixel diameter by a certain factor causes the area to decrease proportionally to the square of that factor. This decrease in sensitivity, however, can be diminished to a degree by manipulation of the laser power.¹⁷ Another fundamental limit on the resolution of the oversampling experiments is the “non-square” (Gaussian in most cases) shape of the laser power distribution within a beam which prevents obtaining sharp borders upon oblation. These and other issues, such as chemical noise and ion suppression, are especially exacerbated when imaging biological tissues, which are inherently complex and lead to the possibility of only fractionally improving spatial resolution, as shown in Fig. 8(II).

VI. CONCLUSIONS

The design goals of building a general purpose vacuum compatible positioning device for MALDI FT-ICR MS imaging have been met and its capabilities were demonstrated. The size, range of motion, ability to operate in vacuum for hours uninterrupted, undetectable levels of RF interference noise, speed, and positional accuracy makes this stage suitable for a wide range of applications ranging from automated offline MALDI MS analysis to the most detailed MALDI MS imaging experiments. Although $0.1\ \mu\text{m}$ positioning accuracy might seem somewhat excessive for the purposes, recent developments in laser optics within mass spectrometry community^{12–16} allows acquiring MALDI with submicron laser spot diameters. Clearly, for such small areas, laser power must be significantly increased causing excessive fragmentation, yet vibrational cooling^{32–38} should prove itself useful to reduce such fragmentation.

In regards to SIMS imaging, the positional accuracy provided by the stage is sufficient for C_{60} primary ion sources, but should be increased by the factor of ten at least for gold or cesium primary ion beams. The current design can be easily modified to these specifications through the use of different optical linear encoders, or by switching to shorter wavelength detecting photo-elements.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support from the National Institutes of Health (Grant Nos. NIH/NCRR P41RR10888, NIH/NHLBI N01HV28178, NIH/NIGMS R01GM078293), and the NSF-PIRE Grant Program (NSF OISE-730072) as well as “Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO),” E.U. Computis project, and the Netherlands Proteomics Centre. Furthermore, the authors thank Dr. Cheng Lin, Nadezda Sargaeva, Dr. David H Perlman, Dr. Chunxiang Yao, Xiaojuan Li, Xiang Yu, Dr. Vera Ivleva, Dr. Parminder Kaur, and Dr. Raman Mathur for help with experiments and useful discussions.

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