Protocol for a Cell Proliferation Assay Using the µ-Slide Angiogenesis

1. General Information 1
2. Background 1
3. Material and Equipment Needed 2
4. Experimental Procedure 2
   a. Seeding Cells 2
   b. Fixation of the Cells in a µ-Slide Angiogenesis 3
   c. Staining of the Cells in a µ-Slide Angiogenesis 3
5. Image Capture of the Wells 3
6. Data Analysis 4
7. Results 5

1. General Information

This protocol describes a cost-efficient technique for analyzing cell proliferation. The example used in this application note is the analysis of the growth rate of MDCK cells in a µ-Slide Angiogenesis, which is shown below.

2. Background

The µ-Slide Angiogenesis contains a total of 15 wells, as shown in Figure 1. Each well is divided in two parts, an upper well and an inner well. The upper well has a volume of 50 µl and the inner well has a volume of 10 µl, which adds up to a total volume of 60 µl per well.

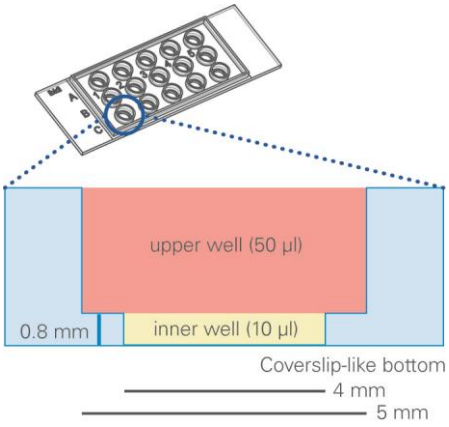

Figure 1: Geometry of a µ-Slide Angiogenesis (ibidi GmbH).

The principle of this cell proliferation assay is that one µ-Slide Angiogenesis will represent the growth of the cells in one day. For this reason, use one µ-Slide Angiogenesis for each day of your experiment. On each day, the cells of one slide will be fixed to stop cell growth. At the end of the experiment, the µ-Slides from all days will be stained with DAPI and images of the wells will be captured with a fluorescence microscope. These images will then be 'stitched' together using ImageJ or FIJI, and the cells in each well will be counted. Finally, a diagram will be created, that will show the growth rate of the cells.

3. Material and Equipment Needed

The following reagents and materials are needed for the given protocol:

Table 1: Material and reagents needed for the cell proliferation assay.

	Name	Concentration for experiment	Company	Order No.
Cells/Material	MDCK cells	1x 10^4 cells/ml	Commercially available	-
	µ-Slide Angiogenesis, ibiTreat	-	ibidi GmbH	81506
Reagents	DMEM	1x	Sigma-Aldrich Chemie GmbH	D5796
	FBS/FCS	10%	Sigma-Aldrich Chemie GmbH	F0804
	PBS	1x	Sigma-Aldrich Chemie GmbH	D8537
	Formalin solution, neutral buffered, 10%	10% formalin (approx. 4% formaldehyde)	Sigma-Aldrich Chemie GmbH	HT50-1-1
	DAPI	0.2 µg/ml	Carl Roth GmbH	6335.1
	Mounting Medium	-	ibidi GmbH	50001

The following equipment and instruments are necessary for the given protocol:

- Cell culture incubator (high humidity, 37°C, 5% CO_2)
- Fluorescence microscope equipped with a DAPI filter set

4. Experimental Procedure

a. Seeding Cells

The first day of the experiment:

1. Prepare a cell suspension (1x 10^4 cells/ml) in DMEM containing 10% FCS.
2. Seed 10 µl of the prepared cell suspension into each inner well (100 cells per well) of all µ-Slides Angiogenesis (prepare one µ-Slide Angiogenesis for each day of the experiment).
3. Place the slides into humidifying chambers (petri dish with a wet sterile tissue, see Figure 2) and incubate them at 37°C until the cells have attached
4. Add 50 µl of growth medium to each well and incubate the slides at 37°C (slide 1 for 24 hours, slide 2 for 48 hours, slide 3 for 72 hours, etc.).

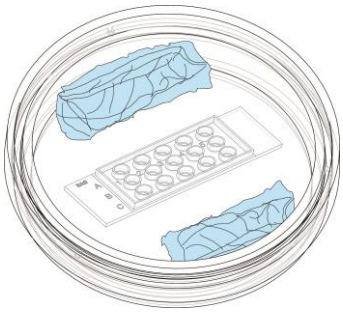

Figure 2: Humid chamber.

b. Fixation of the Cells in a µ-Slide Angiogenesis

Note:
Make a fixation of only one µ-Slide Angiogenesis per day!
The pipette should only touch the upper well (see Figure 3)!

