Fortgeschrittenen-Praktikum Biophysik: Zellpolarität

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Foreword

At the end of the description of the experiments you will find some questions. As preparation we ask you to think about those questions and try to answer them. We also prepared a list of literature that may help you to answere these questions. However, you will also have to look for other literature. Your answers will also appear in your report. Along your answers we ask you to provide the source of your reflections (book title and authors, websites, lectures...). During the experiments you should take notes of what you do since some things might differ from the protocol. This written protocol of yours will be looked at after the FoPra.

Contents

1 Introduction

1.1 Cell polarity

Cell polarity is the term that defines the orientation of a cell in space as well as its inner organization of different organelles. One simple example for living cells which are polar are intestine epithelial cells. These cells' task is to let nutrients pass through the barrier of the intestine to reach the blood circulation. It is important for the entire organism that the exchange of material is in this one direction only. To that end, intestinal cells have a dedicated anisotropic organization and geometry: they are polar.

Another important example of cell polarity is found in cell migration. This is the translocation of cells in space and time. In this process, when a cell moves from one place to another place in the tissue, cells have a particular direction in which they are going. This means, one can define a front and a back of the cell. At a molecular level the front and the back are indeed different in terms of composition and activity: while the front has a whole machinery to protrude new membrane, the back exerts contractile forces to produce the actual movement. On top of that, cells frequently change direction during their migration. This means that they are able to re-define a front-back axis within seconds, meaning that they re-polarize.

When cells are cultivated on 2D substrates and in absence of any chemical cues they spontaneously adopt a random polarity. However it is possible for an experimentalist to play with this polarity with the help of chemicals. Indeed, in vivo cells may find their orientation or direction of migration by the mean of external gradients to which they orient. Briefly, the presence of a particular component will induce changes at the membrane and these changes cascade to eventually recruit specific factors which will induce the reorientation (or the orientation keeping) of the cell.

Since there is a direct correlation between the cell polarity and its shape, researchers were interested in seeing if one could force a cell's polarity by simply forcing it on a specific shape using so-called micro patterns. The main goal of these experiments will be to make a correlation between the cell shape (e.g. the shape of the pattern) and the polarity of the cells, measuring the axis between cellular organelles.

1.2 Micropatterning

For several years now, people have developed techniques to create structured surfaces to control the cell shape. The main goal of all those is to have a "sticky" microstructure on which cells can adhere surrounded by a non-adherent surface so that they can't escape the pattern. Among those techniques, one of the first ones was to use self-assembled monolayers (SAM). The principle is, by use of microfabrication, to mask some part of a surface which is in turn functionalized with a silage on which cells can't adhere. After removal of the remaining photoresist, the native surface is functionalized with another, reactive, silage on which adhesion cues can be grafted.

A much cheaper but somewhat less reliable alternative is to use micro-contact printing. In that case, an adhesion protein is transferred on a glass surface via an elastomer stamp. The remaining glass is then passivated by a non-adherent cue.

In both cases, when cells are cast over the micro patterned surface, they will find small adhering islands with a defined shape on which they will spread without being able to leave.

1.3 Deep-UV micropatterning

For the experiments of this FoPra, deep UV micro patterning will be used. For this technique, a glass coverslip is activated by plasma (creates negative charges on the surface) before being exposed to Poly-L-Lysine grafted Poly(ethylene)glycol (Pall-g-PEG, positively charged). The poly-L-lysine backbone will stick to the surface and expose the PEG chains towards the exterior, leading to a PEGylated surface. PEG is protein repellent so that cells can't create any adhesion point on it. The functionalized surface will then be exposed to Deep-UV through a photomask bearing the shape of the wanted patterns. Deep-UV illumination (wavelength less than 200 nm) is energetic enough to break the C-O-C bond linking the PEG chains to the poly-L-lysine chain, replacing it by a pH sensitive and reactive carboxylate group. The surface is then incubated with an adhesive protein, the fibronectin, at a basic pH to graft the protein to the surface via the carboxylate groups. This results in small fibronectin adhesive islands surrounded by a protein-repellent PEG surface. Figure 1 describes the final state of the preparation.

