A progress report for the Latvian Council of Science project conducted within the Fundamental and applied research projects framework "*Engineered surface platform for immobilization of microorganisms*" (lzp-2018/1-0460) on the work done during the 01.03.2018. – 01.06.2019. time frame

The report includes progress information on the following tasks:

- Development of a sterilizable optically opaque enclosure with precisely controlled air temperature and air humidity dedicated specifically for sample irradiation with UV light.
- Cell immobilization studies on immobilization platforms without surface irradiation with UV light.

Achieved results

Development of a sterilizable optically opaque enclosure

An enclosure for sample irradiation with UV light has been developed. It consists of a sealable 300x300x300 mm^2 HDPE container with a sealable 200x200 mm^2 hatch located on the front face of the container through which samples can be inserted into the container, a separate hatch in the center of the top face of the container to which a light source can be connected and another hatch on the containers back face that connects the container to the air conditioning system of the facility where the container is located. The light sources emitter is fixed in place in a vertical position at a constant distance of 20 cm from the surface of the samples. The temperature and humidity levels are monitored using a thermometer/hygrometer circuit with its output located outside of the box and can be controlled using the facilities thermostat and hygrostat controls that function in the range of 10-30^oC and 15-60 RH%. The outside of the enclosure is covered in copper tape to prevent any outside electromagnetic interference from entering the enclosure. Before the samples can be placed into the enclosure, they are inserted into the previously demonstrated sample holder which is then located in the center of the container. Both the insides of the container and the sample holder need to be sterilized with 96% ethyl alcohol before placing the samples into the container. After inserting the samples into the container and the front face door hatch is shut the temperature and humidity need to reach their set values after which the light source can be turned on and sample exposure to UV light can proceed.

Cell immobilization studies without the application of UV light

Using the cell deposition technique developed during this project cell deposition was performed on 4 samples from 2 groups (39-1-6 and 39-1-10) of immobilization platforms. After deposition, sample imaging was performed using a Zeiss Jena NU-2 optical microscope in reflected light mode at x125 magnification. The microscope is equipped with an ocular-mounted digital camera to enable acquisition of digital images and a custom-made motorized stage to enable movement along the XY-plane in precise

and repeatable increments. The camera provided images with an image size of 1600x1200 pixels^2. The surfaces of all 8 samples were imaged totaling the number of images at 1152. Images taken at the borders of the samples were discarded due to consistent higher concentrations of adsorbed cells leaving 800 images to be analyzed at the present moment.

To determine the overall area taken up by adsorbed cells Fiji – a distribution of ImageJ – was used. Since the number of images was large their analysis was automated using Fiji's macro composition capabilities. For this purpose, a set of dedicated algorithms was written the description of which will be given in this section. The following steps were performed:

1. After loading the image in the Fiji environment, darker areas that correspond to adsorbed cells and microstructure edges – further referred to as "clusters" – were marked. Cluster marking was performed with the function "Image / Adjust / Threshold Color", which is able to highlight areas of the image by 'Hue', 'Saturation' and 'Brightness'. Of those three options highlighting only by "Brightness" proved to give the best fit. Therefore, all pixels in the image with a brightness in the range of 0 to x units were marked and for further processing their brightness values set to zero giving a set of completely black pixels. The value of x depends on the contrast of the available image and should be selected so that all cells in the image are marked and the edges of the structures are minimally marked. For 39-1-6 samples x equals 150 and for 39-1-10 samples its value was 160 units.

2. After the clusters were marked, the image was converted from a 32-bit color image into an 8-bit black-and-white image. For all pixels of the image that did not have a brightness value of zero, this was set to 255 to achieve absolute contrast between adsorbed cells / microstructure borders and the background. This was achieved by first applying the "Image / Type / 8-bit" function and then using the "Image / Adjust / Threshold" function, selecting a value from 0 to 3 After these manipulations, the final image colors were inverted by changing the cell/column cluster color to white and the background color to black.

3. After dividing the image into black and white clusters, the area occupied by the white clusters was evaluated. This was done using the "Analyze / Analyze Particles ..." function, which gives out the number of clusters per image and the size of every counted cluster. The function allows to filter out the counted clusters based on their size, i.e. disregard clusters that are too small to be considered as single cells. Thus, clusters with an area smaller than 100 pixels^2 (<6.76 mkm) were not counted. After counting the clusters their data was tabulated and stored in a .csv document for further processing.

This algorithm was converted into a *macro* and used to extract data from every valid image *en masse*. Later, the gathered data was processed using Microsoft Excel using the following approach:

1. The cluster areas obtained from each image were grouped into columns with data in each column corresponding to all cluster areas derived from a single image. For all images, the total area occupied by clusters was calculated.

2. Since the clusters gathered during the processing stage corresponded both to adsorbed cell area and the microstructure area the additional area taken up by the microsctructures had to be subtracted. The way the microstructures were marked, however, differs for both sample groups

used during this study. Therefore, a dedicated approach to data analysis should be used for samples in groups with different microstructures: 39-1-6 and 39-1-10 (Table 1).

a. **Processing of 39-1-6 sample data**. The average size of a column cluster without attached cells was found then multiplied by the number of columns per each image and subtracted from the total cluster area of that picture. Since most images had few attached cells it was easy to calculate the average area of a column cluster which was in the range of 2000 to 3000 pixels^2. This value corresponds to the microstructure sizes attained from samples that didn't undergo cell deposition, thus the value can be considered as reliable. Since the microstructures are distributed on the wafer surface in a repeating grid-like pattern and the area of each image is the same it is possible to calculate the number of microstructures per image which was about 240 structures per image. Then, by multiplying the average number of columns by the average column cluster area for each image, it was possible to obtain the area occupied by the columns in each given image. In the end, this area was subtracted from the total cluster area to get the area of the attached cells for each image. Since the area of a structure was calculated as the total area of an enclosed particle and then subtracted, it was impossible to count the cells that were attached to the plateaus. Thus, only the area taken up by cells inside of the valleys was calculated.

