Cell & Systems Biology
UNIVERSITY OF TORONTO

April 5th, 2021

Dear Dr Myers,

We are hereby re-submitting our manuscript entitled "Control of cell geometry through laser assisted micropatterning" (manuscript number: JoVE62492) to JoVE. We appreciate the consideration of the paper and the reviewers' helpful suggestions and insightful criticisms. We are encouraged by the positive comments and have worked hard to address the major concerns in the revised manuscript. We truly believe that the changes inspired by the reviewers have made the manuscripts substantially better. In this letter, we provide a brief summary of the revisions made to the original manuscript as well as detailed point-by-point responses to the reviewers' comments.

As per the editor's comments and suggestions, we carefully proofread the manuscript to ensure a logical and comprehensible flow between statements. Additionally, the protocol was thoroughly revised to further parallel the spoken directions within the video, homogenizing the video and manuscript. Only a select few directions and notes were verbally omitted from the video as they are visually demonstrated. Furthermore, all sound edits and visual suggestions were successfully integrated.

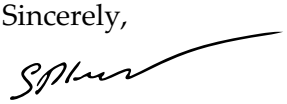
To address the reviewers' concern of a narrow range of imaging system that are compatible with our protocol, we included a note section with a detailed description of how the overall workflow described in the protocol can be reproduced on a multi-photon microscope from a different vendor. We also provided several lines of evidence to convince the reviewers that our protocol is fully compatible with any multi-photon imaging system controlled by NIS Elements and will retain the compatibility for the foreseeable future.

We revised the macros and Python scripts provided with this protocol to make them more interactive and user friendly. In accordance with the reviewers' suggestions, we revised the Python scripts to ensure their compatibility with various computer platforms and operating systems. The updated scripts work correctly on Windows 10 and MacOS.

Additionally, we refined the text of the manuscript according to the reviewers' suggestions. We revised the manuscript with the intention of avoiding any overinterpretations and misleading statements, which were present in the initial submission, and to clarify and unclutter the protocol. We hope we have achieved these goals. We also slightly expanded the Introduction section to provide a more comprehensive review of published micropatterning techniques.

We are immensely grateful for the reviewers and their comments, and we hope that our detailed point-by-point responses provided below alleviate reviewer's concerns and make the manuscript acceptable for publication in JoVE.

Sincerely,



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Detailed responses to the reviewer's comments and questions

Reviewer 1:

1.6 The link on proweb.org is dead.

We apologize for the dead link for the coverslip spinner description. The link in the revised manuscript was updated as follows (page 4):

> *"1.6 Spin dry coverslips for 30 s using a custom-built coverslip spinner. A detailed description of the coverslip spinner has been published{Inoué.Spring.1997} and is available online (https://mullinslab.ucsf.edu/home-built-coverslip-drierspinner/). Activated coverslips can be stored for up to one month at +4 °C in a box with dividers so that they stand apart from each other."*

4.1.1 use GIMP or ImageJ or Photoshop.

We agree with the reviewer that commercial image editors are not essential for generating the ROI masks. The following Note was included in the manuscript to elaborate on the available free options (page 7):

> *"4.4.1. NOTE: A number of commercial and free image editors can be used to generate ROI masks for laser assisted micropatterning. Although we use Adobe Photoshop to generate the masks, GIMP and ImageJ/Fiji are also available as alternative free option."*

5.3 why is the water needed? How is it possible to have water on an inverted microscope?

The requirement to add water or other immersion media is strictly determined by the objective used for micropatterning. We optimized the protocol for 25x/1.1NA water immersion objective, but dry and oil-immersion objectives with an appropriate immersion media can also be used for micropatterning. We stated this in the following Note on page 8:

> *"NOTE: The protocol described here is optimized for 25x/1.1 NA water immersion objective. If a dry or oil-immersion objective is used for micropatterning, water should be replaced with an appropriate immersion media."*

Water immersion objectives are routinely used on both upright and inverted imaging systems - surface tension of water is sufficient to hold it in place on the objective. But water evaporation can, in fact, be an issue for printing over a large coverslip area. Thus, we included the following statement in the Note on page 8:

> *"When water immersion objective is used to generate a large micropattern array, evaporation might become an issue. If this is the case, water should be replaced with GenTeal, an over-the-counter eye lubricant available from a pharmacy."*

6.3 why is the pluronic needed here?