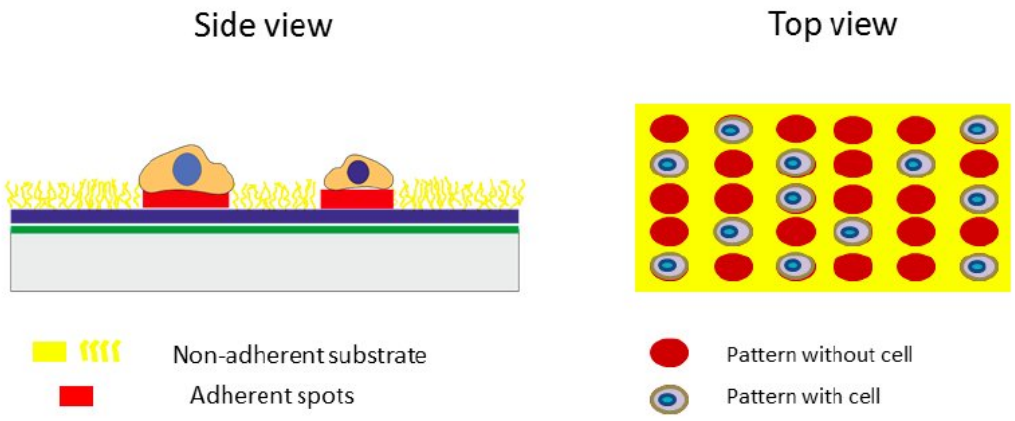

Figure 1: Top and side view of a micropatterned surface with cells adhering on some of the patterns.

1.4 Cell polarity determinded by the organisation of cell organelles

Defining the polarity of a cell can be done in different ways, depending on interest. There are specific markers of polarity that trigger the (re)organization of cells. In the experiments of this FoPra, we will focus on spread cells (well adhered to the surface) and determine their orientation by investigating the position of their nucleus and their 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 FoPra you will prepare micro patterned surfaces with four different geometries: circles, triangles, ellipses with a ratio between their main axis of 2 and ellipses with a ratio of 3. Cells will be seeded on those surfaces. They spread over a time course of 3 to 4 hours. Images of cell nuclei and the microtubule network will then be recorded during the rest of first day using fluorescent microscopy.

On the second day, data will be analyzed and evaluated. Images will be treated so that we can compare them with each other and that we can detect the nucleus and MTOC. The output data will be the angle formed by the axis drawn between those organelles and one chosen axis of the pattern. The distribution of these angles will then be represented on comprehensive graphs in order to check if the shape of a cell has an influence on its inner organization. Ideas, how such comprehensive graphs might look like are supposed to come from you.

2 Protocol

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

2.1 PEGylation of the coverslip

1. Clean glass coverslips with ethanol. Dry them.
2. Cut a Parafilm piece that will be stuck in a large Petri dish.
3. Deposit 40 µl drops of a solution of Pll-g-PEG at a concentration of 0,1 $\frac{mg}{ml}$. There should be enough space between drops to fit the coverslips. Do one drop per coverslip.
4. Plasma activate the coverslips for 30 seconds (max power).
5. Lay down the coverslips on the PEG solution, activated surface facing the PEG solution.
6. Let incubate for 1 hour.

7. Rinse the coverslip with water. Add 1 ml of water close to the coverslip so that it flows between the coverslip and the Parafilm. If the water doesn't enter, use a tweezer to lift the coverslip.

8. Continue rinsing the coverslip in water several time then dry it. Remember to always know which side is PEGylated.

The coverslips can be stored in that state for several days, preferably at 4 °C.

2.2 Preperation of fibronectin solution

Before irradiation of the coverslip the solution of fibronectin needs to be prepared. Fibronectin will be dissolved in a carbonate buffer (pH 8,7). The concentration of the stock solution is at 1 $\frac{mg}{ml}$. The working concentration is at 25 $\frac{\mu g}{ml}$.

2.3 Irradiation of the coverslip

During this process, it is really important to respect the security rules. First, deep-UV light can be harmful for your vision. You always have to wear protection glasses during this step. Second, oxygen under deep-UV illumination forms ozone. This is the reason why the lamp is under a chemical hood. This hood should be operated properly during the whole process. Last, wear gloves.

1. Take time to recognize the different sides of the mask. One side is golden and will be further called chrome side. The other one is glass like and will be called quartz side.

2. Clean the mask. Use acetone and ethanol for that purpose. Pay attention not to scratch the mask to avoid replication of those scratches on the patterns.

3. Start the lamp and the fans. These fans help to reduce overheating within the cavity which would lead to evaporation.

4. Illuminate the chrome side of the mask for 5 minutes. This step ensures a partial activation of the mask to make the chrome more hydrophilic, which will be important for the next step.

5. Take the mask out of the lamp and let it cool down. Deposit a 4.5 µl drop of deionized water in the center of each section you want to reproduce.

6. Lay down the coverslip on the droplet. The PEG side should go in contact of the chrome. With a plastic pipette tip, press on the coverslip to remove excess of water and trapped bubbles. The small quantity of water will allow the glass coverslip to stick on the mask.

7. Place the mask holding the coverslips in the illumination system, this time the quartz side facing the light.

8. Illuminate the mask for 6 minutes.

2.4 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 have to be filled with an adhesive protein with which cells will interact.

