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Live-cell Imaging of Single-Cell Arrays (LISCA) – a versatile technique to quantify cellular kinetics

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TITLE

Live-cell Imaging of Single-Cell Arrays (LISCA) – a versatile technique to quantify cellular kinetics

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KEYWORDS

Single-cell analysis, micro-structured arrays, time-lapse microscopy, cellular kinetics

SUMMARY

We present a method for the acquisition of fluorescence reporter time courses from single cells using micropatterned arrays. The protocol describes the preparation of single-cell arrays, the setup and operation of live-cell scanning time-lapse microscopy and an open-source image analysis tool for automated preselection, visual control and tracking of cell-integrated fluorescence time courses per adhesion site.

ABSTRACT

Live-cell Imaging of Single-Cell Arrays (LISCA) is a versatile method to collect time courses of fluorescence signals from individual cells in high throughput. In general, the acquisition of single-cell time courses from cultured cells is hampered by cell motility and diversity of cell shapes. Adhesive micro-arrays standardize single-cell conditions and facilitate image analysis. LISCA combines single-cell microarrays with scanning time-lapse microscopy and automated image processing. Here, we describe the experimental steps of taking single-cell fluorescence time courses in a LISCA format. We transfect cells adherent to a micropatterned array using mRNA encoding for enhanced green fluorescent protein (eGFP) and monitor the eGFP expression kinetics of hundreds of cells in parallel via scanning time-lapse microscopy. The image data stacks are automatically processed by newly developed software that integrates fluorescence intensity over selected cell contours to generate single-cell fluorescence time courses. We demonstrate that eGFP expression time courses after mRNA transfection are well described by a simple kinetic translation model that reveals expression and degradation rates of mRNA. Further applications of LISCA for event time correlations of multiple markers in the context of signaling apoptosis are discussed.

INTRODUCTION

In recent years, the importance of single-cell experiments has become apparent. Data from single cells allow the investigation of cell-to-cell variability, the resolution of intracellular parameter

correlations and the detection of cellular kinetics that remain hidden in ensemble measurements[1-3]. In order to investigate cellular kinetics of thousands of single cells in parallel, new approaches are needed that enable monitoring the cells under standardized conditions over a time period of several hours up to several days followed by a quantitative data analysis [4]. Here, we present Live-cell Imaging of Single-Cell Arrays (LISCA), which combines the use of microstructured arrays with time-lapse microscopy and automated image analysis.

Several methods for generating microstructured single-cell arrays have been established and published in literature[5,6]. Here, we briefly describe Microscale Plasma-Initiated Protein Patterning (µPIPP). A detailed protocol of the single-cell array fabrication using µPIPP is also found in reference[7]. The use of single-cell arrays enables alignment of thousands of cells on standardized adhesion spots presenting defined microenvironments for each cell and thus reduces a source of experimental variability (**Figure 1A**). Single-cell arrays are used to monitor the time courses of fluorescent markers purposed to indicate a variety of cellular processes. Long-term microscopy in scanning time-lapse mode allows for monitoring a large area of the single-cell arrays and hence sampling single-cell data in high-throughput over an observation time of several hours or even days. This generates time-line stacks of images from each position of the array (**Figure 1B**). In order to reduce the large amount of image data and to extract the desired single-cell fluorescence time courses in an efficient way, automated image processing is required that takes advantage of the positioning of cells (**Figure 1C**).

The challenge of LISCA is to adapt the experimental protocols and computational tools to form a high-throughput assay that generates quantitative and reproducible data of cellular kinetics. In this article we provide a step-by-step description of the individual methods and how they are combined in a LISCA assay. As an example, we discuss the time course of enhanced green fluorescent protein (eGFP) expression after artificial mRNA delivery. eGFP expression following mRNA delivery is described by reaction rate equations modeling translation and degradation of mRNA. Fitting the model function for the time course of eGFP concentration to the LISCA readout of the fluorescence intensity for each individual cell over time yields best estimates of model parameters such as the mRNA degradation rate. As a representative result we discuss the mRNA delivery efficiency of two different lipid-based transfection agents and how their parameter distributions differ.

[Place Figure 1 here]

PROTOCOL

[Place Figure 2 here]

1. Microstructured single-cell array fabrication (Figure 2A)

1.1. Prepare the materials needed for µPIPP array fabrication.

1.1.1. Prepare sterile phosphate-buffered saline (PBS) at pH 7.4.

1.1.2. Prepare sterile ultrapure water with a resistivity of at least 18 MΩcm at 25 °C.

1.1.3. Prepare PLL(20 kDa)-g[3.5] PEG(2 kDa) (PLL-PEG) working solution with a 2 mg/mL concentration of PLL-PEG in ultrapure water containing 150 mM NaCl and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

1.1.4. Prepare an extracellular matrix protein solution for surface coatin: 1 mg/mL fibronectin (FN) in PBS.

1.1.5. Prepare a silicon wafer with a micropattern fabricated by photolithography[8] that functions as a reusable master. The micropattern consists of squares with an edge length of 30 µm, a depth of 12 µm and an inter-square distance of 60 µm, arranged in six stripes each having a width of 6 mm and a height of 18 mm.

1.2. Mix a polydimethylsiloxane (PDMS) monomer with 9% crosslinker (mass %) using a silicone elastomer kit and degas it for about 30 min until it is bubble free using a desiccator. Cast the silicon wafer with a roughly 3-5 mm thick PDMS layer and degas it again for about 30 min until it is bubble-free.

1.2.1. Put the silicon wafer with the PDMS in a baking oven at 50 °C to cure the PDMS for at least 4 h.

1.3. Cut the PDMS stamps.

1.3.1. Use a scalpel and cut out of the PDMS layer one PDMS masterpiece that contains the six micropattern stripes.

1.3.2. Place the PDMS masterpiece on a bench with the micropattern facing upward.

1.3.3. Cut each of the six micropattern stripes of the PDMS masterpiece with a razorblade into a PDMS stamp. Take care that the edges of the PDMS stamps are open by cutting off some of the patterned area.

1.4. Place the PDMS stamps on a coverslip of a six-channel slide (**Figure 3-1**).

1.4.1. Use an uncoated coverslip and mark the channel positions of the six-channel slide by carefully scratching the protection foil of the coverslip. Then place the coverslip on the bench with the protection foil facing downward.

1.4.2. Place the PDMS stamps with the micropattern facing downward on the coverslip at the marked channel positions using tweezers.

1.4.3. Check the attachment of the PDMS stamps under a microscope. If a PDMS stamp is fully

attached to the coverslip, the squares in contact appear darker than the interspace. The attachment of the PDMS stamp to the coverslip is crucial for the micropattern quality.

1.5. Place the coverslip with the six PDMS stamps on it into a plasma cleaner and treat it with oxygen plasma (pressure 0.2 mbar, ~40 W for 3 min) to make the surfaces between the PDMS stamps and the coverslip hydrophilic (**Figure 3-2**).

1.6. Carry out all further steps of the micropattern fabrication in a biosafety cabinet. Use 15 µL of the PLL-PEG solution and pipette one drop of it next to each PDMS stamp so that the PLL-PEG solution is absorbed into the hydrophilic pattern of the PDMS stamp (**Figure 3-3**). Let the PLL-PEG incubate for 20 min at room temperature.