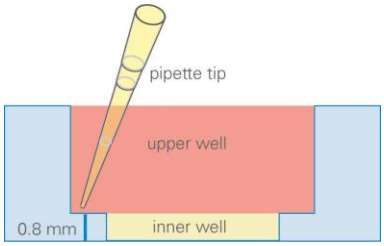

Figure 3: Using the pipette in one well of the µ-Slide Angiogenesis.

The following days of the experiment:

5. Remove 50 µl of medium from each well of the µ-Slide Angiogenesis.
6. Wash the cells twice with 50 µl of 1x PBS per well, and remove the 1x PBS again.
7. Apply 30 µl of Formalin 10% (contains 4% Formaldehyd) to each well, incubate for 5 minutes, and then remove Formalin.
8. Wash the cells twice with 50 µl of 1x PBS per well, and remove 1x PBS again.
9. Apply 50 µl of PBS to each well and store the µ-Slide Angiogenesis at 4°C.

c. Staining of the Cells in a µ-Slide Angiogenesis

The last day of the experiment:

10. Prepare a 1:5 dilution of 1 µg/ml DAPI in 1x PBS (e.g., for one µ-Slide Angiogenesis prepare 180 µl of 1µg/ml DAPI + 720 µl of 1x PBS).
11. Remove the 1x PBS from each well. Do not let the well dry out after aspirating the liquid!
12. Apply 50 µl of the DAPI mixture to the wells of every µ-Slide Angiogenesis.
13. Incubate for 1 hour at 4°C.
14. Remove the DAPI mixture and apply 50 µl of Mounting Medium.

5. Image Capture of the Wells

If the microscope software you are using does not have an automatic image-stitching tool, you can also manually stitch several single images together, as described in this chapter.

Note:
Find out how many images you need to capture with the respective camera of your microscope to give you an overall picture of one well.

Observe the cells under a fluorescence microscope (e.g., Nikon Eclipse Ti-E) with the appropriate filter sets for a DAPI-staining.

With the camera used for this protocol, nine images must be captured to create an overall picture of one well. Make sure that the images are overlapped. It is useful to save the images as “well xy” (Figure 4).

