Please see below our point by point response to editorial and reviewers comments. We have colour coded the text in our revised manuscript to make it easier to see the modifications we have made.

Response to Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Spelling and grammatical errors have been corrected and are shown in red in the manuscript.

• **Protocol Language:** Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) For example: Step under section 1 needs to be re-written in imperative voice.

Step 1 has been re-written. We also removed commercial sounding language from this paragraph and now refer to the software we used to design the photomask in the table of materials.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

1) 2.1.3: mention % of acetone, isopropanol.

We added the percentage (100%)

2) 3.2: which cell line, which media?

We added a note at the beginning of this section to refer the reader to the table of material where media composition and details about the specific cell line used in this study can be found. Note also that we decided not to include details about mouse ESC culture procedures for 2 reasons:

- They are extensively described elsewhere as mentioned in the note (a reference is provided).
- We would like to emphasise that the method is adaptable to any cell line in principle.

3) 7.8, 7.9: unclear what is done. Mention all button clicks and software selections.

This section has been substantially extended to include sub-tasks in the PickCells software in order to achieve the desired results. We highlighted steps 7.8 and 7.10 as they should provide good examples of how to generate custom data types in the software.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE’s instructions for authors for

more clarification.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.
5) R scripting in section 8 is too vague to filming, please unhighlight.

We have carefully reviewed highlighted text and are confident that the chosen steps will form a coherent narrative.

Please note that due to format conversion issues between libre office and microsoft office, some numbers may appear in yellow, however we confirm that if a sentence is not fully highlighted in yellow, this means that we do not wish to include the step in the video.

With respect to point 5 of the comment above, we are unsure why section 8 would be too vague for filming. This step consists in running the R script from a graphic user interface in order to obtain the final result of the protocol. We believe this step should be easy to screen capture and would help users with no experience with R to reproduce our protocol.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have made small changes to the discussion as a result of reviewers comments. We believe the discussion now meets the above requirements.

• **Figures:**
1) Please reference figures in the order of listing. Fig 2 is currently called out before 1.

Thank you for pointing this mistake, the figure number has now been changed

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are LayoutEditor (https://layouteditor.com/), parafilm, ibidi, Pluronic F-127, PickCells
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We have replaced commercial sounding language as follows:
- LayoutEditor: replaced with "layout editor software"

- Parafilm : replaced with “laboratory film”
- Pluronic F-127: replaced with “Poloxamer 407”
- Ibidi: We removed mentions to the ibidi company and simply refer to the material as “hydrophobic plastic slide”
- PickCells: This software, like ImageJ, is not commercial but is our own open-source, public funded and free software. We have kept mentions of PickCells in our text and hope that this will be reasonable to the editors.

• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as cell lines, media, etc

We have added the full composition of mESC medium, a reference to the cell line used in this study and references to the layout editor softwares used to design the photomask.

Response to Peer-Reviewers:

Reviewer #1:

Manuscript Summary:
Understanding self-organisation of cells has gained attention in recent years due to its promise to reveal the inherent ability of cells to undergo patterning during development. In vitro techniques of culturing pluripotent cells have been crucial in this. Micro-patterns are particularly useful since they allow precise spatial control of the patterning by controlling the location where cells can adhere and grow through regionalised deposition of extra cellular matrix. In this paper, Wisniewski and colleagues describe their adaptation of micropatterning approach that bypasses the use of specialized equipment. They describe their approach of generating the design, creating photo-patterned chip, deposition of matrix, seeding of cells, imaging and the image analysis. The strength of the manuscript lies in (a) making the manufacturing of the chips accessible to a standard cell biology laboratory (b) developing an analysis pipeline PickCells that relies completely on free and open-source software (c) adapting the software to Linux, Windows and MacOS with standard RAM and disk storage requirements, the running of which does not require knowledge of command line or programming. Overall the manuscripts describes a potential technique to assess the influences of parameters such as physical cues, cell density, endogenous or exogenous signaling, etc on a given patterning process in a quantitative manner.

We thank this reviewer for taking the time to review our manuscript.

Major Concerns:
None

Minor Concerns:
It will be useful to have a summary figure that describes the overall pipeline in a pictorial manner.

We agree with this reviewer and so we have added a new figure (fig. 1) showing the overview of the method. A note at the beginning of the protocol now refers the reader to this new figure.