1.7. Rinse 1 mL of ultrapure water over the coverslip with the PDMS stamps on it and remove the PDMS stamps using tweezers (**Figure 3-4**). Then rinse the coverslip a second time with 1 mL of ultrapure water and let it dry.

1.8. When the coverslip has dried completely, stick a six-channel sticky slide to the coverslip (**Figure 3-4**). Take care that the micropatterned areas align with the bottom of the channels.

1.9. Functionalize the adhesion squares with FN.

1.9.1. Fill 40 µL of PBS into each channel.

1.9.2. Prepare a 100 µg/mL FN solution in PBS.

1.9.3. Add 40 µL of the FN solution to each channel (**Figure 3-5**). Mix the FN solution with the PBS in the channel thoroughly by removing 40 µL from one reservoir and adding it to the opposite reservoir of the same channel for 3 times to generate a homogeneous solution. Incubate the FN solution for 45 min at room temperature.

1.9.4. Wash each channel three times with 120 µL of PBS (**Figure 3-6**).

1.10. In order to check for the pattern quality, us a fluorescently labeled FN in step 9.2. (**Figure 4A**).

NOTE: We recommend preparing the µPIPP array not more than one day before cell seeding as PLL-PEG and FN are not covalently bound to the substrate and the quality of the pattern may decrease over time. Store the prepared µPIPP array in the fridge.

[Place Figure 3 here]

2. Cell seeding (Figure 2A)

NOTE: For the following washing steps, add the respective liquid to one reservoir and then

remove an equal volume of liquid from the opposite reservoir of a channel.

2.1. Wash each channel with 120 µL of 37 °C fully supplemented cell growth medium. Before adding the cell suspension, ensure that only the channels are filled with medium but not the reservoirs.

2.2. Detach HuH7 cells from a cell culture flask following your standard protocol for cell passaging and adjust the cell suspension concentration to 4 x 10^5 cells/mL.

2.3. Add 40 µL of cell suspension and mix the cell growth medium with the cell suspension by removing 40 µL from one reservoir and adding it to the opposite reservoir of the same channel for 3 times to reach a homogeneous cell distribution (**Figure 4B**).

2.4. Remove 40 µL of suspension from the channel so that only the channel is filled with cell suspension.

2.5. Put the slide in an incubator and check cell adhesion 1 h after seeding using a phase-contrast microscope.

2.6. Add 120 µL of 37 °C warm cell growth medium.

2.7. But the slide back in the incubator for further 3 h to enable cellular self-organization on the micropattern (**Figure 4C**).

[Place Figure 4 here]

3. Perfusion system (Figure 2A)

NOTE: The use of a perfusion system is only required if reagents or fluorescent markers need to be added during the course of the time-lapse measurement. Depending on your needs, you can connect each channel to a separate perfusion system or connect several channels in series to the same perfusion system. The number of perfusion systems corresponds to the number of independent experimental conditions. Connect the tubes under sterile conditions in a biosafety cabinet and avoid the inclusion of air bubbles in the perfusion system. If no perfusion system is used, add the reagents/markers in a biosafety cabinet before the time-lapse measurement. The perfusion system is in-house fabricated, the used material is listed in the **Table of Materials**. The assembly of the perfusion system has been described previously[9].

3.1. Use a 1 mL syringe (with replacement sporn) and fill the syringe with 1 mL of 37 °C cell growth medium.

3.2. Connect the syringe to the inlet tube using the valve and fill the tube with medium.

3.3. Connect the inlet tube to a reservoir of a channel and make sure that no air bubbles are

trapped.

3.4. To connect another channel in series to this perfusion system, connect a serial connector to the reservoir opposite to the inlet tube of the current channel. Proceed to the next channel and connect the free end of the serial connector to one of its reservoirs.

3.5. Repeat the previous steps until the required number of channels are connected in series.

3.6. Connect the outlet tube directly to the free reservoir of the current channel. Fill the connected tube with medium in order to check that the perfusion system does not leak.

3.7. Repeat the previous steps until all six channels of the slide are connected to a perfusion system.

3.8. Place the slide with the connected perfusion system(s) back in the incubator until further use or place it directly in the heating chamber of the microscope pre-warmed to 37 °C for time-lapse measurement.

4. Time-lapse microscopy (Figure 2B)

NOTE: For long-term measurements, maintain a stable temperature of 37 °C and a stable CO_2 level. As an alternative to CO_2-dependent cell growth medium, use L15 medium for which no gas incubation system is required.

NOTE: For quantitative imaging, use cell growth medium without phenol red during the time-lapse measurement to reduce background fluorescence and use the same settings of the time-lapse protocol as well as the same microscope for technical replicates.

4.1. Set up a time-lapse protocol for recording a phase-contrast image and a fluorescence image with exposure times of 750 ms (depending on the camera), 10 min time interval between consecutive loops through the position list, and an observation time of 30 h, using a 10x objective and appropriate fluorescence filters.

4.2. Put the six-channel slide with the cells on the single-cell arrays in the sample holder of the 37 °C warm heating chamber. If perfusion systems are connected to the six-channel slide, fix the tubes to the stage using some tape to ensure that the six-channel slide is not moved during liquid exchange. Insert the free ends of the outlet tubes through a hole of a 15 mL reaction tube to collect the liquid waste.

4.3. Set the position list for the scanning time-lapse measurement. Ensure that the number of positions can be scanned within the defined time interval between consecutive loops through the position list. With a 10x objective, 10-30 positions per channel can be set to scan the total micropattern area depending on the camera chip size.

4.4. Start the time-lapse measurement. For a better image quality of long-term measurements, use an automated focus correction system.

5. Fluorescent marker – mRNA transfection (Figure 2B)

NOTE: For a transfection in two channels connected by a tubing system, a total volume of 600 µL transfection mix is needed (300 µL for one channel). The indicated volumes refer to a transfection in two connected channels.

5.1. Prepare a transfection agent solution by diluting 1 µL of transfection agent in 200 µL of serum-reduced medium and let the solution incubate for 5 min at room temperature.

5.2. Prepare a mRNA solution by diluting 300 ng of mRNA encoding for eGFP in 150 µL of serum-reduced medium.

5.3. Prepare the transfection mix by adding 150 µL of the transfection agent solution to the mRNA solution and mix it well. Let the transfection mix incubate for 20 min at room temperature.

5.4. Flush the tubing system with 1 mL of 37 °C warm PBS using a syringe during incubation of the transfection mix. When flushing the tubes, make sure that the microscope stage does not move. Pause the time-lapse measurement if necessary.

5.5. Dilute the transfection mix to the final mRNA concentration of 0.5 ng/µL by adding 300 µL serum-reduced medium.

5.6. Flush the tubing system with the transfection mix using a syringe and let the mRNA lipoplexes incubate for 1 h (pause the time-lapse measurement if necessary).

5.7. Stop the transfection incubation and flush out the unbound mRNA lipoplexes by washing with 1 mL of 37 °C warm fully supplemented cell growth medium using a syringe (pause the time-lapse measurement if necessary).

6. Image analysis and fluorescence readout

6.1. When running the image analysis for the first time, install version 0.1.5 of the open-source software "Automated Microstructure Analysis in Python" (PyAMA) from the cited location[10] according to the instructions provided there.

