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TITLE:

Mapping the Emergent Spatial Organization of Mammalian Cells using Micropatterns and Quantitative Imaging

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Patterning, micropattern, quantitative, stem cells, emergent behavior

SUMMARY:

The method presented here uses micropatterning together with quantitative imaging to reveal spatial organization within mammalian cultures. The technique is easy to establish in a standard cell biology laboratory and offers a tractable system to study patterning in vitro.

ABSTRACT:

A fundamental goal in biology is to understand how patterns emerge during development. Several groups have shown that patterning can be achieved in vitro when stem cells are spatially confined onto micropatterns, thus setting up experimental models which offer unique opportunities to identify, in vitro, the fundamental principles of biological organisation.

Here we describe our own implementation of the methodology. We adapted a photo-patterning technique to reduce the need for specialized equipment to make it easier to establish the method in a standard cell biology laboratory. We also developed a free, open-source and easy to install image analysis framework in order to precisely measure the preferential positioning of sub-populations of cells within colonies of standard shapes and sizes. This method makes it possible to reveal the existence of patterning events even in seemingly disorganized populations of cells. The technique provides quantitative insights and can be used to decouple influences of the environment (e.g., physical cues or endogenous signaling), on a given patterning process.

INTRODUCTION:

In mammalian systems, patterning is an emergent property of the collective behavior of cells and so, patterns can form in vitro if appropriate cues are provided to the cells¹⁻⁶. One way to reveal

the intrinsic ability of the cells to self-organize in vitro is to force the cells to form groups/colonies of a defined shapes and sizes^{7–10}. A technique which enables this is micropatterning¹¹. Micropatterning makes it possible to precisely define the location where extracellular matrix (ECM) molecules are deposited on a surface. This, in turn dictates where the cells can adhere and therefore controls how cells spatially organize.

Micropatterning is a technique with numerous applications, for example, micropatterning enables standardization of initial conditions prior to differentiation¹². Importantly, micropatterning makes it possible to easily control the size, shape and spacing of cell colonies and this property can be used to devise experiments aimed at interrogating the collective response of the cells to morphogen or to physical cues^{7, 8, 10, 13–17}.

Several micropatterning methods have been developed¹¹. Photopatterning techniques are perhaps the easiest methods to establish¹⁸. These approaches also have the advantage of precision as they can be used to control the shape of single cells^{18–20}. However, they also require expensive specialized equipment including a spin coater, a plasma chamber and a UVO (UV-Ozone) cleaner which are generally not readily available in standard biology laboratories. To facilitate adoption of the technique, we adapted the protocol to necessitate only the UVO lamp. We start from commercially available plastic slides which can be cut with scissors or with a hole punch to the desired format.

One important utility of micropatterns is the ability to standardize colonies in order to compare individual colonies across multiple replicates. This makes it possible to ask to what extent pattern formation within these colonies is reproducible, and to explore factors that influence the robustness of the patterning process. Importantly, quantification of “averaged” patterns across multiple standardized colonies can also reveal patterning processes that would not otherwise be apparent. The advantage of being able to quantify patterning on standardized colonies depends on being able to accurately measure protein expression, ideally at the single cell level. However, cells on micropatterns are often tightly packed, making them difficult to segment with high accuracy. Cells also often organize themselves in three rather than two dimensions, and it can be challenging to detect and preserve three-dimensional (3D) information during segmentation. Once cells have been successfully segmented, computational methods are needed for extracting patterning information from the resulting datasets.

We have developed segmentation and image analysis tools to help overcome these problems. This analysis method only uses free and open-source software and does not require knowledge of command line or programming to implement. To illustrate the method here, we use mouse embryonic stem (mES) cells which spontaneously express a marker of early differentiation brachyury (Tbra)^{21, 22}. While no apparent spatial arrangement is visually detectable, the method allows the creation of a map of the preferential positioning of T+ cells in colonies. We also show that Tbra patterning contrasts with the absence of a preferential localization of the cells expressing Id1, a direct readout of the bone morphogenetic protein (BMP) pathway²³. We also discuss the current limitations of the method and how this technique may be adapted to other experimental systems.

89
90 **PROTOCOL:**

91
92 NOTE: An overview of the method is provided in **Figure 1**.
93

94 **1. Mask design**

95
96 1.1. Design the photomask according to the guidelines described in Azioune et al.¹⁸. See the **Table**
97 **of Materials** for a reference to the software and mask manufacturer used for this study.
98

99 NOTE: Multiple geometries, sizes or spacing between shapes can be created on one mask. The
100 UV lamp can fit a 15 cm photomask which may contain up to 49 different designs (assuming 2 cm
101 x 2 cm chips).
102

103 **2. Micropattern manufacturing procedure**

104
105 2.1. Prepare the necessary materials.
106

107 2.1.1. Prepare a solution of 0.1% poloxamer 407 (10 mg for 10 mL) in phosphate buffered saline
108 (PBS) and leave on a shaker at room temperature. The poloxamer 407 will take about 20 min to
109 dissolve.
110

111 2.1.2. Lay laboratory film (see the **Table of Materials**) in the bottom of a 10 cm square Petri dish.
112 This will be used as the chamber for matrix deposition.
113

114 2.1.3. Clean the surface of the photomask, first with 100% acetone, then with 100% isopropanol
115 and finally with ddH₂O. If possible, air dry the photomask or otherwise dry the mask with clean
116 paper towel.
117

118 2.1.4. Prepare a square, rigid and opaque piece of plastic with the exact same size as the
119 photomask (later on referred as 'holder').
120

121 NOTE: This will be used to maintain plastic coverslips in contact with the photomask during the
122 illumination step.
123

124 2.1.5. Turn the UVO lamp on and run a warming illumination for 10 min.
125

126 2.2. Create photopatterned chips.
127

128 NOTE: It is possible to adapt the procedure to create chips of any desired size. For simplicity, we
129 describe here the procedure to generate a 12 mm round micropatterned chip.
130

131 2.2.1. Using a 12 mm hole punch, cut hydrophobic plastic slides to create 12 mm round coverslips
132 and place them in a clean new Petri dish.

CAUTION: Use gloves at all time to avoid skin contact with the surface of the plastic as this may damage the surface treatment.

2.2.2. Carefully remove the protective film from the coverslips with tweezers.

NOTE: Avoid damaging the plastic surface as this may influence the placement of the cells on the chip during the seeding procedure (section 3).

2.2.3. Place the photomask on a clean and stable surface (e.g., photomask box), chrome side facing up, and add a 2 μ L drop of ddH₂O at the position of the desired chip design.

2.2.4. Lay a coverslip onto the drop of ddH₂O and press gently.

NOTE: Ensure that the plastic side which faces the photomask is the side which was protected by the film that was removed in the earlier step.

2.2.5. Place the holder on top of the plastic slides and carefully fix this sandwich with clamps in order to maintain the plastic pieces in contact with the photomask.

NOTE: Place the clamps as close as possible to the location of the plastic slides in order to ensure that the plastic slides are perfectly maintained in contact with the surface of the photomask.

2.2.6. Place the assembly in the UVO lamp at approximately 2 cm from the light source and illuminate for 10 min.

NOTE: The power of the light is estimated to be 6 mW/cm² at 254 nm of wavelength when the chip is placed at a distance of 2 cm from the source.

2.2.7. Hold the sandwich with the photomask at the bottom and carefully remove the clamps while maintaining pressure with one hand to prevent the slides from moving about while disassembling the sandwich. Remove the holder, ensuring that all plastic pieces are still on the mask and not stuck to the holder.

2.2.8. Add ddH₂O on top of the chips and gently detach the chips from the photomask.

NOTE: If the plastic chip is stuck to the photomask, detach the chip using a plastic pipette tip to push the chip while holding tweezers slightly above the chip in case the chip detaches suddenly.

2.2.9. Finally, place the photopatterned chips within the matrix deposition chamber.

NOTE: Ensure that the illuminated side of the chip is facing upwards.

2.3. Deposit the matrix.

NOTE: All procedure in this section should be performed in a tissue culture hood.

2.3.1. Filter the poloxamer 407 solution through a 0.22 μm polyethersulfone (PES) filter.

2.3.2. Prepare the ECM coating solution by mixing 500 $\mu\text{g}/\text{mL}$ of sterile filtered poloxamer 407 and 1 mg/mL of gelatin.

NOTE: See also **Table 1** for additional information regarding other possible ECM molecules.