Reviewer #2:

Manuscript Summary:

This paper describes 2 useful methods for studying spatial organization of multicellular colonies of mammalian cells

The first part of the paper describes an adaptation of existing photo patterning methods to pattern tissue culture plastic. As mentioned by the authors, several micropatterning methods have been described before, the method proposed here could be useful to labs not equipped with microfabrication tools as it relies only on a UV lamp and no other special equipment

The second part of the paper describes a software suite to segment and analyze the spatial patterns of expression of markers of differentiation. It relies on using a nuclear envelope stain rather than the usual DNA stains. The methods gives really impressive results on dense 3D samples that are notoriously hard to segment.

Overall the protocols are sufficiently detailed and gives enough info for somebody to repeat them.

We thank this reviewer for taking the time to review our manuscript and for the positive comments.

Minor Concerns:

2.1 measure of the light output intensity of the UVO lamp would help somebody willing to repeat the experiment but not having the same lamp, or willing to build a home made one

Unfortunately we do not have a UV meter in our lab and so were unable to measure the light intensity of the UV lamp. However we have added a note at step 2.2.6 to specify the expected light intensity:
"The power of the light is estimated to be 6 mW/cm^2 at 254 nm of wavelength when the chip is placed at a distance of 2 cm from the source."

4.2 maybe mention to adapt fixation time to thickness of the sample.

We have added the following note: "If colonies appear particularly thick (more than 5 cell layers), it may be necessary to adjust fixation time to 20 min."

6.4 maybe gives an example of a imaging set up. 20x confocal spinning, 2000x2000 sCMOS camera.

We addd the following note to step 6.4:
Note: For example, in this study, we used an inverted scanning confocal microscope with a 40X objective (numerical aperture equal to 1.3), an image size of 1024 by 1024 without digital zoom and a z-step size of 0.5 µm. This gave us the following voxel size: 0.38×0.38×0.5 µm.

6.8 note. In case of stitching, illumination roll off correction might be necessary

The note has been modified to add this precision.

7 image analysis. I have downloaded the software and tried it on the provided sample stack (E875). And I followed the video tutorial provided on the nessys webpage. Using the provided classifier (E70) I was able to obtain a really impressive segmentation on such a dense 3D tissue sample. To my knowledge, It wouldn't be impossible to achieve similar results by using dapi stain segmentation.

I also tried to train my own classifier, but I couldn't obtain a segmentation this way and I haven't been able to understand what was done wrong in that case. I would recommend that parameters for the different steps of the process would be explained and that example of good and bad parameters choice be given for each step be given. This would ensure that the software can be used on other types of samples in the most straightforward way.

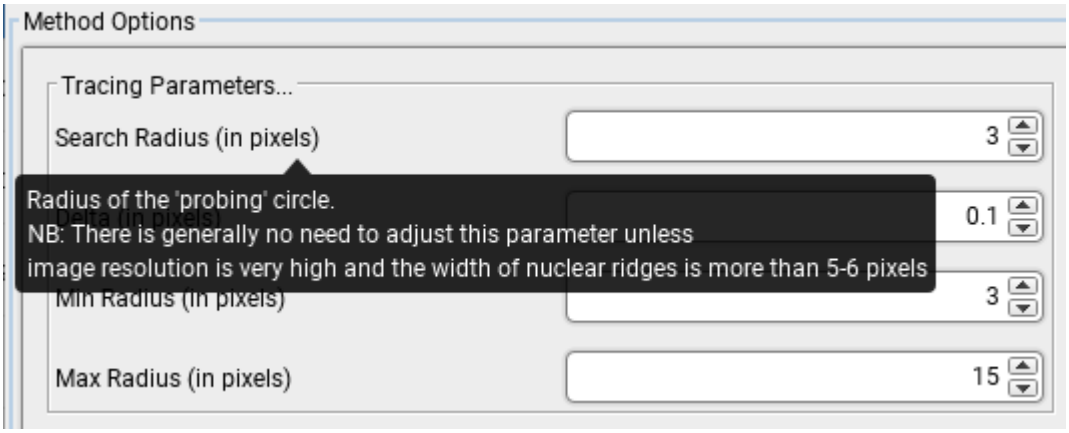
Specific points:
- Extra step "denoising" Not mentioned in the video tutorial. Tips should be given about how to choose the Gaussian filter parameters unclear if xyz radii are in microns or pixels
- Step "ridge enhancement" maybe give tips about how to evaluate the quality of the output. Image examples of when the scale is too high/too low
- Step "slice by slice segmentation" tracing parameters in pixels or in microns? This is important to help adapt the parameters to another data set