6.2. Ensure that the image channels (phase-contrast and fluorescence) are available as multi-image 16-bit TIFF files. If necessary, convert them accordingly.

6.3. Start PyAMA and click on **Open stack…** to open images for analysis.

6.4. For each multi-image TIFF file to open, click on **Open** and select the file so that it is

displayed in the list of loaded files on the left side of the dialog (**Figure 5-1**).

6.5. Mark the channels to include in the analysis. For each channel, perform the following steps.

6.5.1. Select in the list of loaded files the TIFF file containing the channel.

6.5.2. In the section **Add new channel**, select the index of the channel in the TIFF file. Indexing is zero-based; the first channel has index 0, the second channel has index 1 and so on.

6.5.3. Select the channel type. Select **Phase contrast** or **Fluorescence** for the corresponding image channels and Segmentation for a binary channel indicating the cell contours.

6.5.4. Optionally, enter a label of the channel for distinguishing different fluorescence channels: eGFP and DAPI.

6.5.5. After configuring the channel, click **Add**.

6.6. When all added channels are displayed in the channel list on the right side of the dialog, click **OK** to load the stack.

6.7. To perform segmentation using PyAMA's built-in segmentation algorithm for cell recognition based on the phase-contrast images (**Figure 5-2**), go to **Tools | Binarize**… and enter a file name for the NumPy file with the binarized channel.

NOTE: In the current version, loading the binarized channel requires reloading all channels.

6.8. To perform a background correction[11] on a fluorescence channel (Figure 5(3)), ensure that the fluorescence channel and a segmentation channel are loaded. If no segmentation channel is loaded, ensure that a phase-contrast channel is loaded for automatic segmentation. Go to "Tools > Background correction…" and select a file name for the resulting TIFF file with the corrected fluorescence channel.

NOTE: In the current version, loading the background-corrected channel requires reloading all channels.

6.9. Inspect the pre-selected cells (**Figure 5-4**) and their integrated fluorescence signal (**Figure 5-5**) by scrolling through the time frames, viewing the channels listed in the channel menu on the left side and clicking on cells to highlight their fluorescence time courses (**Figure 1C**). Use the cell selection to exclude cells that are not viable, not confined to an adhesion spot or attached to another cell from further analysis. Toggle the selection of cells for readout by pressing **Shift** and clicking on the cell, or by highlighting the cell and pressing **Enter**.

6.10. Save the single-cell time courses for the cell area and the integrated fluorescence (**Figure**

5-6) by clicking on **File | Save** and selecting a directory to save to.

[Place Figure 5 here]

7. Single-cell time course analysis

7.1. To analyze the translation kinetics after mRNA transfection, fit a translation model based on biochemical rate equations to each single-cell time course as described previously by Reiser et al.[12]. The data and code used in that study are publicly available[13].

7.2. For each single-cell time course, retrieve the estimated fitting parameters of the translation model that represent the mRNA degradation rate and the time point of translation onset. An example data set is discussed in the representative results section.

7.3. Perform further analysis on the distributions of best estimates of the parameters for varied experimental conditions to investigate the cell-to-cell variability within the cell populations.

REPRESENTATIVE RESULTS

The LISCA approach enables to efficiently collect fluorescence time courses from single cells. As a representative example we outline how the LISCA method is applied to measure single-cell eGFP expression after transfection. The data of the LISCA experiment is used to assess mRNA delivery kinetics, which is important for the development of efficient mRNA drugs.

In particular we demonstrate the different impact of two lipid-based mRNA delivery systems with respect to the time point of translation onset and the expression rate at the single-cell level. We cultured cells and divided the batch into two populations. One subpopulation was transfected with lipoplexes as described in the protocol section. The other subpopulation was transfected using the same mRNA with the same final mRNA concentration, but with lipid nanoparticles (LNP) as delivery system, which were produced using microfluidic mixing[12]. Due to a different lipid composition and the different fabrication of the mRNA delivery systems of the lipoplexes and the LNPs we expect an impact on the translation kinetics as the uptake kinetics should be influenced. Using the LISCA method we quantify the time point t_0 of translation onset after the transfection and how strong the cells express eGFP, which depends on the product of transfected mRNA molecules m_0 and the translation rate k_{TL}. To obtain these two parameters we fit a three-stage translation model as sketched in **Figure 6A**. After successful release of the mRNA molecules m at time point t_0 in the cytosol, the mRNA is translated with rate k_{TL} into unmaturated eGFP G^*, which is non-fluorescent. The unmaturated G^* matures with rate k_M to eGFP G, the fluorescence intensity of which is measured during the time-lapse measurement. The mRNA as well as the (unmaturated and maturated) eGFP degrade over time with respective degradation rates δ and γ. The model is described by ordinary differential equations and the analytical solution for G is used as a model function for parameter estimation. The model function is fitted to each of the single-cell time courses as shown in **Figure 6B** with example time courses (grey) and the respective fits (green). In **Figure 6C** we show the histograms of the time point t_0 of translation

onset and the expression rate $m_0 k_{TL}$ of lipoplex transfected cells. As both parameters are estimated for each cell, the correlation of these parameters can be analyzed as shown in the scatterplot (Figure 6D blue data) and can be compared to cells transfected with LNPs (red). As shown in Figure 6D, cells transfected with LNPs show less cell-to-cell variability compared to cells transfected with lipoplexes and the population average shows a faster onset of translation as well as a higher expression rate (thick dots with black outline).

These two data sets are just one example how LISCA can be used to study translation after mRNA transfection. Further investigations can be made for example with regard to mRNA stability dependent on mRNA sequence modifications [14], varied reporter protein stabilities [15], or siRNA mediated mRNA degradation [16].

[Place Figure 6 here]

Figure 1: Representation of the LISCA workflow combining (A) micro-patterned single-cell arrays (B) scanning time-lapse microscopy and (C) automated image analysis of recorded image series. The single-cell arrays consist of a two-dimensional pattern of cell-adhesive squares with a cell-repellent interspace leading to an arrangement of the cells on the micropattern, as can be seen in the phase-contrast image as well as the fluorescence image of eGFP expressing cells (**A**). The entire microstructured area is imaged in a scanning time-lapse mode repeatedly taking images at a sequence of positions (**B**). Recorded image series are processed to read out the fluorescence intensity per cell over time (**C**). Scale bars: 500 µm (**A**), 200 µm (**C**).

Figure 2: Data acquisition combining single-cell microarrays (A) with scanning time-lapse microscopy (B). As preparation of the time-lapse experiment, a single-cell array with a 2D micropattern of adhesion squares is prepared (1), followed by cell seeding and the alignment of the cells on the micropattern (2) as well as the connection of a perfusion system to the six-channel slide, which enables liquid handling during the time-lapse measurement (3). A scanning time-lapse experiment is set up (4) and the cells are transfected on the microscope by injecting an mRNA lipoplex solution through the perfusion system during the time-lapse experiment (5). Scale bars: 200 µm.