2.3.3. Add 200 μL of the coating solution onto each illuminated chip. The laboratory film will prevent the drop from falling outside of the chip.

2.3.4. Add a 3 cm Petri dish filled with ddH₂O in order to limit evaporation and place it with the chip at 4 °C overnight.

3. Seeding procedure

NOTE: The steps described below have been optimized for CGR8 mouse embryonic stem cells (mESC)²⁴ using standard mESC medium (see also the **Table of Materials**). However, it is possible in principle to adapt the procedure for any cell type. Note also that conventional cell culture of mouse embryonic stem cells is not described here as extensive documentation can be found elsewhere²⁵.

3.1. Aspirate the coating solution and incubate the chips twice for at least 5 min with sterile PBS.

3.2. In the meantime, prepare a cell suspension of 5.5×10^5 cells/mL in warm medium.

3.3. Pipet 200 μL of cell suspension onto each chip ($\sim 100,000$ cells/cm²).

3.4. Close the seeding chamber and leave the cells to adhere for 1 h in the incubator.

3.5. After 1 h, fill the wells of a multiwell plate (4-well or 24-well plate depending on the number of chips) with 500 $\mu\text{L}/\text{well}$ of warm medium and transfer the chips into the plate with sterile tweezers.

3.6. Shake the plate vigorously in order to detach non-adherent cells. Aspirate the medium and immediately replace with fresh warm medium. Check under the microscope to see if patterning is visible (**Figure 2a**).

NOTE: Adhesion time may need to be optimized when using cell lines other than mESC or other matrix proteins (see also **Table 2**).

3.7. Repeat this step until patterns become clearly visible as shown in **Figure 2a**.

NOTE: This step is **critical** and determines the success of the procedure. A washing procedure which is too intense may detach the cells, in contrast insufficient washing may result in cells remaining attached in between the patterns (**Figure 2c,d**).

4. Fixation

NOTE: After 48 h in culture the cells should form dense colonies which rigorously follow the shape of the patterns (as shown in **Figure 2b**).

4.1. Leaving the chips in the plate, remove ~90% of the medium, leaving just enough medium to prevent the chips from drying.

NOTE: It is important that the chip never dries to avoid staining artefacts and to prevent cell detachment from the surface. Due to the hydrophobicity of the chip surface between adhesive patterns, the chip may have a tendency to de-wet. At this stage, the fixative may cause large domed colonies to detach from the chip. Washes should be very gentle, ideally performed by pipetting liquid on the side of the well and not directly onto the chip.

4.2. Add at least 500 μ L of paraformaldehyde (PFA)-based fixation solution per well and incubate for 10 min.

NOTE: If colonies appear particularly thick (more than 5 cell layers), it may be necessary to adjust fixation time to 20 min.

4.3. After fixation, wash 3 times with the washing solution (PBS with 0.01% poloxamer 407). An extra wash using 50 mM NH_4Cl diluted in washing solution may be intercalated to quench residual PFA cross-linking activity.

4.4. Incubate the samples for at least 30 min in blocking solution.

NOTE: At this stage, samples may be stored at 4 $^{\circ}\text{C}$ for about a week prior to staining. If so, seal the plate with laboratory film to prevent evaporation.

5. Immunostaining

5.1. Prepare a staining chamber by placing a sheet of laboratory film in the bottom of a 10 cm square Petri dish.

5.2. Prepare antibody solutions (See **Table 3** for a list of antibodies and dilutions used in this article).

5.3. Place the chip into the staining chamber with the side supporting the cells facing upwards and immediately add 100 μ L of primary antibody solution onto the chip.

CAUTION: At this stage, chips should not easily de-wet as in step 4.1. However, care should still be taken because it is important that the chips do not dry. If multiple chips have to be processed, apply step 5.3 to each chip sequentially.

5.4. Incubate for 1 h on a rotating platform at room temperature.

NOTE: If the cells have formed large 3D structures, a longer incubation time may be required to allow for uniform staining of the sample. Incubation time may be increased up to 24 h. However, the staining chamber must contain a 3 cm dish filled with water and the staining chamber must be sealed with laboratory film to prevent evaporation.

5.5. Transfer the chips into a fresh multiwell plate and wash 3 times with the washing solution.

5.6. Perform incubation with secondary antibodies as described in step 5.3 and 5.4.

5.7. Mount the chip on a microscopy slide using 20 μL of any standard mounting medium (e.g., Mowiol).

6. Imaging

NOTE: Imaging can be performed on a standard confocal microscope. Here we only provide recommendations to ensure an image quality which will be sufficient for the subsequent quantitative analysis.

CAUTION: To avoid any operator bias, the colonies to image should only be chosen using the nuclear envelope signal (to see if a colony properly follows the shape of the pattern). Avoid checking the signal of markers of interest except when adjusting microscope settings.

6.1. Ensure that the acquisition bit depth is 12 or 16 bits.

6.2. Identify the appropriate settings to maximize the dynamic range for each image channel. In particular, avoid image clipping.

6.3. Include a channel to image the micropattern autofluorescence (see **Figure 3**).

6.4. Adjust Image size and zoom factor to obtain voxel sizes ranging between 0.1 and 0.6 μm in the x- and y-axes and ranging between 0.2 and 2 μm in the z-axis.

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 x 1024 pixels without digital zoom and a z-step size of 0.5 μm . This resulted in a voxel size of 0.38 μm x 0.38 μm x 0.5 μm .

6.5. For each colony, define the minimum and maximum position along the z-axis to ensure that

the entire colony is acquired. At least one plane with low to no signal should be included below and above the colony.

6.6. Ensure that z-stack orientations are acquired consistently (either always top to bottom or always bottom to top)

6.7. Adjust the scanning speed, image resolution, frame averaging, and detector gains to identify an optimum between image quality and imaging time. As an indication, imaging time for one colony as shown in **Figure 3** was approximately 2–3 min. Perform the image acquisition.

CAUTION: All images, in order to be comparable, must be acquired on the same microscope with the same objective and acquisition settings.

6.8. At the end of the acquisition, save all the images and assign a unique naming convention to identify the experimental condition that each image represents.

NOTE: See **Figure 4** as an example, this convention will be used later during the analysis procedure. Note that images may be saved to any format that is supported by BioFormats²⁶. If colonies are larger than the field of view, the stitching plugin of ImageJ may be used²⁷. Note also that in case of stitching, illumination roll off correction might be necessary.

7. Image analysis

NOTE: The recommended computer specifications for this procedure is: 16 GB RAM, a multi-core 3.33 GHz CPU, and at least 50 GB of disk space (or more depending on the number of chips that have been imaged). The software has been tested on Linux, Windows and MacOS. PickCells is a cross-platform image analysis application with a graphic user interface dedicated to the analysis of the collective organization of the cells in complex multidimensional images (Blin et al, in preparation). Note that more information on PickCells as well as documentation for the specific modules mentioned here can be found online: <https://pickcellslab.frama.io/docs/>. Note also that the interface is subject to change as we keep improving the software. If the interface differs from what is shown in the figure or video, please refer to the online manual.

7.1. Install and run PickCells following the documentation available online.

7.2. Import images and verify the accuracy of the provided information (**Figure 4-1**).

7.3. Document the name of each channel.

7.4. Segment nuclei based on the nuclear envelope signal using the Nessys module²⁸ (**Figure 4-2**) and provide a prefix (“nuclei” for example) which will be used to name the generated segmented images.

NOTE: Documentations about usage and parameter adjustments can be found at

<https://framagit.org/pickcellslab/nessys>.

7.5. Inspect and edit segmentations if necessary, using the segmentation editor module (**Figure 4-3**)

NOTE: If for any reason the segmentation process did not provide satisfactory results, manually delete images in the database folder and also delete the 'segmentation result' node in the **MetaModel** View. Then, repeat step 7.4 and 7.5. If the segmentation of only a small subset of images did not provide satisfactory results, then use the Nessys standalone application (see the link in 7.4), attempt segmentation on the 'faulty images' and replace the corresponding file in the database folder).

7.6. Segment the pattern autofluorescence signal using the basic segmentation module (Figure 4-4)

7.6.1. Provide a prefix ("pattern" for example) which will be used to name the generated segmented images.

7.6.2. Select the channel containing the autofluorescence signal.

7.6.3. Apply noise reduction; generally using a gaussian filter with a kernel size of 10 x 10 x 0.5 voxels gives satisfactory results.