We thank this reviewer for the suggestions. The following improvements to the documentation have been made:

- We created a new page on our website to document individual parameters and to provide advice on parameter adjustments. This page also mentions the denoising step and gives an example of expected outputs from the ridge enhancement step.

  https://pickcellslab.frama.io/docs/use/features/segmentation/nessys/

- We improved the labels of parameters to add the unit that is expected (pixels)
- We have improved tooltips to better explain what each parameter does. An example is shown in the screenshot below:



Please note that we have decided to add these details on our website or in the user interface directly rather than in the text of the present manuscript because the software is subject to change. The documentation of the website is kept under the same version control as the code which helps us maintaining documentation in sync with changes to the software.

Discussion section:
I don't understand the following sentence: "We suggest to fix and permeabilise the cells in one step (step 4) as this can improve antibody penetration" As in step 4 fixation and permeabilisation seem to be done sequentially.

Fixation and permeabilisation are in fact performed in one step as the fixative solution contains Triton X-100. We thank this reviewer for pointing out that this was not clear from our protocol. We have added the following sentence to the “representative result” section right after the sentence that this reviewer mentions:

“We suggest to fix and permeabilise the cells in one step (step 4) as this can improve antibody penetration. This is the reason why our fixative solution contains a detergent.”

Reviewer #3:

Manuscript Summary:

The article by Blin et al. describes a method to generate micropatterns for confining stem cells to study developmental questions, as has been used in recent years by a number of groups. The authors describe a simple and inexpensive method of photo printing the patterns, which will help to make the method more accessible. They also provide downstream analysis software which is meant to facilitate quantitative analysis for users with little or no computational background.

The protocol is detailed and carefully written, and a welcome addition to the literature. The software provided is comprehensive and may be useful for researchers with no resources to develop their own analysis pipelines. I have a few comments, but overall believe that this will make timely contribution to an important topic.

We thank this reviewer for the constructive and positive comments.

Major Concerns:
None

Minor Concerns:

* There is a similar methods paper by Deglincerti et al in Nature Protocols, Nat Protoc. 2016 (11):2223-2232, using commercial micropatterns, which the authors should compare their approach with.

The reference is now cited and commented in a new paragraph of the discussion “Significance with respect to other methods”.

We have also included a reference to Ostblom et al. published in Plos Computational Biology during the revision of the present manuscript

* (3.4-3.6) I am a bit surprised at the 1h adherence time for the cells. I would assume significantly less time would be sufficient, and that the vigorous shaking of the plates wouldn't be necessary with a shorter coating time.

In our hands this protocol gives the best results we could obtain for our particular cell line and ECM coating procedure but we agree that adhesion time would need to be adapted for other cell lines/ECM combinations. We have added the following note at step 3.6:

“adhesion time may need to be optimised when using other cell lines than mouse ESC or other matrix proteins (see also Table 2)”

* (6.7) "CAUTION: All images, …" there is a "same" missing in front of "objective"

We thank the reviewer for pointing out this mistake, this has been corrected.

* Advantages and limitations/Future directions: The authors mention the FUCCI line, but don't comment on the prospect of imaging live reporters. It would be useful to discuss the possibilities and limitations of their system for live imaging.

We have expanded the last paragraph of the discussion to comment on the prospect of being able to quantify population dynamics with live reporter. In particular we emphasise that live tracking at single cell resolution is highly desirable if we wish to better understand how self-organisation emerges. We also mention that tracking is challenging but that recent developments from us and others are bringing us a step closer to this goal.

* As the authors recognize themselves, the software will need some improvement in the future to be more accessible to users. They have definitely taken the right steps (e.g. using Java to make it run easily on different platforms), but I wonder how many people are actually going to use it in its current form, e.g. with the additional exporting step to R (which, for the target audience of a researcher with less computational experience, can be a significant obstacle). The software is already quite advanced in its current state, so I would encourage even more development towards user-friendlyness in the future.

We agree with the reviewer that usability may be improved in the future, our main ideas to improve usability are:

- To enable our software to communicate with R directly so that the entire analysis workflow presented in this manuscript may be done from within the user interface.
- To by-pass the need for parameter adjustment for the nuclei segmentation step by using a few hand-drawn shapes for automated parameter adjustments.

We have created dedicated feature requests for these purposes, and hope to be able to address these in the near future.