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(Advanced practicals)

Area: Biophysics

Experiment: Cell polarity

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Advanced practical course in biophysics: cell polarity

Preface: At the end of the instructions you will find some questions that you should think through in preparation and answer as well as possible. We have provided you with some literature suggestions to help you answer the questions. However, you will also need to refer to other literature. The answers to the preparatory questions (including references) should appear later in the formulated evaluation.

During the internship, you are expected to write down exactly what you do. Some things may be different from the protocol or are not yet described in detail. The log you keep will be checked afterwards.

Introduction

Cell polarity is defined as the orientation of a cell in space and the organization of the different organelles. A simple example of living cells that are polar are intestinal epithelial cells. The function of these cells is to transport nutrients through the intestinal barrier into the bloodstream. It is important for the organism as a whole that the exchange of substances only takes place in one direction. For this purpose, intestinal cells have a dedicated anisotropic organization and geometry: they are polar.

Another important example of cell polarity is cell migration: the displacement of cells in the spatial directions over a certain period of time. During this process, the movement of a cell from one place in the tissue to another, the cell migrates in a certain direction. This means that the front and back of the cell can be determined. At the molecular level, the front and back of cells are actually different in terms of composition and activity: while the front has all the machinery to advance new membranes, the back exerts contractile forces to perform the actual movement. In addition, cells often change direction during their migration, i.e. they are able to redefine a horizontal axis within seconds, i.e. they polarize anew.

When cells are cultured on 2D substrates and in the absence of chemical signaling agents, they spontaneously adopt a random polarity. Nevertheless, it is possible for an experimenter to play with this polarity using chemicals. In fact, cells can find their orientation or migration direction in vivo by means of external gradients to which they orient themselves. In short, the presence of a particular component will induce changes to the membrane that cascade to eventual accumulation of specific factors, which will induce reorientation of the cell (or maintenance of orientation).

Since there is a direct correlation between the polarity and shape of the cell, researchers are interested in whether the polarity of a cell can be enforced by simply forcing it to a certain shape using so-called micropatterns. The main aim of these experiments is to investigate whether there is a correlation between the cell shape (or the shape of the pattern) and the polarity of the cells. This is measured via the axis between cell organelles.

Microstructuring

A few years ago, techniques were developed to form structured surfaces in order to predetermine the cell shape. The main objective is to obtain a "sticky" microstructure to which cells, surrounded by non-adherent surfaces, can adhere so firmly that they cannot get away from the pattern. Among

the techniques, one of the first was the use of self-assembling monolayers (SAM), the principle of which is to partially cover a surface using microfabrication. The uncovered surface is then functionalized with a coating to which cells cannot adhere. After removing the remaining photoresist, the rest of the surface is functionalized with another reactive coating to which adhesion molecules can be applied. A cheaper but slightly less reliable alternative is to use micro-contact printing. In this case, an adhesion protein is transferred to a glass surface via an elastomer imprint. The remaining glass is then made non-adhesive by suitable passivation.

In both cases, the cells are placed on the microstructured surface. As soon as they come close to these small "sticky" islands with a defined shape, the cells will spread out on them without being able to leave these shapes again.

Deep UV microstructuring

In this FoPra, so-called deep-UV microstructuring is used. For this technique, a cover glass is activated by plasma, which generates negative charges on the surface. It is then treated with poly-L-lysine dissolved in poly-(ethylene) glycol (Pll-g-PEG, positively charged). The poly-L-lysine backbone adheres firmly to the surface and the PEG chains are directed outwards, creating a PEGylated surface. PEG is protein-repellent so that the cells cannot adhere at any point. This functionalized surface is then exposed to deep UV, not directly but through a photomask with the patterns of the desired shape. Illumination in the deep UV range (wavelength less than 200nm) is energetic enough to break the C-O-C bond that links the PEG chains to the poly-L-lysine chain and replace it with a pH-sensitive and reactive carboxylate group. The surface is then incubated in alkaline with an adhesive protein, fibronectin, to attach the protein to the surface via the carboxylate groups. The result is small fibronectin-adherent islands surrounded by a protein-repellent PEG surface. Figure 1 describes the final stage of this experiment.