7.6.4. Set the lower threshold so that the background appears in blue while the foreground appears white. Set also the upper threshold to its maximum value to avoid high intensities being excluded from the final result (red areas).

7.6.5. Select **skip** for the last step.

7.6.6. Click **finish and wait till all images are processed.**

7.7. As for nuclei, segmentation results can now be visually inspected and corrected if necessary using the segmentation editor module (**Figure 4-5**).

7.8. Create nuclei objects and compute basic object features.

7.8.1. Launch the **intrinsic features module from the task bar on the left of the main interface (Figure 4-6).**

7.8.2. Close the **Ellipsoid Fitter and **Surface Extractor** panels to keep only the **Basic Features** panel open.**

7.8.3. Choose **Nucleus as object type and choose the prefix given in step 7.4 for "segmented images".**

7.8.4. Press **Compute** and wait till all the images have been processed.

NOTE: After this step, it will not be possible to edit the nuclei segmentations again.

7.9. Create pattern objects and compute basic object features. Repeat steps 7.8.1 to 7.8.4, only this time choose **Custom Type** as object type and the prefix given in step 7.6.1 for **segmented images**.

NOTE: After this step, it will not be possible to edit the pattern segmentations again.

7.10. Store the name of the image each nucleus belongs to as a nucleus attribute.

7.10.1. Click on **Data > New Attribute** and select **Nucleus** in the popup dialog and click **Ok**.

7.10.2. Select **Collect data from other objects connected to the node** and click **Next**.

7.10.3. In the left panel, select **Image** and then double click on the interrogation mark under the **Finish** flag in the **Path definition** panel to set the image node as target of the path.

7.10.4. Expand the **Available Attributes** pane on the left panel and select the **name** attribute.

7.10.5. Expand the **Reduction operation** pane and select **Get One**, then click on the **change** button and click **Next**.

7.10.6. Type "Image Name", press the tab key and click **OK**.

7.11. Create a "normalised coordinate" attribute in nuclei objects (**Figure 4-7**).

7.11.1. Adapt steps 7.10.1 to 7.10.6 to store the coordinates of the pattern centroid as a nucleus attribute. Name this new attribute "Pattern Coordinate".

7.11.2. Then, click on **Data > New Attribute**, select **Nucleus** and click **Ok**.

7.11.3. Select **Define a function between spatial or directional vectors of the node** and click **Next**.

7.11.4. For **Type of function** select **Array Operation**, for V1 select **Item Vector** and then **Centroid**, and for V2 select **Item Vector** and then **Pattern Coordinate**.

7.11.5. Click **Next**, Type "Normalised Coordinate" in the **Name** field and click **Finish**.

7.12. Export the data to a tab separated value file.

8. R analysis

8.1. Download and install Rstudio.

NOTE: Software information and download links are available at <https://www.rstudio.com/>.

8.2. Download the R scripts required for this analysis.

NOTE: Scripts can be downloaded from the GitLab repository: <https://framagit.org/pickcellslab/hexmapr>.

8.3. Open Rstudio.

NOTE: If running the scripts for the first time, install the required R packages (ggplot2 and scales).

8.4. From Rstudio, open the `binnedmap_template.R` script.

8.5. Set the working directory to the source file location.

8.6. Follow instructions provided in the script to adapt the script to any given dataset in order to obtain spatial maps as shown in **Figure 5**.

8.7. Run the script to generate density maps.

REPRESENTATIVE RESULTS:

The photo-patterning method described here makes it possible to precisely organize cultured cells into colonies of defined shapes and sizes. The success of this procedure should be clearly apparent immediately after the cell seeding procedure (step 3.7) as adhering cells will cluster according to the photomask design as shown in **Figure 2a**. At 1 h after cell seeding, individual patterns may not be fully confluent (only a few cells per pattern), however, as the cells proliferate over time, patterns will become fully colonized with only very few cells outside the adhesive surfaces (**Figure 2b**). The exact appearance of the culture will be cell line dependent. For example, mESC form domed-shape colonies¹⁰. A chip where patterning is not clear 1 to 2 h after cell seeding indicates failure of the procedure (**Figure 2c,d**).

Large and thick colonies can sometimes be challenging to stain homogeneously. We suggest to fix and permeabilize the cells in one step (section 4) as this can improve antibody penetration²⁹. This is the reason why the chosen fixative solution contains a detergent. **Figure 3** shows the fluorescence signal which is expected after immunostaining. Notice that bright Id1 positive cells are found within dense regions (bright NPC regions) of the colonies (**Figure 3A**). Hints such as this are useful to assess the quality of the antibody staining procedure. Notice also that the micropatterns created with the present technique are autofluorescent. This signal (**Figure 3A,B** left most images) is useful during the analysis stage to spatially register colonies with one another

and create the results shown in **Figure 5**. The autofluorescence signal is generally the brightest when the sample is excited with a 405 nm laser and this channel should be left without staining for this purpose. **Figure 3** also shows how the cells are precisely constrained on patterns of different shapes.

The analysis of imaging data is performed in PickCells, a free and open-source software developed in our lab (Blin et al., in preparation). This software includes the image analysis modules to read and sort confocal images (**Figure 4-1**), to segment (**Figure 4-2,4-4**) and curate segmented objects (**Figure 4-3,4-5**), to compute object features such as coordinates or average intensity (**Figure 4-6**) and to export the data (**Figure 4-7,4-8**). Importantly, we developed a robust nuclear segmentation method called Nessys²⁸ which is particularly suited for dense and heterogeneous populations of cells such as cells grown on micropatterns (**Figure 3**). **Figure 4-2** shows a representative output of the Nessys module where each individual cell is accurately given a unique color identity. Only minimal editing should be necessary, however editing is feasible should the user decide so (**Figure 4-3**). Finally PickCells provides a number of visualization modules to visualize the data. An example is given in **Figure 4-6**: a ring-shaped colony is rendered in 3D where nuclei are color-coded according to their position along the z-axis. Once the analysis is validated in PickCells, data can be exported to create the spatial maps in R using the scripts available at (<https://framagit.org/pickcellslab/hexmapr>) as shown in **Figure 5**³⁰.

We have shown recently that spatial confinement of mESC on small (30,000 μm^2) disc or ellipse micropatterns guides the patterning of a subpopulation of cells expressing the mesodermal marker Tbra¹⁰. Thus, to illustrate our method here, we ask if patterning of Tbra may be influenced by BMP signaling in larger colonies (90,000 μm^2). **Figure 5A** shows that when mESC are grown on large disc micropatterns, Tbra+ cells are preferentially restricted to the periphery of the pattern (Tbra+ density map), where the local cell density is the lowest (see the blue map on the left of **Figure 5A**). This patterning of Tbra is confirmed by the map of mean Tbra intensity.

These data demonstrate that the method can reveal sub-visual information. Indeed, from **Figure 3**, visual inspection of one colony is not sufficient to identify any form of spatial organization in Tbra expression. This is notably explained by the important colony to colony variability which is quantified and shown in the right-most panel of **Figure 5A**.

The technique also shows that no detectable patterning exists for Id1 (a target of BMP signaling) which may indicate that T patterning is not driven by BMP signaling in this context.

Micropatterning makes it possible to force colonies to adopt almost any desired geometry. This is particularly useful to interrogate how the system responds to various geometries. For example, we may reason that if a morphogen gradient builds up at the center of the colony, creating a hole in the colony would disrupt this gradient. Interestingly we still observe patterning on a ring micropattern albeit in a less robust manner (**Figure 5B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the method. The diagram showing the main steps of the method. For each step, the estimated amount of time is indicated under the name of the task and a schematic illustrates the purpose of the procedure. A reference to a relevant figure is also provided when available.

Figure 2: Culture appearance 1 h and 48 h after seeding the cells on micropatterns. Brightfield images of mESC seeded onto micropatterns. (a) Expected cell organization 1 h after seeding, patterns should be clearly identifiable. (b) Expected result after 48 h of cultures. mESC have proliferated and are still strictly confined to the pattern shapes. (c–d) Possible non-optimal outcomes, either very few cells adhere to the plastic except at the periphery of the slide (c) or cells adhere in between the patterns (d). See **Table 2** for a troubleshooting guide.

Figure 3: Representative confocal images of immunostained colonies grown on micropatterns. Representative mESC colonies after immunofluorescence for (A) Id1 and Nuclear Pore Complex or (B) Tbra and LaminB1. For each staining, a colony grown on a disc micropattern and a colony grown on a ring micropattern is shown. Individual channels are provided as gray scale images. Notice the clear auto-fluorescence signal of the micropattern (405 nm laser excitation). Scale bar represents 50 μm .