In our hands Pluronic is not essential for coating the micropatterns with ECM proteins. It has been reported that Pluronic decreases non-specific protein absorption to the patterned surface, but even in the absence of pluronic we did not observe cell attachment outside patterned islands. Thus, we revised the results section of the manuscript as follows (page 10):

> *"6.3. Dilute fibronectin (FN) in PBS to a final concentration of 10 μg/mL. Incubate the coverslip in FN for 1 h at +37 °C.*

NOTE: *If substantial nonspecific binding of ECM protein to the substrate is observed, FN can be diluted in PBS containing 0.1% Pluronic F-127{Doyle.Doyle.2009}."*

7.2 should recommend EDTA/Versene instead of trypsin for easier reattachment.

As the reviewer suggested, we expanded the list of cell detachment techniques mentioned in Step 7.2. The following text was added on page 10:

"7.2 NOTE: For cells that adhere weakly to the substrate, non-proteolytic dissociation with versene (0.48 mM EDTA), or a proprietary enzyme-free buffer that may increase cell attachment to the patterns should be considered. "

9.2 Authors must not assume an Anaconda environment but must make the script runnable everywhere. See comments below

We appreciate the reviewer's suggestion to improve code implementation in other environments. In accordance with the suggestions, we have added a requirements.txt file listing the required packages and have added instructions. For the purpose of this protocol, we recommend an Anaconda environment because it contains most packages that are required for the codes, with only a small number of packages requiring additional installation. We have added the following Note on page 11 for clarification:

"NOTE: The .py scripts can be run in any environment with the appropriate packages identified in the requirements.txt file installed. We recommend Anaconda because it contains most of the required packages for the codes below."

In order to allow as many labs to use this technique, the protocol should also be available for MicroManager and MetaMorph softwares. This protocol is too specific to the particular microscope and software of the authors. An effort should be made to allow users with different setups to use it.

We agree with the reviewer that developing a micropatterning protocol optimized (or even compatible) with an open-source imaging software like MicroManager would be extremely useful for the cell biology community. Unfortunately, neither MicroManager nor Metamorph support any laser scanning microscopes (including multi-photon microscopes). To the best of our knowledge, every commercial laser scanning system from the Big Four microscope manufactures comes with a proprietary software and is not compatible with an open-source or competitor's software. Because of that, it is just not feasible to develop a universal, cross platform micropatterning protocol compatible with a range of commercial multi-photon systems. However, to address the reviewer's concern and expand an application of these protocol beyond Nikon A1R+ MP imaging system, we included a description of how the overall workflow described in the protocol can be reproduced on other imaging systems. The following text was added (page 14):

"Although the macros in our protocol enable automation of micropatterning on our system, we understand that every commercial laser scanning microscope comes with their own proprietary software that is rarely compatible with others, making it difficult to implement our exact protocol on other systems. However, the overall workflow can be well adapted to other commercial systems to facilitate automated micropatterning, namely the process of focusing on each individual FOV, loading the mask, ablating PVA, and moving the microscope stage to a new FOV."

Temperatures must have sign (+4°C instead of 4°C)

As the reviewer suggested, we added the sign to temperatures throughout the manuscript.

Repository is missing a LICENSE file along with a copyright stated in the files.

We apologize for missing the license file for provided scripts. The license was added to the repository.

The README.md is missing installation and usage instructions.

We appreciate reviewer's suggestion to provide installation and usage instructions in the Readme.md file. The file now includes an overview of the scientific question and technique, a description of the macros and .py scripts, an explanation of the workflow, and detailed explanations of how to use the codes. Due to the syntax of the README.md file, we do not quote the updated file here but refer the reviewer to the Github repository.

No requirements.txt nor Pipfile.lock are provided, making installing the required dependencies hard. Dependencies must be listed in such file and instructions must be given on how to make the code run in a virtual environment. Along with version lock.

As the reviewer suggested, we added a requirements.txt file that includes usage instructions and the version of all packages used.

Python code MUST use the pathlib.Path python library to work well on all operating systems instead of assuming Windows path separators.

As the reviewer suggested we revised the script. The updated script uses pathlib.Path library to construct filenames.

Stage_Movement file is missing an extension and contains hardcoded paths that might not work everywhere. Paths should be configurable at the beginning of the file. Same with Pattern_Simulation: remove the hardcoded path and make it clear that this needs to be adjusted. If possible, make the application ask the user to input a file path through a GUI interface.