Figure 3: Single-cell microarray fabrication by µPIPP. (1) PDMS stamps with a three-dimensional micropattern structure on the surface are arranged on a coverslip of a six-channel slide. (2) The coverslip with the PDMS stamps on it is treated with oxygen plasma to make the surfaces hydrophilic. (3) PLL-PEG is added. It is absorbed into the microstructure by capillary forces and makes the surfaces not covered by the PDMS stamp cell-repellent. (4) The coverslip is rinsed with water to remove the remaining PLL-PEG. Then, the PDMS stamps are removed and a six-channel sticky slide is stuck unto the coverslip. (5) Fibronectin, a protein of the extracellular matrix, is added to make the areas without PLL-PEG cell-adhesive. (6) The six-channel slide is washed with phosphate-buffered saline.

Figure 4: Cellular self-organization and quality control of µPIPP array. (**A**) The microstructured surface consists of squared FN-coated adhesion spots shown in red surrounded by a cell-repellent

polymer. (**B**) After cell seeding, the HuH7 cells are randomly distributed and (**C**) adhere mainly on the adhesion spots over a time period of 4 h. Reprinted with permission [7]. Scale bars: 200 µm.

Figure 5: Automated image processing of time-lapse image series using PyAMA. (1) Phase-contrast and fluorescence image series for each imaging position are imported. (2) Cell contours are determined by segmentation on the phase-contrast image stack. (3) A background correction is applied to the fluorescence images. (4) The cell contours are tracked over time and pre-selected for export. (5) The fluorescence intensity is integrated based on the tracked cell contours. (6) Single-cell cell areas and integrated fluorescence intensities are evaluated and time courses for each cell are exported. Scale bars: 100 µm.

Figure 6: Data analysis of single-cell eGFP translation kinetics. (**A**) The translation kinetics of the reporter protein eGFP after mRNA delivery can be described by a three-stage reaction rate equation with the respective parameters. (**B**) The model is fitted (green traces) to each single-cell eGFP expression time course (gray traces) to estimate the model parameters such as the time point (t_0) of transfection onset and the expression rate (m_0k_{TL}), two parameters to quantify mRNA delivery efficacy. (**C**) Histograms of the parameter distribution for the transfection onset time and the expression rate. (**D**) As the parameters are estimated for each cell, a scatter plot of these parameters shows parameter correlation. The small dots represent the parameters of a single cell. The plot shows cells transfected with mRNA LNPs (red) and mRNA lipoplexes (blue). The thick dots with black outline correspond to the respective population average.

DISCUSSION

Here we described LISCA as a versatile technique to follow cellular kinetics of intracellular fluorescent labels at the single-cell level. In order to perform a successful LISCA experiment, each of the described steps of the protocol section must be established individually and then all steps must be combined. Each of the three major aspects of LISCA feature crucial steps.

Single-cell microarray fabrication

The quality of the microarray is crucial as the cellular alignment on the microarray is not only important for all further experimental steps but also has influence on the data quality. For this reason, the geometry of the pattern and the fabrication method must be adapted with respect to the used cells. The representative results discussed in this article have been generated with the liver carcinoma cell line HuH7 aligned on a $(30\ \mu m)^2$ square pattern. This pattern geometry is also suitable for other cell lines such as A549 or HEK293. For larger cells such as the BEAS-2B, a larger square pattern with 35 µm edge length and an inter-square distance of 80 µm can be used. The described seeding procedure is optimized for the HuH7 cells but it can be adapted to many other adherent cells[17]. For example, some cell lines need different sizes of the adhesion spots or need larger spacing between the adhesion spots to avoid cell-cell contacts through elongated cells.

The micropattern should be adjusted such that the adhesion site area meets roughly the average area of a cell in standard culture dishes. The geometry can be round or squared and has no measurable effect of cell viability. Cell motions seem to be more restricted on a squared pattern

compared to round pattern, where cells are often seen to rotate. Separate viability testing is recommended when new cell lines are used for the first time. Seeding density needs to be adjusted for specific cell lines to reach a high occupancy of the adhesion spots and to minimize double occupancies of adhesion spots at the same time. Typically, the resulting occupancy of number of cells per adhesion site follows Poisson statistics and is either zero, one or two. Hence total occupancy between 60% and 80% should be aimed at to avoid double occupancies[17]. The seeding protocol may need to be adapted regarding the number of seeded cells and the time duration between seeding and the first washing step. For example, a washing step 30 min after seeding instead of 1 h (see steps 5 and 6 of protocol section 5) will reduce the number of double-occupied adhesion spots but will also lower the total number of occupied adhesion spots for HuH7 cells.

As the connection of a tubing system to the channel slides is not mandatory, it is easier to establish the use of a reagent or fluorescent marker without using a tubing system to check for the best suitable concentration and incubation time. After a suitable protocol is established for the reagent/marker of interest, the tubing system can be included in the workflow.

Time-lapse microscopy

Another crucial step are the settings of the time-lapse measurement. For example, the exposure time for the fluorescence marker must be chosen carefully to avoid photobleaching of the fluorophore and phototoxicity effects but still ensure a good fluorescence signal. Another important parameter of the time-lapse setup is the best suitable combination of spatial and temporal resolution, which heavily depends on the observed cellular kinetics. If a high temporal resolution is needed, only a smaller number of positions in the microarray can be scanned between two time points, which reduces the scanned observation area and, thus, also reduces statistics. The observation area is furthermore not only dependent on the number of scanned positions but also on the objective and the size of one field of view of the camera. In the given example, a time resolution of 10 min is sufficient using a 10x objective to observe the translation kinetics. This combination allows to scan 70-100 positions per time point depending on the size of the camera chip, the speed of the microscope stage, and the number of imaging channels (e.g., phase-contrast and eGFP fluorescence).

Image processing

The analysis of the total fluorescence per cell is facilitated as the cells are positioned in an array. Yet, the quality of the single-cell fluorescence time courses, in particular the signal-to-noise ratio (SNR), depends on the algorithm used for the integration of cell fluorescence intensities from the image series. The image processing steps of the software tool PyAMA are shown in **Figure 5**. The determination of the integration areas for the single-cell fluorescence readout is particularly important because the cell contour of living cells varies with time. PyAMA integrates the fluorescence intensity over a cell contour, which can be determined by a built-in segmentation algorithm. An alternative would be to integrate over fixed boundaries based on the micropattern geometry[12,16]. The current version of PyAMA performs image segmentation based on a threshold on the standard deviation of pixel neighborhoods of the phase-contrast image channel. Segmentation results can be imported from external software as well. For future versions, native

support for segmentation based on machine learning and for integration over fixed boundaries is planned.

PyAMA offers to filter out outlier cells or anomalous time courses using an interface that allows for visual inspection of both the fluorescence image as well as the fluorescence time courses of selected cells (**Figure 1C**). Examples of cells that may be excluded from analysis are cells that undergo division or apoptosis or that are located outside of an adhesion site. Aggregations of multiple cells erroneously recognized by the tracking algorithm as a single cell also need to be filtered out to ensure that the time courses originate from single cells. PyAMA performs a pre-selection of cells to reduce the amount of manual interaction required for filtering out anomalous cells. The pre-selection can be inspected and corrected by hand before exporting the time courses of the selected cells to compensate for inaccuracies of the pre-selection. The pre-selection of the current version of PyAMA is based on a threshold for the cell size to deselect cell aggregations. For future versions, an additional machine-learning based pre-selection is planned, which allows to account for further criteria including the examples for anomalous cells described above.