Figure 4: Flow chart of the image analysis procedure. A list of 3D confocal images is imported into PickCells for analysis (1). This example shows an experiment with two distinct shapes (discs and rings as in **Figure 2**). The image naming convention is shown on the left and PickCells interface on the right. Then, the Nessys module is used to automatically segment nuclei (2). In the screenshot, each individual nucleus is given a unique color indicating accurate segmentation. The autofluorescence of the pattern is also segmented, this time, using the ‘basic segmentation’ module (4). Background appears in blue and white signal will be defined as the pattern shape. Segmented shapes are then visually inspected to ensure accurate segmentation, and edited if required using the segmentation editor module (3–5). The screenshots show the outline of the detected shapes. The pink and yellow shapes have been edited. Finally, objects features are computed and exported to file to be later processed into R (6–7). A screenshot of a colony rendered as a 3D view is provided (6). For steps 2 to 6 the icons found in the PickCells interface at the time of writing this article are given next to the step index.

Figure 5: Representative results for two different transcription factors and micropattern shapes Binned spatial map for mESC grown for 48 h on (A) disc shaped micropatterns or (B) ring shaped micropatterns. For each micropattern shape, the map of cell density, irrespective of cell phenotype, is shown on the left with a blue color scale. Then for each marker (Tbra on the top row and Id1 on the bottom row), three distinct maps are provided, from left to right: cell density map of marker expressing cells only (threshold based analysis), the map of the average marker intensity (\log_2) and the map of the standard deviation of the marker intensity. Intensities are given as arbitrary fluorescence units. For each map, the micropattern shape is given as a white outline.

Table 1: Tested concentrations of poloxamer 407 and ECM. This table provides an overview of

the concentrations of ECM and poloxamer 407 that we tested in our lab. For each ECM/poloxamer 407 combination, the cell type for which patterning was successfully achieved is shown as well as whether the culture contained serum or not. mESC = mouse embryonic stem cell, mEpiSC = mouse epiblast stem cell.

Table 2: Troubleshooting guide. This table provides an overview of possible sub-optimal outcomes. The potential sources of the problems are also listed together with recommended solutions.

DISCUSSION:

Here we describe a method for analyzing emergent patterning in cultures of cells. A simplified micropatterning approach is used for standardizing the shape and size of cell colonies, and we present image analysis tools and R scripts that enable the detection and quantification of patterns within these colonies.

The pipeline we propose is similar to some extent with a previously published method³¹ where the authors focus on the culture conditions, using commercially available micropatterns, to obtain reproducible germ layer formation in ESC colonies for the study of early gastrulation events in vitro. Our aim is more focused towards providing a generalizable pipeline for the discovery of pattern formation in vitro where the collective organization of the cells may only become apparent after statistical analysis. For this reason, we provide a robust image analysis workflow which enables the accurate identification and analysis of nuclear position in 3D space over multiple colonies (see also the ‘Advantage and limitations of the pattern detection method’ section of this discussion). We also decided to develop a simple in-house micropatterning approach which offers a more flexible and cheaper alternative to commercially available solutions in the long term which we hope will be useful to the community.

Finally, we note that during the revision of this manuscript, a new package for the analysis of patterning in vitro similar to our R scripts has been released³². This new package accepts tables of cell features as input which can be obtained from high throughput imaging platforms. We believe that the table of nuclei features generated at step 7 of our protocol could in principle serve as an input to this new package although we have not tested this possibility ourselves.

Adaptability of the method to other cell types and colony geometries

We present this approach in the context of studying emergence of mesodermal transcription factors in cultures of pluripotent cells in the presence of serum. However, the method is readily adaptable to other cell types and to serum-free cultures although it may be necessary to optimize ECM/poloxamer 407 concentrations (see **Table 1** for tested concentrations and **Table 2** for a troubleshooting guide). The method can also be adapted to larger or smaller sizes of micropatterns and to a wide range of shapes according to the needs of the user. However, while establishing the method, it is important to be aware that not all shape/cell type combinations are optimal. For example, mESC express high levels of E-cadherin^{33, 34} enabling these cells to form collective structures spanning areas that are devoid of ECM. These cells do not strictly follow

geometries with sharp angles or that include small holes in the pattern. Notice for example that on the ring of **Figure 3B**, the cells are in the process of colonizing the central area. In our hands a smaller central area did not force mESC to form ring-shaped colonies. It is therefore highly recommended to include a diversity of geometries while designing the photomask to be able to test and identify the optimal sizes and curvatures which will be suited for the cell type of choice.

Another important factor to take into consideration is the length of the experiment and the proliferation rate of the cells. For some rapidly proliferating cell types (including pluripotent cells) it can be difficult to maintain cells on micropatterns over many days (For mESC three days is a maximum). Also, seeding of cells on micropatterns does not always occur optimally for every colony, so it is advisable to seed an excess of colonies in order to have spares.

Advantage and limitations of the pattern detection method

One particular advantage of the method is the ability to detect “averaged” patterns by combining image analysis results from multiple replicate colonies (**Figure 5**). This can reveal patterning events that are not apparent from inspection of individual colonies. A disadvantage of this ‘averaging’ approach is that it may miss certain types of repetitive patterns, for example small spots or narrow stripes. However, these types of pattern may instead be revealed with a combination of carefully chosen pattern sizes⁸. Also, the image analysis pipeline described here provides quantitative data at both single cell and colony resolution offering the possibility to investigate the level of inter-colony variability (**Figure 5**) or to perform neighbor analysis at multiple scales¹⁰.

Another important advantage of the averaging method is that it offers the opportunity to map the preferential location of many markers without being limited by available fluorophores of detection channels. Indeed, although we make use of only two markers of differentiation in the work presented here, the ability to standardize colonies and extract “averaged” patterns makes it possible to compare the distribution maps from different sets of colonies together in order to reveal the generalized spatial relationships of markers to each other.

Furthermore, although our focus here has been to study markers of differentiation, the analysis method can be extended to study other biological processes for which nuclear markers are available. For example micropatterning of a cell line containing a fluorescence ubiquitination cell cycle indicator³⁵ (FUCCI) would make it possible to study how colony level geometry may influence cell cycle events in the group.

Future directions

The method is amenable to medium throughput image analysis, however, image acquisition is currently not fully automated and can become limiting for very large experiments. The regular arrangements of colonies should make it possible to create fully automatized acquisition routines similar to what has been developed for single cell averaging²⁰. However, because the size of the field required to image a colony is large, possibly requiring mosaicking, and because colonies are

three-dimensional, it is highly desirable to reduce both dataset size and acquisition time by imaging only relevant colonies. Therefore, future efforts may be dedicated to develop an 'intelligent' microscope able to identify relevant colonies and adapt imaging coordinates to each sample. This will not only reduce time and effort but also prevent potential operator biases.

The analysis pipelines may also be made more efficient by reducing the number of steps the user needs to take. We have plans to build a pipeline-building mechanism and to integrate R directly into our software (see also issues pickcells-api#3 and pickcells-rjava#1 in the issue tracker of our code repositories [<https://framagit.org/groups/pickcellslab/-/issues>]). The full automation of the analysis procedure will reduce time and effort and limit potential user errors.

Finally, we note that our analysis method does not yet fully capture the dynamic nature of cellular patterning. Some limited dynamic information can be extracted by examining a time-series of snapshot images^{8, 10, 36}. However, being able to record the history of the cell population is highly desirable if we wish to better understand how patterning emerges. One limitation is that accurate tracking of individual cells in a 3D dense cell population remains a very challenging task³⁷. Our cell detection method uses the nuclear envelope and performs particularly well on dense and overlapping cell populations²⁸. Live reporters of the nuclear envelope are readily available^{28, 38} and one advantage of the micropatterning technique is that it may be used to prevent the cells from moving outside the field of view during long-term imaging. Overall, we are confident that automated tracking of cells will be achievable using a combination of recently established tools^{28, 39, 40} and that this should bring new insights into the fundamental principles of self-organization.