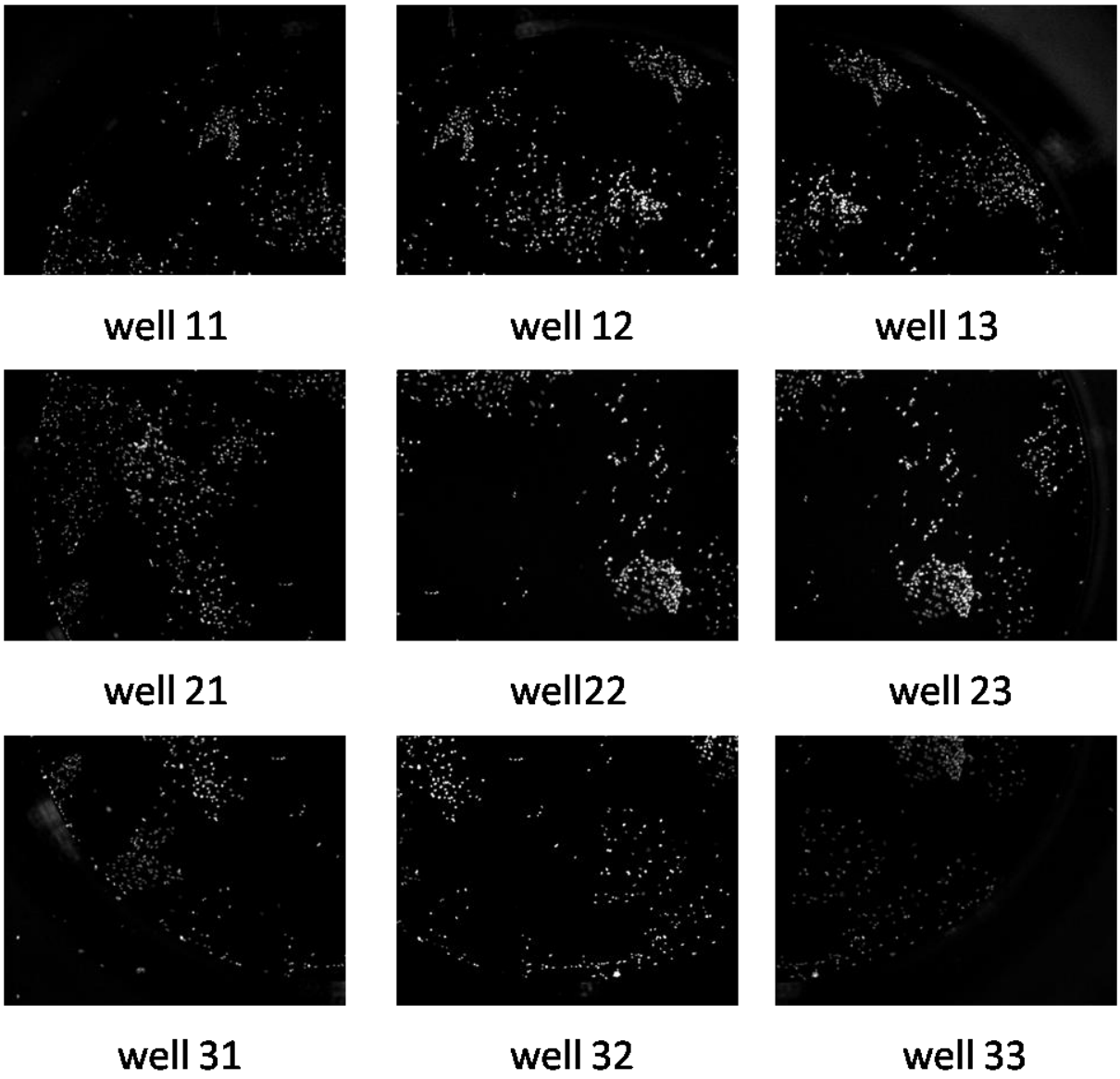

Figure 4: Images of the parts of one well and naming suggestions.

6. Data Analysis

The single images can be “stitched” together with the software ImageJ or Fiji, in order to create one big image for each well. The Fiji software is just ImageJ, with many useful plugins included.

- Download ImageJ here: http://rsb.info.nih.gov/ij/,
  and Fiji here: http://fiji.sc/Downloads.
- Install Fiji or ImageJ and the “Stitching” Plugin.
- Choose: Plugins → Stitching → Pairwise stitching (two images are stitched into one).
- Repeat stitching for all images.

Another plugin that can be used is MosaicJ:

- Install Fiji or ImageJ and the “Stitching” Plugin.
- Choose: Plugins → Stitching → MosaicJ.
- Go to File → Open Image Sequence of all nine images, and then arrange them until the whole well is shown in one image.
- Go to File → Create Mosaic.

7. Results

Figure 5 shows the “stitched-together” pictures. Each picture represents one well at four different time points. These images show how the cells grow within 96 hours.