As the reviewer suggested, we simplified the layout of macros by defining all hardcoded variable right at the beginning of the macro. We also clearly identified hardcoded variables and changed their names to make variables self-explanatory. The section of 'Stage_Movement.mac' where the hardcoded variables are defined was revised as follows (lines 5-13):

> *"//Hardcoded Variables*
> *//The provided macro creates a micoropatterned array of 5x5 fields of views located next to each //other. These values (PatternLength, PatternHeight, StepSize) are hardcoded and should be //adjusted if an array of different size is required. Two other input parameters required for the //macro are file path of a desired pattern ablation protocol and file path of a binary map of the //pattern. These parameters can be either hardcoded or provided through GUI (the default //option).*
>
> *int PatternLength = 5; //Length of the patterned array counted in fields of view*
> *int PatternHeight = 5; //Height of the patterned array counted in fields of view*
> *double StepSize = 532.48; //Microscope stage translocation between subsequent fields of view measured in micrometers"*

We agree with the reviewer, that some users of this protocol might prefer to control the micropatterning macro through a graphical interface. Thus, we revised the macros and added two dialog windows allowing user to select the photoablation protocol and pattern map. We found this feature to be very handy! We greatly appreciate reviewer's suggestion to make the macro interactive.

In python files, use MACRO_CASE (uppercase with underscore) for constants and make it clear which ones can/should be edited. This part should be before any actual code. Also remove any hardcoded paths (like line 33 of Pattern_Averaging_3Channels.py).

As the reviewer suggested, we have changed the layout of python files with clearer instruction on how to change variables. Hardcoded paths have been replaced with a file selection dialog box.

Add a space after comment sign, conform to PEP8 (https://www.python.org/dev/peps/pep-0008/#inline-comments). It's also more readable. A tool like "black" (https://github.com/psf/black) can help formatting the code correctly.

As the reviewer suggested, we have added a space after each comment sign and slightly adjusted the format of the code to improve readability.

Example pattern image should be provided in the repository.

We appreciate reviewer's suggestion to provide pattern masks that we previously tested. Two masks of different shape and size are now available in the repository.

Reviewer 2:

Photomicropatterning using PVA-coating and multi-photon microscopy was introduced in 2009 (J. Cell Biol., https://doi.org/10.1083/jcb.200810041, Current Protocols in Cell Biology, https://doi.org/10.1002/0471143030.cb1015s45). I found it misleading that the abstract did not mention that the current protocol is an improved version of an existing method. I would also find it appropriate to not give the technique a new name (original: "microphotopatterning" here: "laser assisted micropatterning").

We apologize for downplaying the role of seminal papers published by Dr Doyle in the current protocol. In the original submission, we have cited both papers mentioned by the reviewer, and we had no intention to neglect their contribution. In the revised manuscript we cited both papers in the abstract and highlighted their critical role in the development of our protocol. The Abstract was revised as follows:

> *"Micropatterning is an established technique in the cell biology community used to study connections between the morphology and function of cellular compartments while circumventing complications arising from natural cell-to-cell variations. To standardize cell shape, cells are either confined in 3D molds or controlled for adhesive geometry through adhesive islands. However, traditional micropatterning techniques based on photolithography and deep UV heavily depend on clean rooms or specialized equipment. Here we present an infrared laser assisted micropatterning technique (microphotopatterning) modified from Doyle et al. that can be conveniently set up with commercially available imaging systems. In this protocol, we use a Nikon A1R MP+ imaging system to generate micropatterns with micron precision through an infrared (IR) laser that ablates preset regions on poly-vinyl alcohol coated coverslips. We employ a custom script to enable automated pattern fabrication with high efficiency and accuracy in systems not equipped with a hardware autofocus. We show that this IR laser assisted micropatterning (microphotopatterning) protocol results in clear patterns to which cells attach exclusively and take on the desired shape. Furthermore, data from a large number of cells can be averaged due to the standardization of cell shape. Patterns generated with this protocol, combined with imaging and analysis, can be used for relatively high throughput screens to understand molecular players mediating the link between form and function."*

We understand the reviewer's concern with using a new name for the established technique, but we would like to keep this name as it is much more specific than "microphotopatterning" first introduced by Dr. Doyle. With the current development of new photopatterning techniques using various laser sources, we feel that microphotopatterning would not be specific enough a name for the presented technique. However, to avoid any confusion and recognize the contribution of the microphotopatterning technique to this protocol, we have used both the new and old name throughout the manuscript.