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DISCLOSURES:

The authors have nothing to disclose

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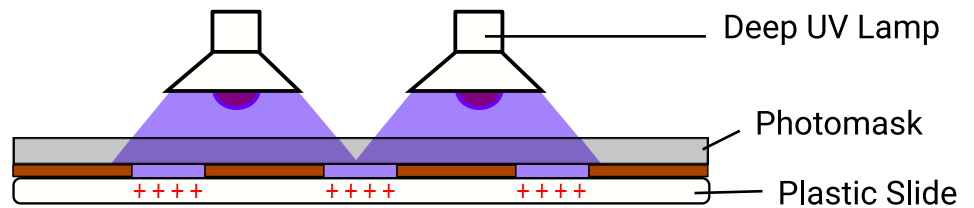
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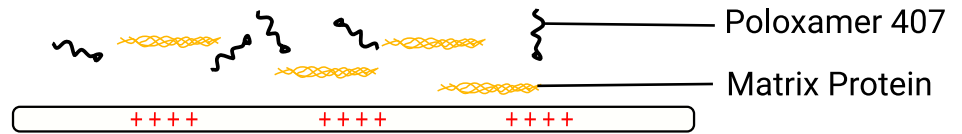
793 Microscopy Data of Developing Embryos. *Developmental Cell*. **36** (2), 225–240, doi:
794 10.1016/j.devcel.2015.12.028 (2016).

Fig. 1

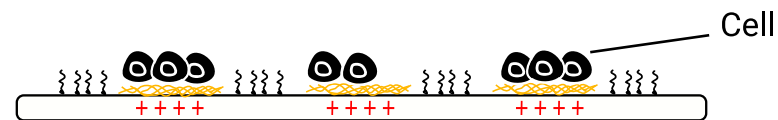
① Photo-patterning (15 min)



② Matrix deposition (Overnight)



③ Cell seeding (~1h)



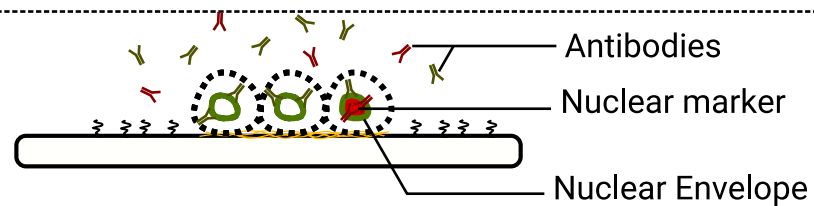
Culture for 48h

See also Fig. 2

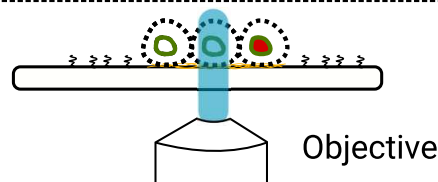
④ Fixation Permeabilisation (20 min)



⑤ Immunostaining (~3h)

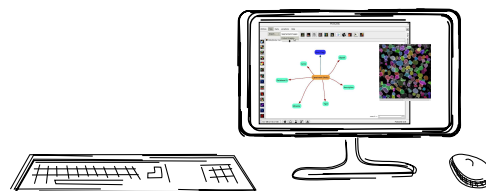


⑥ Imaging (~3min/colony)



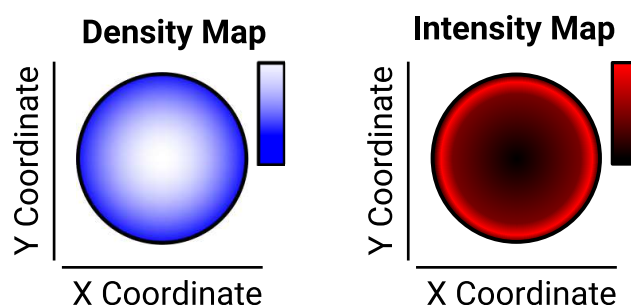
See also Fig. 3

⑦ Image Analysis (~1h)