In summary, the LISCA approach employs single-cell arrays to efficiently collect single-cell fluorescence time courses. Confining cells to microfabricated adhesion sites facilitates tracking and image analysis. Furthermore, cells are cultured in standardized local microenvironments and, hence, are presented with uniform surface area when exposed to agents like lipid nanoparticles for transfection. This aspect is particularly beneficial when cellular heterogeneity within a population is investigated. The µPIPP technique described here is one of many microfabrication techniques that result in regular microarrays of protein patterns. The reader is referred to literature reviewing microcontact printing, photolithographic approaches and soft lithography as alternative fabrication processes[6]. Depending on the cell line, the one or the other patterning technique may be preferable. In our experiments, µPIPP technique showed well-positioned cells after seeding of cells due to cellular self-sorting, which relies on residual cell adhesiveness on the PEGylated area so that cells are able to search in random migration and spread on the protein target arrays with differentially larger adhesiveness[17].

LISCA allows for the acquisition of large numbers of single-cell time courses of arbitrary fluorescence markers. Analysis of fluorescence signals at the single-cell level, in contrast to bulk experiments, yields pristine single-cell time courses and reveals cell-to-cell variability. Cellular heterogeneity plays an eminent role in cell fate decisions such as apoptosis[18-20]. In this context, we recently extended the LISCA approach using two fluorescence channels allowing for analysis of temporal correlations of two cellular events at any one time indicating particular stages within the cell-death signaling cascade[19]. In this field of research, LISCA presents itself as an alternative to flow cytometry measurements. While flow cytometry undeniably provides a faster workflow and typically yields larger statistics, the data are acquired at one particular point in time. Full time dependencies yield estimates of kinetics parameters and reveal temporal correlations between different fluorescence signals, which otherwise are difficult to access. In order to use multiple fluorescence channels, the setup requires automated filter wheels or multiple cameras. In this case also single-cell FRET analysis should be feasible and enable time-resolved studies of

proximity between fluorescently labeled molecules. One drawback of image-based analysis like LISCA is the labor linked to visual controls and outlier detection. Here machine learning tools could ease data processing and allow fully automated data analysis. In the future, using automated microscopy platforms, rapid array microfabrication and artificial intelligence, the throughput and applicability of LISCA could be substantially increased. Furthermore, subsequent analysis of cells that exhibit unusual fluorescence responses using microfluidic extraction [21]and single cell genomics is a frequent requirement in pharmaceutical industry. The protocol presented in this article suits the demand for studying the kinetics of cellular processes with single-cell resolution and adequate statistics.

ACKNOWLEDGMENTS

This work was supported by grants from the German Science Foundation (DFG) to Collaborative Research Center (SFB) 1032. Support by the German Federal Ministry of Education, Research and Technology (BMBF) under the cooperative project 05K2018-2017-06716 Medisoft as well as a grant from the Bayerische Forschungsstiftung are gratefully acknowledged. Anita Reiser was supported by a DFG Fellowship through the Graduate School of Quantitative Biosciences Munich (QBM).

DISCLOSURES

The authors declare that they have no competing financial interests.