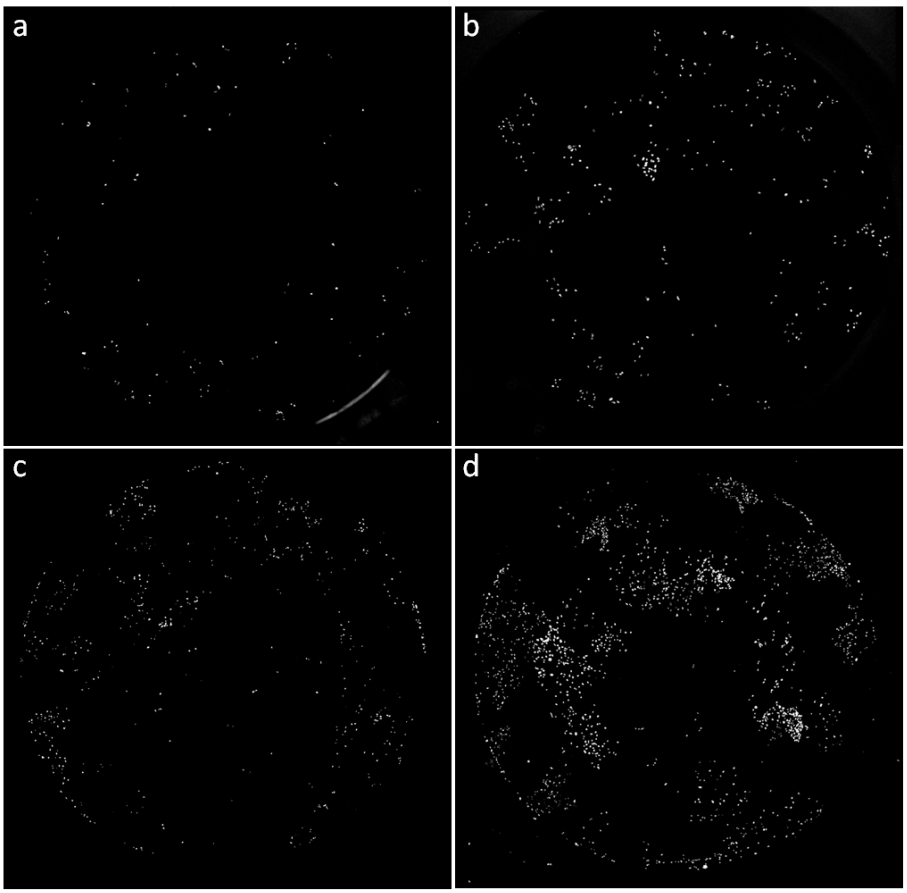

Figure 5: Image of one well of each µ-Slide Angiogenesis. a) One well of the µ-Slide fixed after 24 hours, b) one well of the µ-Slide fixed after 48 hours, c) one well of the µ-Slide fixed after 72 hours, and d) one well of the µ-Slide fixed after 96 hours.

Now these cells can be counted with ImageJ.

- Install the ‘Cell Counter’ plugin for ImageJ.
- Choose: Plugins → Analyze → Cell Counter.
- Count the cells manually.
- The “Results” window will show your total cell number and can be transferred to an excel file, as shown in Table 2.

Table 2: Results of the Cell Counter for total cell numbers.

well	hours			
	24	48	72	96
1	188	371	815	2054
2	181	367	703	1971
3	113	257	803	1735
4	145	297	1047	1830
5	212	358	801	1971
6	209	434	1183	2539
7	247	536	830	1933
8	121	336	1095	2435
9	241	509	861	2595
10	251	502	1156	1992
11	142	351	902	2031
12	167	339	906	1899
13	131	362	1021	2302
14	231	405	798	1997
15	103	393	897	2135
Mean	178.8	387.8	921.2	2094.6
StdDev	51.3	78.5	145.2	257.5

A chart will be created with the values from Table 2 that will show the growth rate of the MDCK cells (Figure 6). Figure 6 confirms that the cell number of MDCK cells continuously increases.

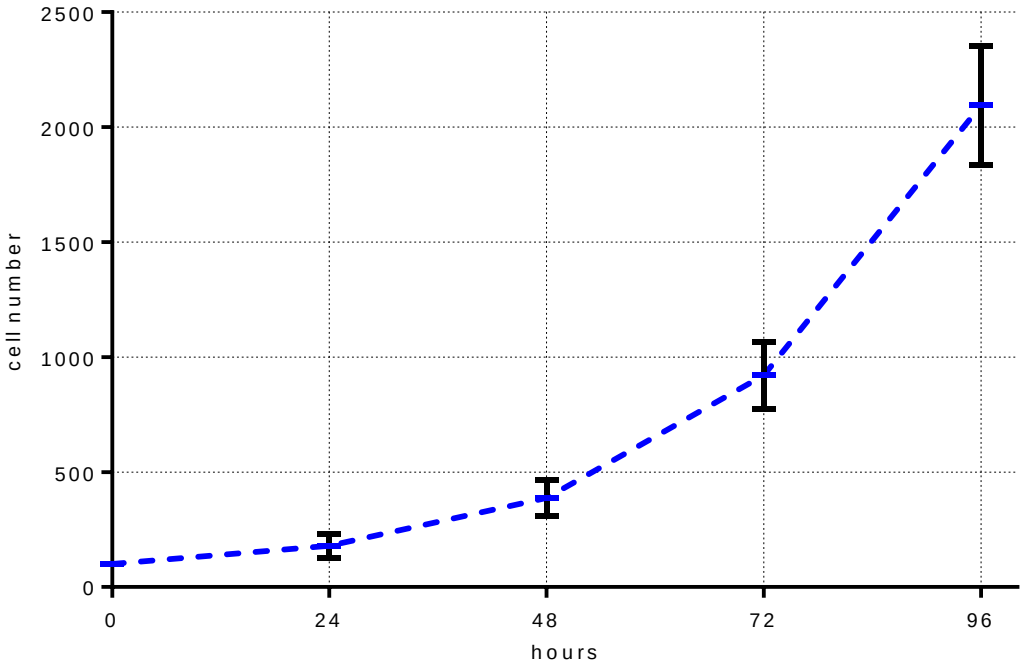

Figure 6: Cell proliferation of MDCK cells.