See also Fig. 4

⑧ Results



See also Fig. 5

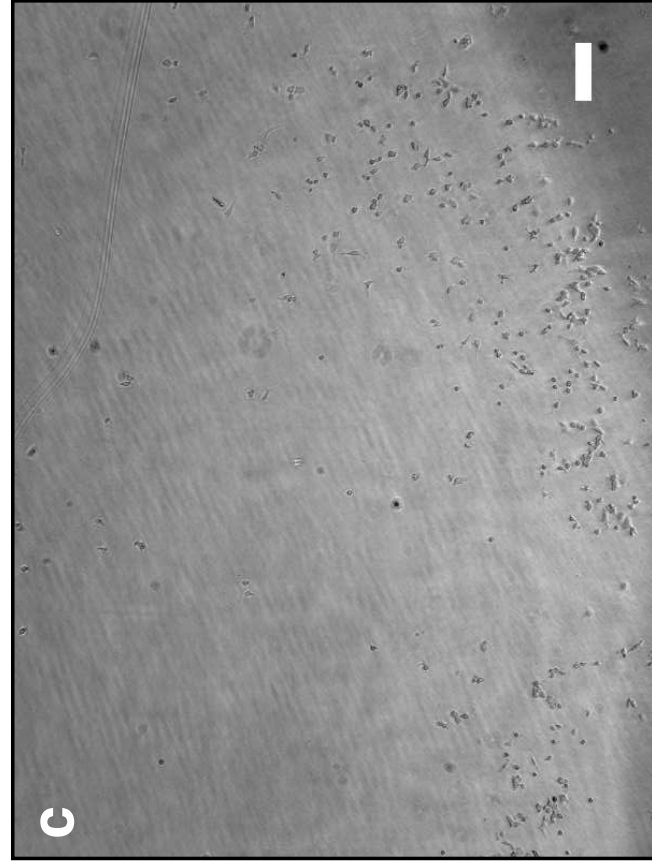
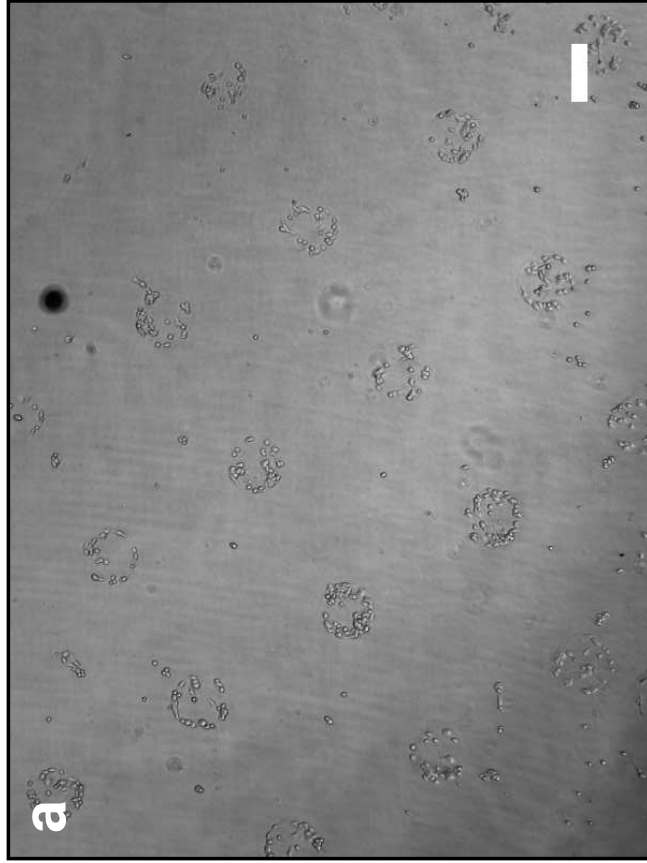
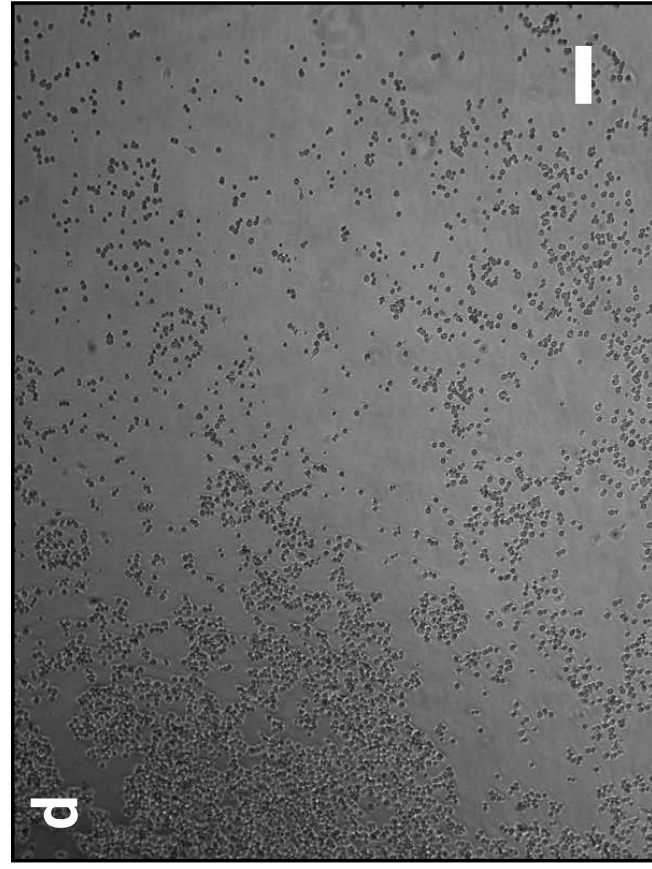
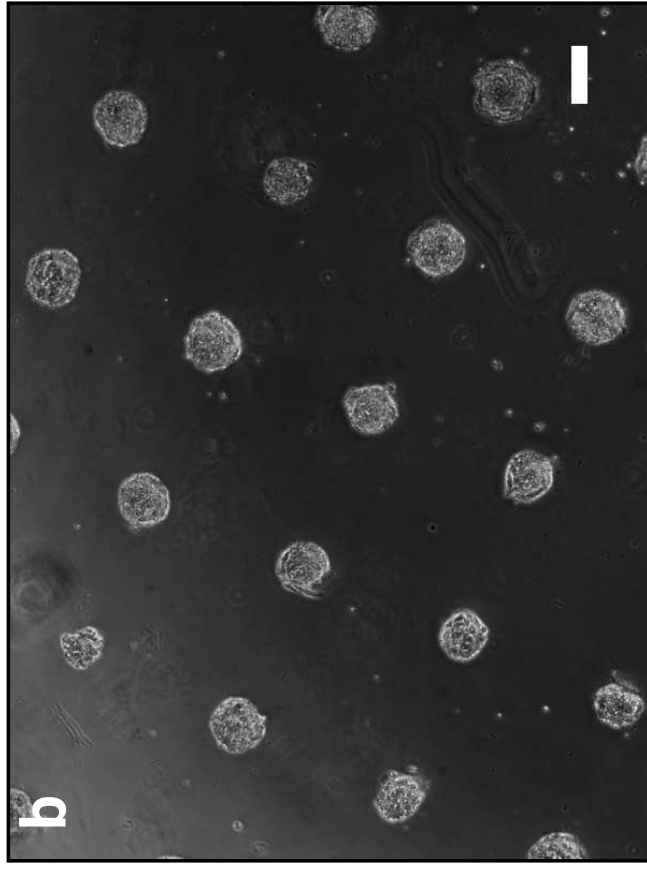
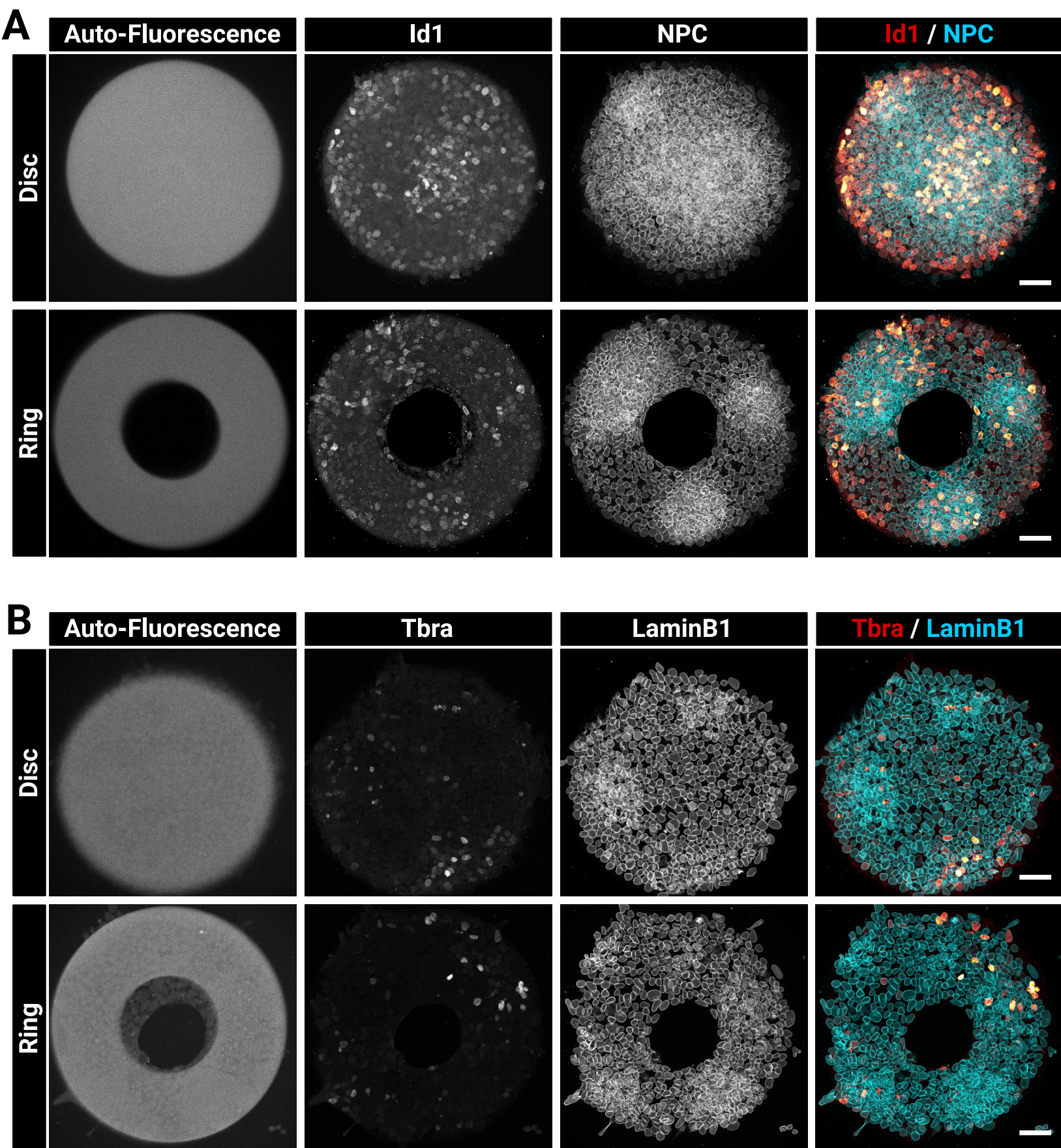
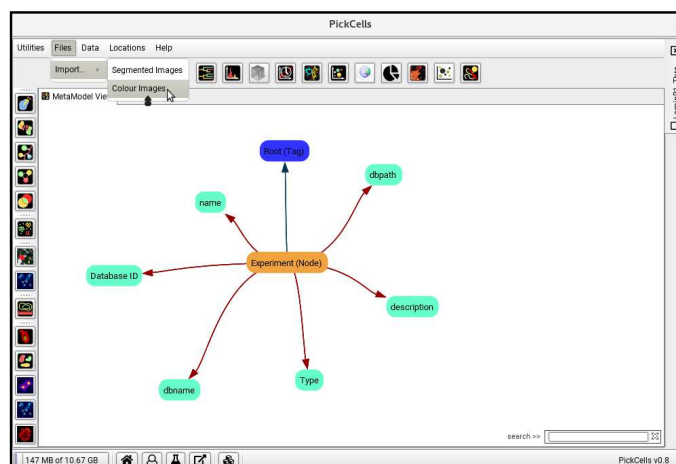
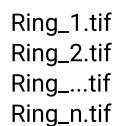
Fig. 2

Fig. 3

List of Confocal Images



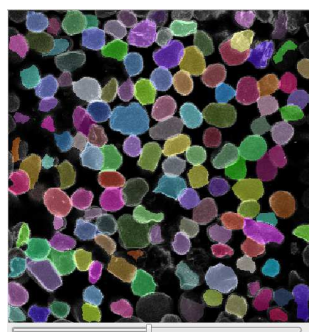
1 Import



②



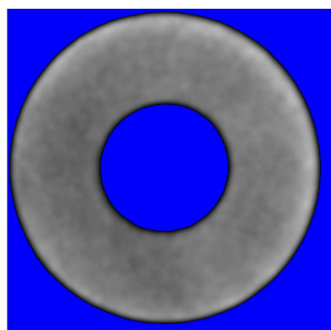
Automated Nuclei Segmentation



4



Automated Pattern Segmentation



③



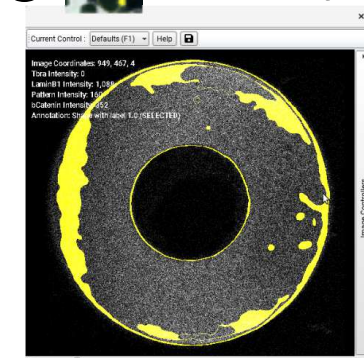
Visual Inspection Manual Editing



5



Visual Inspection Manual Editing

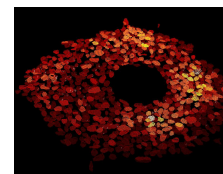


⑥



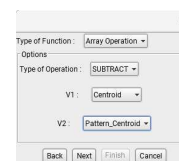
Compute object features

3D Coordinates
Median intensity
Volume...