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Figure 1

Click here to access/download;Figure;Figure1_LISCA_rs.tif

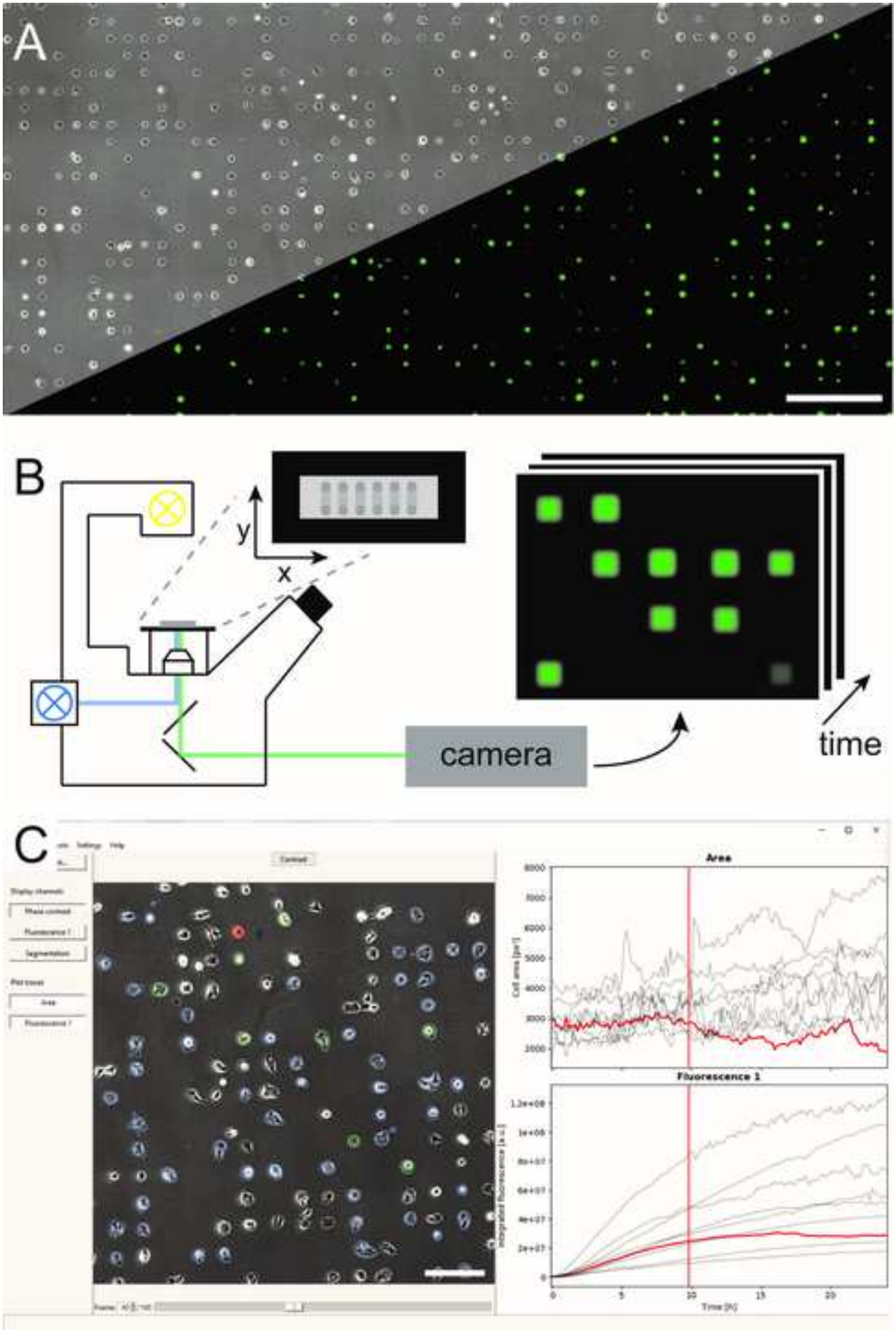

Figure 2

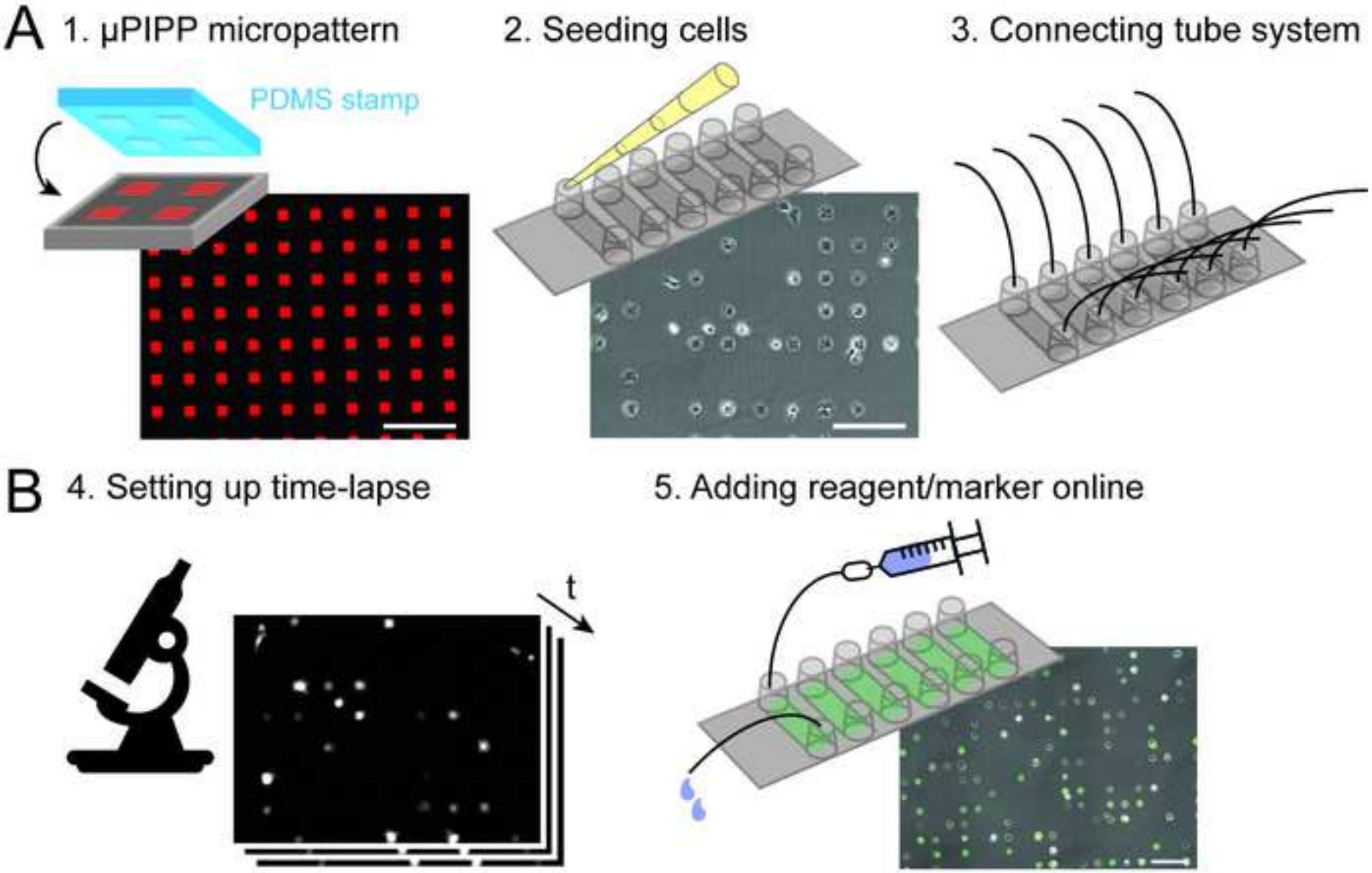

Figure 3

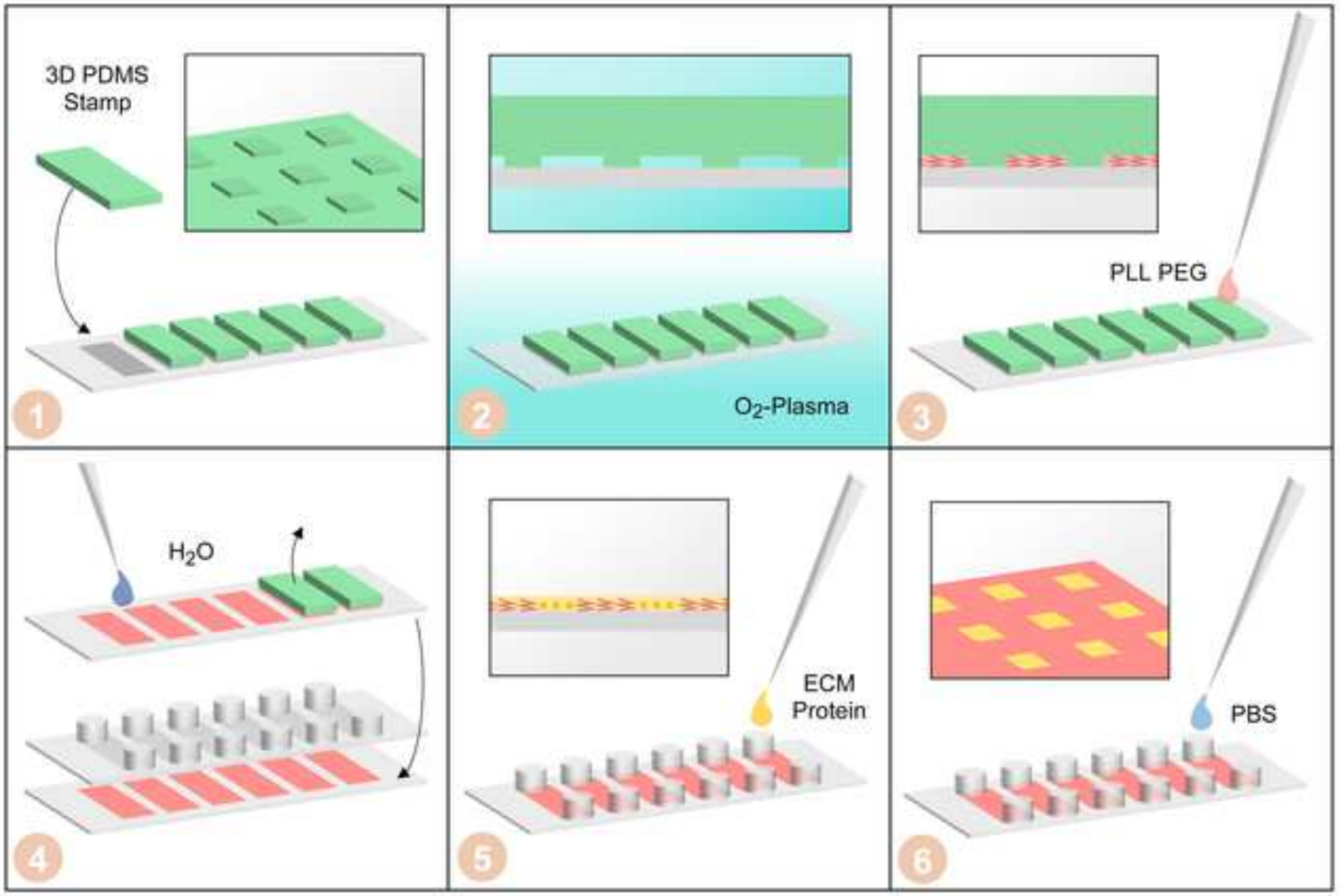

Figure 4

Click here to access/download;Figure;Figure4_LSCA_rs.tif

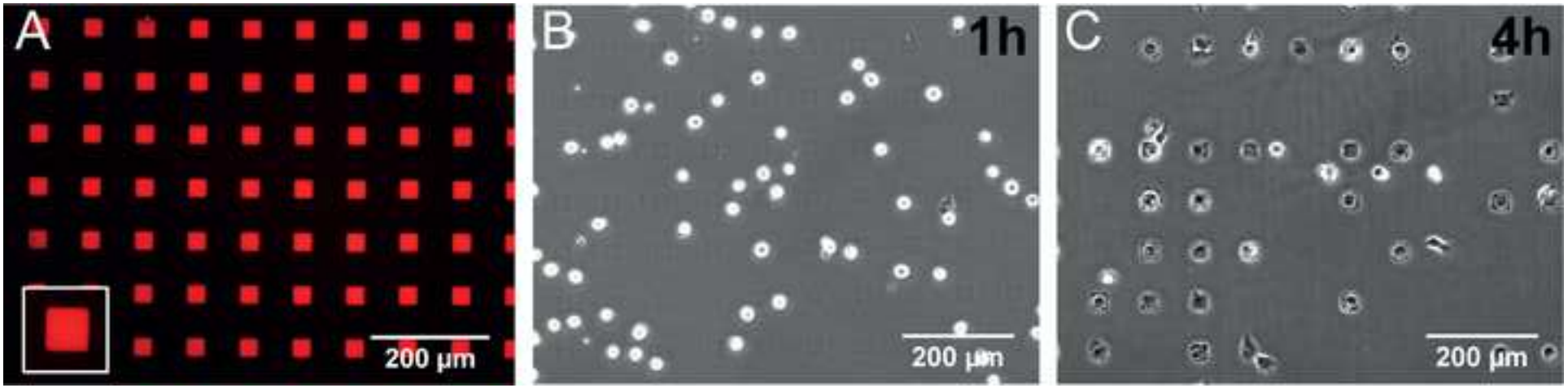

Figure 5

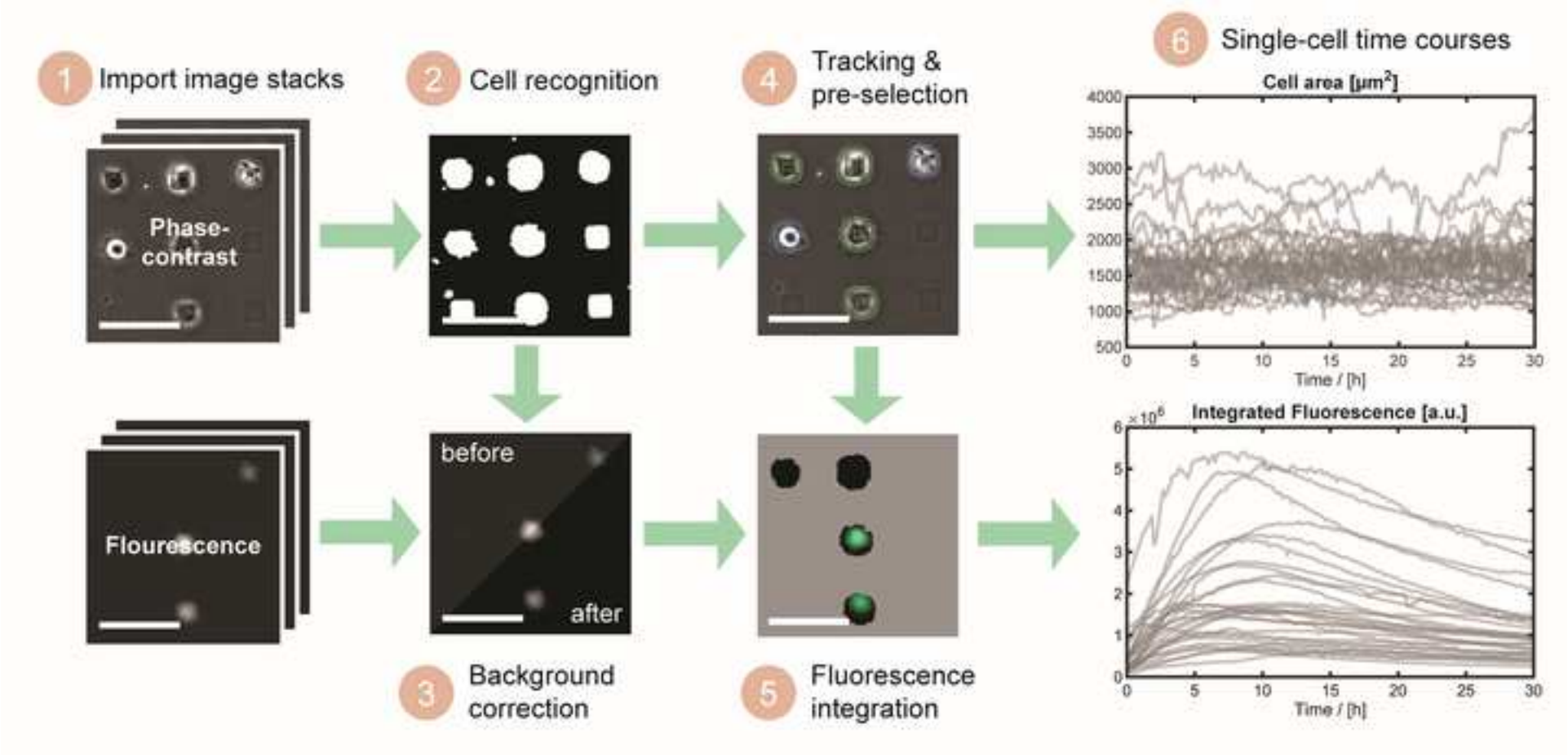

Figure 6

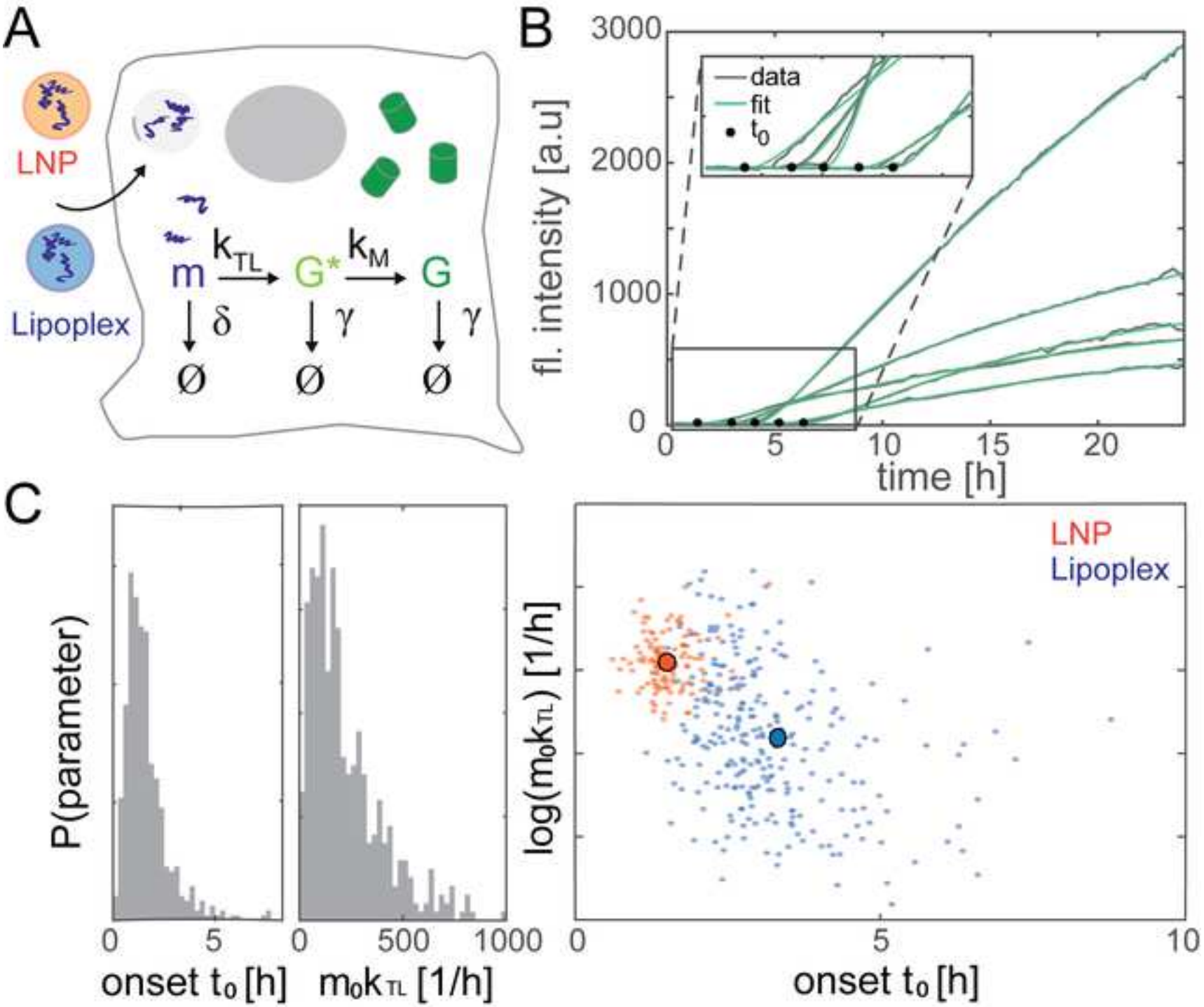

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Adtech Polymer Engineering PTFE Microtubing	Fisher Scientific	10178071	
baking oven	Binder	9010-0190	
CFI Plan Fluor DL 10x	Nikon	MRH20100	
Desiccator	Roth	NX07.1	
Eclipse Ti-E	Nikon		
eGFP mRNA	Trilink	L-7601	
Female Luer to Tube Connector	MEDNET	FTL210-6005	
Fetal bovine serum	Thermo Fisher	10270106	
Fibronectin	Yo Proteins	663	
Filter set eGFP	AHF	F46-002	
Fisherbrand Translucent Platinum-Cured Silicone Tubing	Fisher Scientific	11768088	
HEPES (1 M)	Thermo Fisher	15630080	
Incubation Box	Okolab	OKO-H201	
incubator	Binder	9040-0012	
L-15 without phenol red	Thermo Fisher	21083027	
Lipofectamine 2000	Thermo Fisher	11668027	
Male Luer			in-house fabricated consisting of teflon
Male Luer to Tube Connector	MEDNET	MTLS210-6005	alternative to in-house fabricated male luers
NaCl (5 M)	Thermo Fisher	AM9760G	
Needleless Valve to Male Luer Connector	MEDNET	NVFMLLPC	
NIS Elements	Nikon		Imaging software Version 5.02.00
NOA81	Thorlabs	NOA81	Fast Curing Optical Adhesive for tube system assembly
Opti-MEM	Thermo Fisher	31985062	
PCO edge 4.2 M-USB-HQ-PCO	pco		
Phosphate buffered saline (PBS)			in-house prepared
Plasma Cleaner	Diener Femto	Pico-BRS	
PLL(20 kDa)-g[3.5]-PEG(2 kDa)	SuSoS AG		