b. **Processing of 39-1-10 sample data**. The average size of a column cluster without attached cells could not be found since the column outlines did not form closed-area clusters. Only partial column outlines could be extracted from the images. While some of the smaller values (area <100 pixels^2) could be filtered out during data extraction, the larger values could be mistaken for cell clusters and would get lost among the valid data. Thus, the "average column area subtraction" method used for 39-1-6 could not be used for these samples and an alternative had to be developed. In this new approach several hundred images were taken from 39-1-10 samples using the same microscope and camera settings as were used for samples with cells deposited after which clusters were extracted using the developed algorithm. Since the extracted cluster area corresponds only to the area taken up by the columns it can be averaged over multiple images of clean surfaces and then subtracted from every cluster area sum for images taken from samples that underwent cell deposition providing one with a corrected total deposited cell area. Since this approach subtracted only parts of the microstructures and not whole plateaus some of the cells that were attached at the plateaus were counted together with the cells located inside of valleys, making this approach functionally different from the one used for 39-1-6 samples. However, this difference can be considered as negligible since very few cells were attached at the plateaus.

3. After calculating the surface area taken up by adsorbed cells per image the total area of adsorbed cells per sample was found, after which an average covered area was calculated for each sample group.

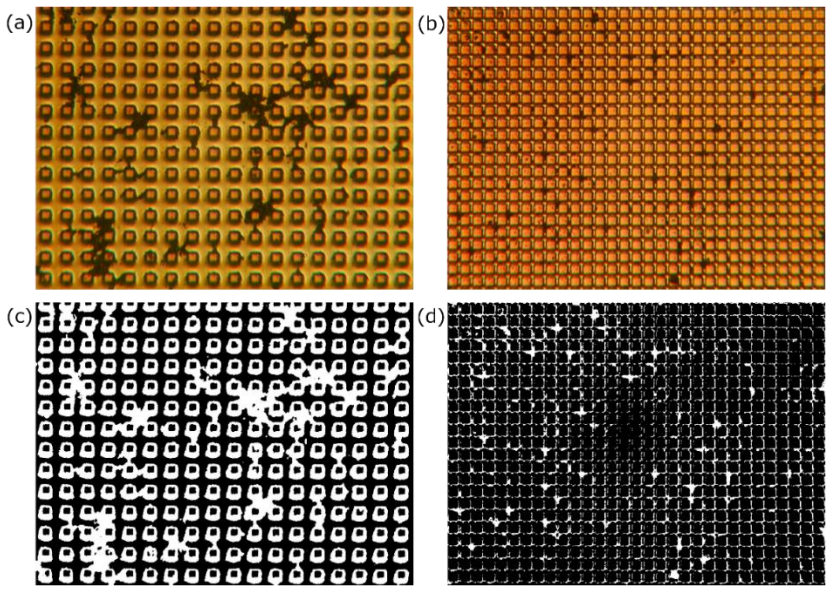

Figure 1. *Image samples of FOVs used during analysis: (a), (c) image of a 39-1-6 sample before and after processing, respectively; (b), (d) image of a 39-1-10 sample before and after processing, respectively.*

The algorithm was used to process images that did not contain any visible traces of the samples edge. A total of 1042 images were processed using this method. Samples of pre-processed and post-processed images can be seen in Figure 1.

For 39-1-6 and 39-1-10 samples the ratio of the average area taken up by adhered cells to the total surface area of an FOV (from this point referred to as cell/surface ratio) was 5±2.66 % and 4.43±3.59%, respectively. The distribution of areas for each sample group is given in Figure 2.

The algorithm is not perfect since a) it doesn't count cells that became attached to the plateaus (as few cells as there were it still makes the data less accurate) and b) it uses two different approaches for processing two different groups of samples. The latter could result in a major discrepancy between the computed results and the actual ones. Therefore, an improved algorithm needs to be developed.

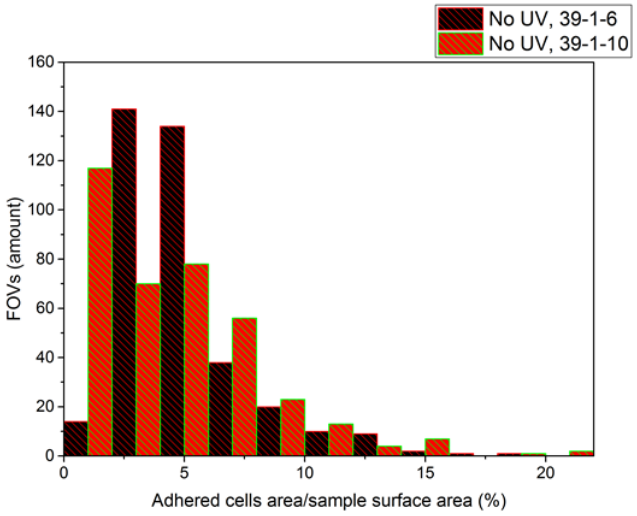

Figure 2. *Distribution of average adhered cells/surface ratio values acquired using Algorithm #1.*

Conclusion

A sterilizable enclosure for performing sample irradiation with UV light has been developed and will be used during sample charging experiments.

Cell deposition experiments on non-irradiated samples have been performed. The results show a difference in the amount of attached cells that depends on the size of the space in between the microstructures present on the platforms surface.