1. Take the mask out of the illumination chamber.
2. Pipette around 1 ml of water around each coverslip. The water will slowly enter between the mask and the coverslip, allowing the coverslip to gently detach from the mask.
3. During this time, prepare a Petri dish cover with Parafilm (just place the parafilm in the cover). Place 40 µl droplets of fibronectin for each coverslip.
4. Once the coverslip floats on top of the given 1 ml drop, lift it gently with a tweezer. Pay attention not to touch the chrome with the tweezer in order not to destroy it. If unsure, add more water to lift the coverslip higher.
5. Remove the excess of water from the coverslip and lay the coverslip on the fibronectin, PEG side against the fibronectin.
6. Wash the mask again with ethanol and aceton.
7. Let the coverslips incubate for one hour at room temperature.

2.5 Cell staining, recovery, counting and seeding

1. At first, place the cover slips into a 6-well plate (fibronectin coated site facing up).
2. Rinse the cover slips three times by adding 1 ml (sterile) PBS into each well and aspirate it.
3. Cover the cover slips with 1 ml medium and place them into the incubator.
4. Aspirate the medium of the cell culture dish with the aspiration system.
5. Give 1 ml PBS in the flask to rinse the remaining medium and aspirate it.
6. Add 1 ml of Trypsin to the cell and place the flask in the incubator for 3 minutes.
7. During that time prepare a tube of medium which will be used for the experiment: add 10 ml of medium in the tube and add Hoechst staining (a fluorophore that binds to DNA to make nuclei fluorescent) at a concentration of 50 $\frac{ng}{ml}$. Stock solution is at 100 $\frac{mg}{ml}$.
8. Check that cells in the flask are detached, knock the flask gently if necessary.
9. Add 1 ml of medium in the flask to stop the action of trypsin. Aspirate the liquid in the cell flask and do several back and forth movements of the liquid to unstick cells which are still attached. During that step be cautious not to form bubbles.

10. Transfer the liquid (cell + trypsin + medium) in a 10 ml tube.

11. Prepare the Malassez-counting cell and deposit 20 µl of the cell solution in one chamber. Calculate the concentration of the cells.

    A Malassez cell is composed of a grid of 25 rectangles of 20 cases (see scheme 2). In order to count a cell solution, count the amount of cells in 10 rectangles. Multiply this number by 10^4 and you have a number of cells per ml.

12. Centrifuge the tube for 3 minutes at 1300 rpm.

13. Aspirate the liquid above the cell pellet that has formed at the bottom of the tube. Be careful not to aspirate the pellet itself!

14. Suspend the pellet in the prepared medium. Put as much medium as needed to obtain a final concentration of cells of 30 000 $\frac{cells}{ml}$. If necessary, make 2 successive dilution.

15. Take the 6-well plate containing the prepared cover slips from incubator. Aspirate the medium.

16. Give 1 ml (=30 000 cells) of cell solution in every chamber. Close the lid and store the 6 well plate back in the incubator.

The cells will need some time to adhere and spread on the patterns. 3 hours should be enough.

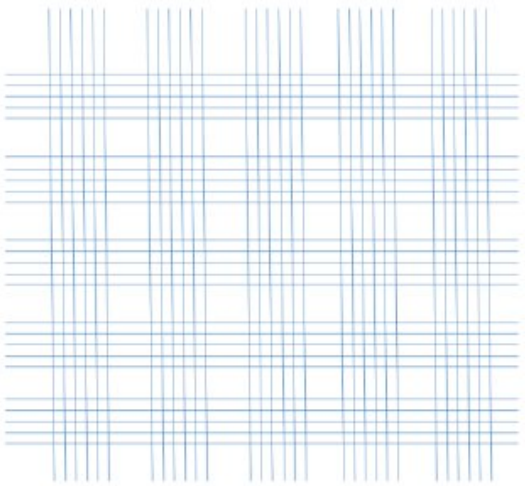

Figure 2: Scheme of a Malassez chamber

2.6 Mounting the magnetic cell chamber

In order to study cells a sample in a form of a Petri dish is preferable to ensure cells having enough nutrients in their surroundings. To that end, the coverslips are mounted on a magnetic chamber which prevents leakage of the cell medium while being compatible with optical microscopy.

1. To mount the chamber, follow the scheme presented on figure 3. All components of the chamber have been sterilized before. To this reason mount them only under the cell culture hood.
2. Place the cover slip with adherend cells facing up.
3. Add 1 ml of PBS to check whether the chamber is well sealed.
4. Aspirate the PBS and replace it with 1 ml cell culture medium.
5. Place all the chamber together in a Petri dish that has been previously wiped with a 70% ethanol in water solution.
6. Place the Petri dish in the cell incubator until you start to take images.