The main difficulty of this protocol is to set up the 2-photon microscope to do something 2-photon microscopes are not normally used to do. The authors provide a very detailed step-by-step protocol for their "Nikon A1R MP+" and Nikon's software package "NIS". Many of the steps describe the use of sub-menus/functions of NIS, i.e. they are very specific for this particular configuration and its version. Similarly, the necessary macros, which the authors provide, are written for NIS. Therefore, the protocol might be useful only for researchers with access to a very similar, if not identical system. Updated versions of NIS might also require changes to the protocol. In its current form, the compatibility of the protocol with 2-photon microscopes of other manufacturers is not discussed and unlikely to be easily transferable. The summary states "This user friendly technique can be set up with commercially available imaging systems". - Unless protocols for other commercial platforms are provided, it would seem suitable to me to mention in title and/or abstract that this protocol is for use with a Nikon A1R MP+ controlled NIS.

We agree with the reviewer that the provided protocol is tailored for a multiphoton microscope controlled by a proprietary Nikon software NIS Elements, but we want to draw the reviewer's attention to the fact that NIS Elements is probably the most open platform on the market. With no intention to advertise Nikon products and no financial interest in sales, we would like to highlight a wide range of third-party microscope components supported by NIS Elements (https://www.microscope.healthcare.nikon.com/products/software/nis-elements/compatibility). The macros we developed would run on a NIS Elements-controlled microscope equipped with a motorized stage from any major manufacture (Prior, ASI, or Ludl, *etc.*), a stepper-motor and/or piezo Z drive from Prior, ASI, Physik Instrumente, or Mad City Labs, an IR laser from Coherent or Spectra-Physics. These macros will literally require no modifications - the device control functions embedded in NIS Elements work with all supported hardware. Thus, we are confident that the described protocol is compatible with any multi-photon microscope controlled by NIS Elements.

While we were developing this protocol, we were also concerned with its compatibility with future versions of NIS Elements. We really like this micropatterning technique and we want to keep it in the lab toolbox and use for multiple projects. Because of that, we decided to minimize the use of device control functions in the macros and use Optical Configurations instead. If new device settings (*e.g.*, dwell time, scan size) are available, the users will be able to implement them, if needed, through a simple and user-friendly graphical interface rather than to modify a myriad of individual functions in the macros. We also put some effort to maximize the use of relative units in the protocol (*e.g.*, maximum zoom, fastest scan speed, smallest scan size), which we believe will make implementation of this protocol for future models of confocal scanners easier for the users. Finally, to expand an application of these protocol beyond Nikon A1R+ MP imaging system, we included a description of how the overall workflow described in the protocol can be reproduced on other imaging systems. The following text was added on page 14:

> *"Although the macros in our protocol enable automation of micropatterning on our system, we understand that every commercial laser scanning microscope comes with their own proprietary software that is rarely compatible with others, making it difficult to implement our exact*

protocol on other systems. However, the overall workflow can be well adapted to other commercial systems to facilitate automated micropatterning, namely the process of focusing on each individual FOV, loading the mask, ablating PVA, and moving the microscope stage to a new FOV."

Another concern regarding this technique is that maintenance of multiphoton systems in costly, resulting in imaging facilities charging significant hourly fees. Since a 3.5x3.5mm area takes about 3h of patterning time, not including set-up time, this might discourage widespread use of this protocol, unless a suitable multiphoton system can be easily accessed.

We agree with the reviewer that a multi-photon microscope is not the most affordable imaging equipment and facility fees for these instruments are usually quite substantial. Nevertheless, for many labs microphotopatterning is the only option to manipulate cell shape. From our experience, multi-photon microscopes are much more available for a generic cell biology lab than micropatterning devices. They also require less training and easier to operate for the lab staff. In the discussion (page 14), we explain that this technique can expand the pool of multiphoton users:

"In fact, as multiphoton microscopes are becoming a more common sight in Biology departments, microphotopatterning expands the applications of the multiphoton microscope and adds to the potential pool of users."

Speaking from experience, our lab has access to a photolithography facility, but we rarely make PDMS stamps for microcontact printing as microphotopatterning is more suited for our current research needs. Therefore, we decided to provide a fair comparison of the patterning methods available for cell biologists in the introduction section of the manuscript and allow the readers to choose the most appropriate technique based on their research, equipment availability, personal preferences, *etc*. In addition, we suggest in the manuscript that finetuning autofocus or decreasing the number of z-plane stimulations can significantly decrease the time required. To clarify, we added the following text on page 15:

"Since IR stimulation is the most time-consuming step, the addition of each stimulation event (~30 sec) significantly lengthens the patterning process. If time is of concern, we suggest fine tuning autofocus by decreasing step size. This facilitates the identification of the best focal plane which will decrease the number of IR stimulation events required. In our experiments, decreasing the number of stimulation events from five to two reduces the time by half (1.5 h)."