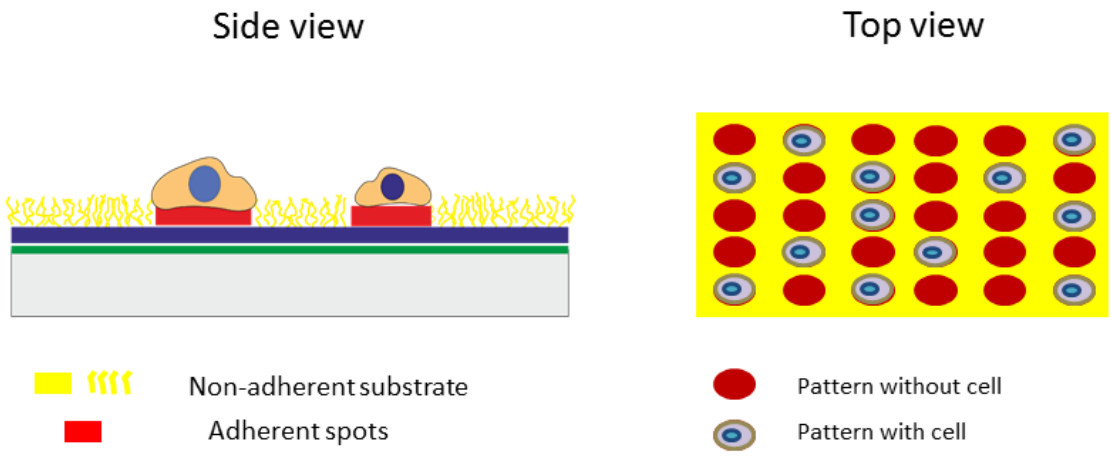

Figure 1: Top and side view of an patterned surface with cells placed on them

Cell organelles

Cell polarity can be defined in different ways depending on the interest. There are specific polarity markers that trigger the (re)organization of cells. In the experiments of this FoPra we will focus on well-spread cells (cells that adhere well to the surface). The orientation of these cells will be investigated by the position of their nucleus and cytoskeleton.

To define an axis of polarity, we will look at the axis between the center of the nucleus and the microtubule organizing center (MTOC). For the experiments of this FoPras you will create microstructured surfaces with four different geometries: Circles, triangles, ellipses with a ratio between their major axes of 2 and ellipses with a ratio of 3. The cells will be seeded on these surfaces. They spread over a period of 3 to 4 hours. Images of the nuclei and microtubule network are then recorded for the remainder of the first day using fluorescence microscopy.

Analysis and evaluation of the data will take place on the second day. Images will be processed so that you can compare them with each other and recognize the core and MTOC can be recognized. The output data will be mapped as angles between the axis on which these organelles lie and a chosen pattern axis. The distribution of these angles is then represented in an understandable diagram to check whether the shape of a cell has an influence on its internal organization of the cell. Ideas on how such comprehensible diagrams could look should come from you.

Protocol

The complete version of the protocol is described here. However, the first part (PEGylation of the cover glass) will not be carried out during the FoPras due to lack of time, but has already been prepared for you.

PEGylation of the cover glass

1. clean the coverslips with ethanol. Dry them.
2. cut a piece of parafilm and stick it in a large Petri dish.
3. place 50 µl-sized drops of a PLL-g-PEG solution with a concentration of 0.1 mg / mL on it. There should be enough space between the drops to accommodate the coverslips. Use one drop per coverslip.
4. Activate the coverslips for 30 seconds in the plasma (max. power).
5. place the coverslips in the PEG solution with the activated surface facing the PEG solution.
6. allow them to incubate for 45 minutes.
7. rinse the coverslip with water: add 1 ml of water near the coverslip so that it flows between the coverslip and the Parafilm. If the water is not absorbed, use tweezers to lift the coverslip.
8. rinse the cover glass several times with water, then dry it. Remember to always know which side is the PEGylated side.

The coverslip can be stored in this condition for a few days, preferably at 4°C.

Preparation of the fibronectin solution

Before the coverslips are irradiated, the fibronectin solution must be prepared. The fibronectin is dissolved in a carbonate buffer (pH 8.7) or in PBS (pH 7.5. The stock solution is present in a concentration of 1 mg/ml. We require a concentration of 25 µg/ml.

Irradiation of the coverslip

During the irradiation of the coverslip, it is really important to follow the safety regulations. Firstly, deep UV light can be harmful to the eyes. You must always wear safety goggles during this step! Secondly, oxygen forms ozone under deep UV lighting. This is the reason why the lamp is placed under a hood. This hood should be used during the entire procedure; also wear gloves.