silicon wafer mit mircorstructures			in-house fabricated
Sola Light Engine	Lumencor		

sticky slide VI 0.4	ibidi	80608	
Sylgard 184 Silicone Elastomer Kit	Dow Corning	1673921	
Tango 2	Märzhäuser	00-24-626-0000	
Ultrapure water			in-house prepared
uncoated coverslips	ibidi	10813	
Injekt-F Solo, 1 mL	Omilab	9166017V	with replacement sporn

Rebuttal letter: Live-cell Imaging of Single-Cell Arrays (LISCA) – a versatile technique to quantify cellular kinetics

Anita Reiser, Daniel Woschée, Simon Maximilian Kempe, Joachim Oskar Rädler

10 November 2020

Dear Dr. Nam Nguyen,

herewith we resubmit our revised manuscript „Live-cell Imaging of Single-Cell Arrays (LISCA) – a versatile technique to quantify cellular kinetics“ for publication in JoVe.

We would like to thank all reviewers for their valuable time and effort reviewing our paper, and their constructive feedback, which has significantly improved the manuscript.

The reviewers wished clarifications and additional information, which we now have included in the revised manuscript. In the following, we have answered the reviewers' comments point-by-point and indicated the changes made. We have revised the manuscript and the protocol for clarity and correct language, as suggested by the reviewers. Also the editorial comments regarding language and formatting have been addressed, and we have revised the protocol section to comply with the editorial policy.

We believe that the revised version of our manuscript is now suitable for publication in JoVe.

Sincerely,

Anita Reiser, Daniel Woschée, Simon M. Kempe and Joachim O. Rädler

Editorial comments

Changes to be made by the Author(s)

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