7

Normalise Nuclei Coordinates

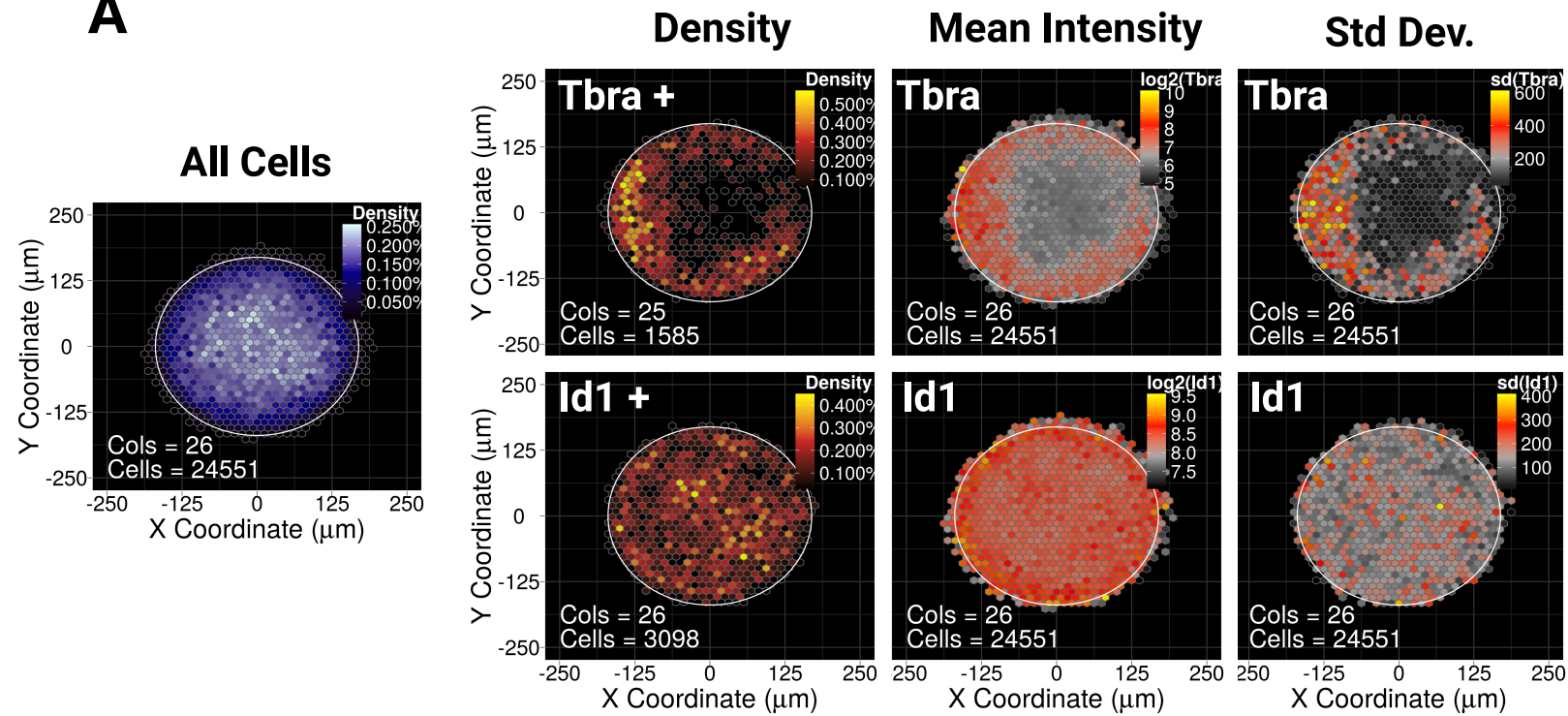
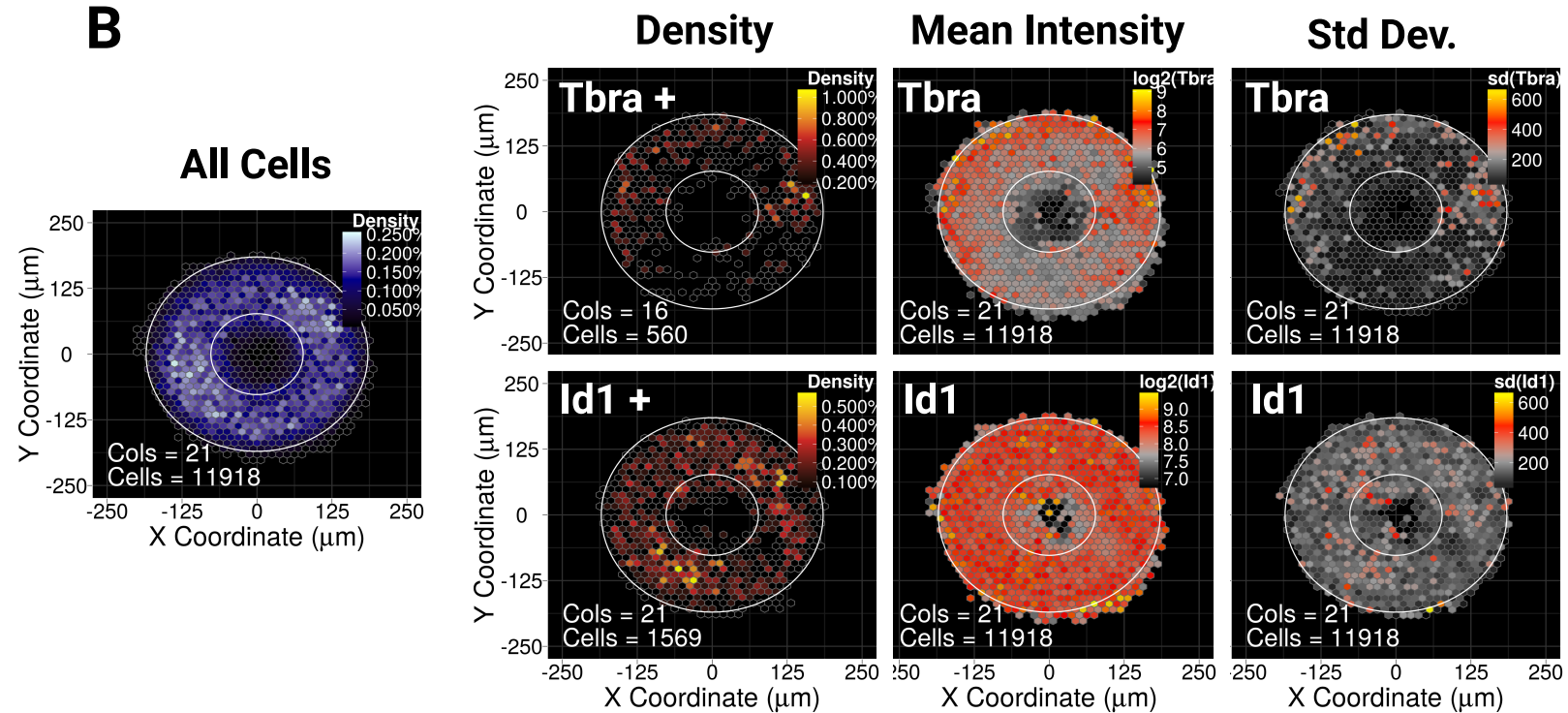