1. take time to recognize the different sides of the mask. The gold side is the chrome side, the glass side is the quartz side.
2. clean the mask. Use acetone and ethanol. Be careful not to scratch the mask, otherwise these scratches will also be shown.

3. start the lamp and the fans. The fans help to avoid overheating inside the cavity, which would lead to vaporization of the liquids.
4. expose the chrome side of the mask for 5 minutes. This step partially activates the mask, making the chrome more hydrophilic, which is important for the next step.
5. remove the mask from the lamp and allow it to cool. Add a 4.5 µl drop of deionized water to the center of each section you want to educe.
6. place the cover slip on the droplet. The PEG side should be in contact with the chrome.
7. press the coverslip with a plastic pipette tip to remove excess water and trapped air bubbles. Due to the small amount of water, the cover glass will stick to the mask.
8. place the mask with the adhering coverslips into the illumination system, this time with the quartz side facing the light.
9. expose the mask for 6 minutes.

Filling the holes with adhesive molecules

Now that the PEG has been removed from the coverslip in the desired shapes, the "holes" in the PEG layer need to be filled with an adhesive protein that the cells can interact with.

1. remove the mask from the illumination chamber
2. pipette approximately 1 ml of water around each coverslip. The water will slowly enter between the mask and the coverslip so that the coverslip carefully detaches from the mask.
3. at the same time, prepare the lid of a Petri dish with Parafilm (place Parafilm in the lid). Place a 50 µl drop of fibronectin on each cover slip.
4. as soon as the coverslip floats above the indicated 1 ml drop, carefully lift it with tweezers. Be careful not to touch the chrome with the tweezers to avoid breaking it. If you are unsure, add more water to lift the cover glass higher.
5. remove the excess water from the coverslip and place the coverslip on the fibronectin with the PEG side on the fibronectin.
6. leave everything at room temperature for approx. 1h.
7. (The following steps will be done under the sterile flow bench. It is mandatory to wear gloves, safety googles and a lab coat). Remove the FN coated cover slips from the parafilm and place them in a so called 6 well plate.
8. Wash each cover slip with PBS 3 times.
9. Fill each well with 2mL of cell medium and place the whole 6 well plate aside until it is needed again.

Cell staining, recovery, counting and seeding

1. aspirate the medium from the cell culture dish using the aspiration system.
2. add 1 ml of PBS to the container to rinse away the remaining medium and aspirate.
3. add 1 ml of trypsin to the cells and place the container in the incubator for 3 minutes.
4. during this time, prepare a tube of medium to be used for the experiment: Pipette 10 mL of medium into a tube and add the Hoechst dye (a fluorophore that makes nuclei bound to DNA fluorescent) at a concentration of 50 ng / mL. The stock solution is present at a concentration of 100 mg / mL.
5. check whether the cells in the container are already detached, otherwise tap the container carefully if necessary.
6. add 1 mL of medium to the container to stop the effect of the trypsin. Take up the liquid and pipette it up and down several times to detach cells that are still attached. Be careful during these steps so that no bubbles form.
7. fill the liquid (cells + trypsin + medium) into a 10 ml tube.
8. prepare the Malassez counting chamber and add 20 µl of the cell solution to a chamber. Calculate the concentration of the cells.

A Malassez cell consists of a grid of 25 rectangles, each consisting of20 boxes (see Figure2). To count a cell solution, count the number of cells in 10 rectangles. Multiply this number by 10^4 and you have the number of cells per ml.