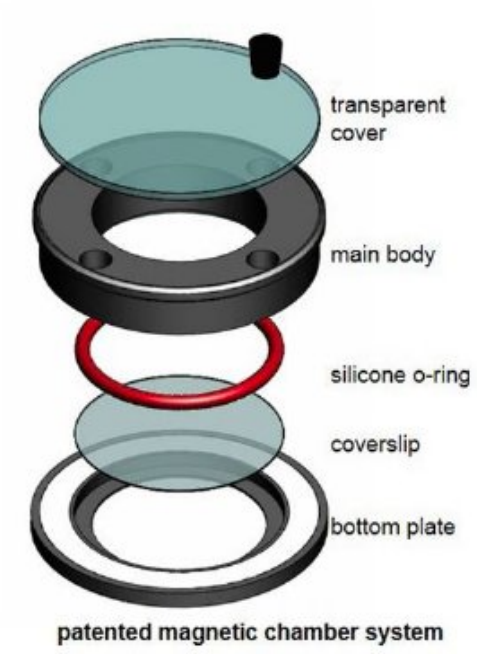

Figure 3: Scheme of a magnetic petri dish (from Chamlide website).

2.7 Imaging cells

The samples will be imaged with a 20 x objective in order to have an optimum between the resolution and the number of cells. You will need to take images of cells in bright field and in fluorescence in order to image microtubules and cell nuclei. During that step you will take as many pictures as possible. There are 4 different patterned shapes. For each shape you need to have a good amount of cases in order to be statistically relevant for the analysis.

3 Analysis

The main goal of this experiment is to investigate whether a correlation between the shape of the cell (or the pattern) and the polarity of the cell exists. The polarity of the cell will be determined by the axis connecting the centrosome (MTOC) and the nucleus. To perform the analysis you have to detect the nucleus and the centrosome on each image. You should bring them in relation to the shape of the pattern. To perform the analysis, the open source software FIJI (Fiji Is Just ImageJ) will be used. The flow of the analysis is:

1. Open the image with its three channels of color.
2. Choose an orientation of the image and rotate it in a direction you will be able to explain. For example, in the case of ellipses, you may choose the orientation of the long axis.
3. Work with the LUT to see how you can play with the contrast and brightness for each color.
4. Specifically on the red channel (microtubule), use the thresholding tool in order to only see the most intense spot which represents the centrosome.
5. Using the line and measure tools, find the direction of the axis going from the center of the nucleus to the centrosome.
6. Represent your results in an understandable manner, showing a comparison for each geometry of pattern, as well as the statistics of this representation. The different possibilities will be discussed during the analysis.

During the analysis try to discuss following questions:

1. How is the axis of nucleus and centrosome correlated to the forced cell shape? Can you find a preferred orientation? To what extend differs the orientation to a random orientation?
2. Where do you find the centrosome and the nucelus relative the center of the cell? What could be a reason?

3. Try to formulate a hypothesis why there should be a connenction of the cell polarity and the cell shape - or why not. (There is no right or wrong hypothesis, however, it should be logically deducted from your results and you should give arguments.)

4 Questions

1. How is polarity defined in physics? How in biology? What are differences or paralles?
2. Find or draw a scheme explaining deep uv micropatterning.
3. Briefly explain composition and function of the nucleus.
4. Give a brief introduction to the cytoskeleton: Which functions does it fulfill? How is it composed? What are the differences of the "cell's cytoskeleton" to the human cytoskeleton.
5. Now give information on microtubule in little more detail. How are they composed? What are their functions?
6. Explain the basic concept of fluorescence? How can one make use of it within cells? What are potential problems?
7. Make a scheme of the set-up of a fluorescence microscope. Think about which additional components are necessary for imaging of living cells.
8. Briefly explain how cells can adhere to a surface. Especially focus on integrin based adhesion and which role stress fibers do play.
9. What are morphological differences of cultured adherent cells (meaning those raised under artificial living conditions in a cell flask) compared to adherent cells inside the body. From your results try to condcut why it could be of interest to investigate cells on micropatterns.
10. Try to find hints why the shape of the cell could be connected to the organization of the cytoskeleton.
11. Calculate which volume of fibronectin stock solution is needed to obtain the working concentration for a total volume of 800 µl.
12. Calculate the volume of the Hoechst solution needed for 10 ml medium.
13. Assumed that you counted 50 cells in the Malassez couting chamber. What is the resulting cell concentration? In how much medium do you need to resuspend the cell pellet in order to get 30 000 $\frac{cells}{ml}$.

5 Litearture recommondations

- Thery, M. (2010). "Micropatterning as a tool to decipher cell morphogenesis and functions." J Cell Sci 123(Pt 24): 4201-4213.
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- Wolpert, L. (2013). "Cell polarity." Philosophical transactions of the Royal Society of London. Series B, Biological sciences 368(1629): 20130419-20130419.