Normalised = Nucleus Centroid - Pattern Centroid

8

Export as .tsv

Nucleus (Node) (preview)				
Database ID	Image Name	Normalised 0	Normalised 1	Median Intensity 0
9	Disc_L14.ids	-81.13832444166898	147.5289654890947	64.0
8	Disc_L14.ids	130.01029261570113	-87.66688474807083	32.0
7	Disc_L14.ids	124.63001033534829	102.07762273244722	96.0
6	Disc_L14.ids	-55.223977002704544	152.90756904738313	96.0
5	Disc_L14.ids	59.42617897937299	75.17858799100534	64.0
49	Disc_L14.ids	112.78183637818591	-43.28194193346965	32.0
48	Disc_L14.ids	35.392439367627134	37.72704978648568	32.0

Fig. 5**A****B**

Table_1_ratios

	ECM type	Gelatin	Fibronectin	Basement Membr
Concentration	ECM concentration	1 mg/mL	20 µg/mL	200 µg/mL
	Poloxamer 307 concentration	500 µg/mL	400 µg/mL	1 mg/mL
Tested with	mESC	YES	YES	YES
	mEpiSC	NO	YES	YES
	Serum free medium	NO	YES	YES

brane Matrix

Table_2_Troubleshooting	
Procedure	Observation
Micropatterning	Low cell attachment
	Cells adhere between the patterns
	Cells do not strictly follow the pattern shape
	Non-homogeneous staining
Staining	Detaching colonies during the staining procedure Colonies appear sheared under the microscope

Table_2_Troubleshooting

Possible Issue

inappropriate ECM/poloxamer 307 concentration ratio
Cell attachment time too short

Washes too intense (step 3.6)

inappropriate ECM/poloxamer 307 concentration ratio
Cell attachment time too long
Washes ineffective (step 3.6)

'Incompatible' cell type and pattern geometry

Non-optimal photo-patterning, this can be diagnosed by observing the sharpness of the autofluorescence signal. Pattern boundaries should look sharp as in Fig. 2. If the borders of the patterns appear blurred then the photo-patterning step needs to be improved.

antibody incubation time too short
colonies flattened during the mounting procedure

Dewetting of the chip

colonies were sheared while mounting the chip on the microscopy slide

Solution

Increase the ECM/poloxamer 307 concentration ratio

Increase incubation time to give enough time to the cells to properly adhere to patterns (step 3.4). To optimise this step, checking the cells under a microscope can help detect a change in cell morphology indicating that the cells have started to adhere.

When replacing medium, avoid pipetting medium directly onto the chips. Instead, gently pipet the medium on the walls of the well instead

Decrease the ECM/poloxamer 307 concentration ratio

Decrease incubation time (step 3.4).

Shaking the plate vigorously is usually sufficient to detach the cells in excess. For cell types which tend to adhere strongly to the chip, pipetting directly onto the chip may improve the outcome. Increasing the number of washes may also help, notably to ensure that no cell remain floating in the medium after this step.

Plan/design multiple geometries/sizes to be added onto the photomask to be able to test and identify the optimal pattern size for a given pattern shape and cell type. Please, see the 'Limitations' section in the discussion

Blurred pattern edges indicate that the plastic slide was not close enough to the surface of the mask during the illumination step. Ensure that the pieces holding the slides to the photomask are even and that a constant and sufficient pressure is applied to the assembly during the illumination procedure.

Increase antibody incubation time (up to 24h at room temperature)

Mount micropattern slides in a chamber such as chamlide or cytoochambers to perform both immunostaining and imaging without the need for mounting the cells. This will better preserve the colonies 3D-structure.

Leave enough medium or use 2 pipettes, one to remove the medium and the other to add the fresh solution

Be very gentle when mounting the chips. Alternatively, mount micropatterns slides in a chamber such as chamlide or cytoochambers to perform both immunostaining and imaging without the need for mounting the cells. This also preserves the colony ultrastructure.

Table_Of_Materials

Name of Material/ Equipment	Company
2-mercaptoethanol	Gibco
1x Master quartz anti-reflective chromium photomask	Toppan Photomask
24-well plates	Corning
4-well plates	Nunclon Delta
5% Donkey Serum	Sigma
Anti Nuclear pore complex	Abcam
Anti-I δ 1	Biocheck
Anti-Lamin B1	Abcam
Anti-Tbra	R&D
Blocking Solution	NA
CGR8 mESC	NA
Fixation solution	NA
Foetal bovine serum	Gibco
Gelatin	Sigma
Glasgow Minimum Essential Medium	Sigma
Laboratory Film	VWR
Layout Editor Software	LayoutEditor
Layout Editor Software	Klayout
L-glutamine	Gibco
LIF	Millipore
MEM NEAA	Gibco
mESC Culture medium	NA
NH ₄ Cl 50mM	Sigma
Paraformaldehyde	Sigma
PBS (immunostaining)	Sigma
PBS (tissue culture)	Gibco
Plastic slides	Ibidi
Poloxamer 407	Sigma
ProLong Gold Antifade Mountant	Molecular Probes
Sodium Azide	Sigma
Sodium pyruvate	Gibco
TritonX-100	Sigma
Trypsin	Gibco
UVO cleaner	Jetlight, USA
Washing Solution	NA

Table_Of_Materials

Catalog Number
31350-010
Custom design
3526
176740
D9663
ab24609
37-2
ab16048
AF2085
NA
NA
NA
10270-106
G1890
G5154
291-1212
NA
NA
25030-024
ESG1107
11140-035
NA
9718
158127
P4417
11140-035
IB-10813
P2443
P36930
8591
11360070
T8532
25200056
42-220
NA

Table_Of_Materials

Comments/Description
handle in fume hood
Mouse monoclonal, use 1:1000
Rabbit polyclonal, use 1:200
Rabbit polyclonal, use 1:1000
Goat polyclonal, use 1:400
0.1% TritonX-100
0.01% Pluronic F-127
5% Donkey Serum
0.003% Sodium Azide
In PBS
Reference: Mountford et al. PNAS, 1994
4% PFA diluted in washing solution
Serum batches must be tested to ensure compatibility with ESC maintenance
https://layouteditor.com/
https://www.klayout.de/
GMEM
10% FCS
100 U/ml LIF
100 nM 2-mercaptoethanol
1x non-essential amino acids,
2 mM L- glutamine
1 mM sodium pyruvate
CAUTION: Toxic, handle undiluted stocks in fume hood and wear protective equipment
Dilute in 200ml of ddH ₂ O
CAUTION: Toxic, handle in fume hood and wear protective equipment
CAUTION: Follow manufacturer's safety recommendations
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Mapping the emergent spatial organization of mammalian cells using micropatterns and quantitative imaging

Author(s):

Darren Wisniewski, Sally Cowell, Guillaume Blin

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Signature:		Date: 30/12/2018

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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 manuscript 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/cm² 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 add 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.

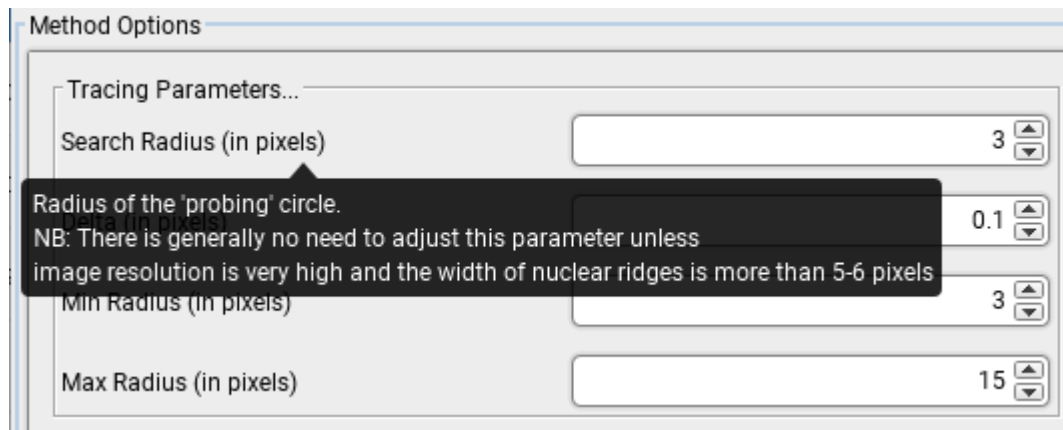
I also tried to train my own classifier, but I couldn't obtain a segmentation this way and I haven't been able to understand what was done wrong in that case. I would recommend that parameters for the different steps of the process would be explained and that example of good and bad parameters choice 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-friendliness 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.