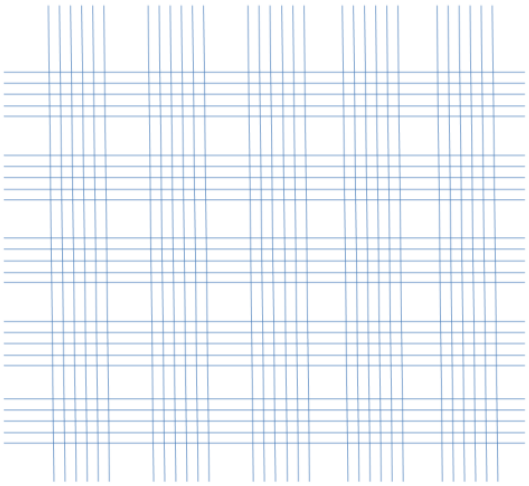

Figure 2: Schematic of a Malassez chamber

9. Centrifuge the tube for 3 minutes at 1300 rpm. 10.
10. aspirate the liquid above the cell pellet that has formed at the end of the tube. Be careful not to aspirate the pellet itself!
11. dissolve the pellet in the prepared medium. Add enough medium to obtain a final concentration of 1 Mio cells per ml. If necessary, dilute 2 times in succession.
12. aspirate the medium from the chambers.
13. add 100,000 cells (=100µl) of the cell solution to each chamber. Close the lid and place all chambers back into the incubator. The cells will need some time to adhere to the pattern and spread. 3 hours should be sufficient

Imaging of cells

The samples are imaged with a 20 x objective to obtain an optimum between the resolution and the number of cells. To obtain images of microtubules and cell nuclei, you need images of cells in Brightfield and in fluorescence. During this step you should take as many images as possible. There are 4 different structured shapes. For each shape you need a good number (at least 10) to be statistically relevant for the analysis.

Data analysis

The main aim of this experiment is to investigate whether there is a correlation between the cell shape (or the shape of the pattern) and the polarity of the cells. This is measured via the axis between cell organelles.

During the analysis, the nucleus and centrosome of each cell will be captured on each image and correlated with the shape of the pattern. The open source software FIJI ('Fiji is justImageJ') is used to perform this analysis. The course of the analysis is as follows:

1. open the image with its three color channels.
2. select an orientation of the image and rotate it in a direction that you can explain. For example, in the case of ellipses, you could possibly choose the orientation of the long axis.
3. work with the LUT to see how you can edit the contrast and brightness for each color.

1. 4 In particular, use the threshold tool on the red channel (microtubules) so that you only see the strongest spot (the centrosome).
4. find the axis from the nucleus center to the centrosomes using the line and measure tool.
5. present your results in an understandable way, comparing each geometry of patterns and the statistics of these representations. The different possibilities will be discussed during the analysis.

Try to discuss the following questions during the analysis:

1. how does the arrangement of nucleus and MTOC depend on the shape of the forced cell shape? Is there a preferred orientation?
2. where are the MTOC and nucleus located relative to the center of the cell? What could be the reason for this?
3. try to formulate a hypothesis as to why the polarity of the cytoskeleton might - or might not - be influenced by the forced shape of the cell. (There is no right or wrong hypothesis here, it should only be logically related to your results and justified).

Preparatory questions

1) How is polarity defined in physics? How is it defined in biology? What are the differences/similarities?

2) Find or sketch a diagram that explains the technique of deep UV microstructuring.

3) Briefly explain the structure and function of the nucleus.

4) Give a brief introduction to the cytoskeleton: What is its function? How is it structured? How does the "skeleton" of the cell differ from that of a human being? What do they have in common?

5) Now explain in more detail what microtubules are. How are they structured? What are their functions?

6) Explain the basic principle of fluorescence? How is it used in cells? What could be problems?

7) Briefly sketch the structure of a fluorescence microscope. Consider what other components a microscope should have to enable images of living cells to be taken.

8) Explain how cells can adhere. In particular, explain integrin adhesion and the importance of stress fibers.

9) What are the morphological differences between adherent cells in culture (i.e. under artificial living conditions in a cell culture flask) and adherent cells in the body? Try to deduce why it might be interesting to look at cells for micropatterns.

10) Try to find clues as to how the shape of the cell and the organization of the cytoskeleton might be related.

11) Calculate the volume of fibronectin stock solution required for a total volume of 800 µl.

12) Calculate the volume of Hoechst dye required for 10 ml of medium.

13) Assume that you count 50 cells in the Malassez cell. What cell concentration does this correspond to? In how many ml of medium must the cell pellet be resuspended to obtain a concentration of 30,000 cells per ml?

Literature suggestions:

Thery, M. (2010). "Micropatterning as a tool to decipher cell morphogenesis and functions." J Cell Sci **123**(Pt 24): 4201-4213.

Thery, M., et al. (2006). "Anisotropy of cell adhesive microenvironment governs cell internal organization and orientation of polarity." Proc Natl Acad Sci U S A **103**(52): 19771-19776.

Wolpert, L. (2013). "Cell polarity." Philosophical transactions of the Royal Society of London. Series B, Biological sciences **368**(1629): 20130419-20130419.