The abstract mentions that "traditional micropatterning techniques based on photolithography and deep UV require clean rooms or specialized equipment", and in the introduction it is stated that "…deep UV light sources are not readily accessible in Biology departments and require special training to handle." There are in fact small deep UV light sources available for deep UV-based micropatterning that are quite affordable and do not require any specialized training (such as the ones sold by "4Dcell").

We appreciate the reviewer's insight on this matter. We have softened the statement to include such information and pointed out the advantages of our protocol with respect to this method. The following text was added on page 2-3:

"This method avoids the use of cleanrooms and photolithography equipment and requires less specialized training. However, the requirement for photomasks still poses a substantial hurdle for experiments that require readily available changes in patterns."

The current protocol uses a 25x water dipping lens. What other lenses/configurations is the protocol compatible with (immersion type, magnification, etc.)?

We appreciate reviewer's suggestion to discuss the selection criteria for the microscope objective. In fact, we believe such Note in the protocol would be extremely helpful as many imaging facilities provide multi-photon microscopes equipped with one or two objectives only. Thus, we added the following Notes on page 4-5 and 8:

> *"3.1. Turn on the microscope software. Ensure that the "Apo LWD 25X/1.10W DIC N2" objective is mounted on the microscope.*
> ***NOTE:** The protocol described here is optimized for a 25x/1.1 NA water immersion objective, but other objectives can also be used for patterning. Readers should be aware that pattering with a high-magnification objective (e.g., 40x and 60x) takes longer time as it significantly decreases the number of patterns ablated in each FOV. Low magnification objectives can be used for patterning as long as they provide uniform illumination across the FOV and laser power sufficient to ablate the PVA layer."*

> *"5.4. Lower the objective and add water onto the coverslip.*
> ***NOTE:** The protocol described here is optimized for a 25x/1.1 NA water immersion objective. If a dry or oil immersion objective is used, water should be replaced with an appropriate immersion media. When a water immersion objective is used to generate a large micropattern array, evaporation might become an issue. If this is the case, water should be replaced with GenTeal, an over-the-counter eye lubricant available from pharmacies."*

Section 1.6: The link provided for a necessary instrument, a coverslip spinner box (Section 1.6), is broken.

We apologize for the dead link for the coverslip spinner description. The link in the revised manuscript was updated as follows (page 4):

> *"1.6 Spin dry coverslips for 30 s using a custom-built coverslip spinner. A detailed description of the coverslip spinner has been published{Inoué.Spring.1997} and is available online (https://mullinslab.ucsf.edu/home-built-coverslip-drierspinner/). Activated coverslips can be stored for up to one month at +4 °C in a box with dividers so that they stand apart from each other."*

Section 2 - PVA coating. Since the coverslips are immersed in solutions throughout their preparation process, are they in fact coated with PVA on both sides, but only the side facing away from the objective lens is being micropatterned? If yes, does the presence of PVA affect the optical properties of the coverslip? Can they subsequently be used on microscopes with oil-immersion lenses without damaging them?

We have never observed an ablation of the PVA layer on the surface facing the objective. We are confident that both surfaces of the coverslips are coated with PVA, but only one of them (the one we focus on) is patterned. We would expect an objective with extremely low NA to allow us pattering on both surfaces, but the output power of our laser is not sufficient to test it experimentally.

We routinely image cells attached to regular coverslips and PVA-coated patterns using dry, water- and oil-immersion objectives and we did not notice any difference in image quality that can be attributed to the PVA layer. We believe that thin layer of PVA (with a thickness of about 1/3 of the excitation wavelength for GFP) has very little impact on beam propagation through the coverslip. We also did not

notice any damage of the oil-immersion objective lenses – the chemical link between PVA and glass surface through APTMS and glutaraldehyde seems to be stable enough to sustain immersion oil. The following Note was added on the page 9 to clarify to the readers that PVA layer on the coverslips does not affect their optical properties and allows imaging with a wide range of objectives:

> *"Although both surfaces of the coverslip are coated with PVA, the optical properties are not significantly altered. We routinely image such coverslips with dry, water- and oil-immersion objectives and did not find the non-patterned PVA surface to interfere with imaging."*

The cover letter appears to belong to a different manuscript.

We apologize for submitting a wrong cover letter with the manuscript.