> **Answer:** The manuscript has been thoroughly proofread. Spelling and grammar issues have been corrected. Abbreviations have been defined at first use.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Sylgard 184 Silicone Elastomer Kit, Lipofectamine 2000, Opti-MEM,

> **Answer:** Commercial language has been removed from the manuscript.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

> **Answer:** The protocol section has been adapted to only contain imperative tense. Text that cannot be written in imperative tense has been canceled, written as a "Note" or moved to the discussion section. Unnecessary "Notes" have been removed.

4. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

> **Answer:** A one-line space has been added between protocol steps. Protocol text for inclusion in the protocol section of the video has been marked yellow.

5. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

> **Answer:** The scale bars have been inserted and indicated appropriately. The scale labels cannot be removed from Figure 3 because Figure 3 is copied from another publication.

Reviewer #1

Minor Concerns

1. Maybe the authors can add some more thoughts and discuss how this procedure might be expanded for the usage of ratiometric biosensors e.g. FRET-based probes.

> **Answer:** FRET-based probes are discussed now at the end of the article.

2. The authors might also add some discussion on how the nature/geometry of the micropatterning might impact cell signaling, function, and fate.

> **Answer:** The effect of micro-pattern on cell viability has been studied by us and others. In short we did not observe adverse effects using viability assays and also did not find changes in division times. We have addressed this issue in the text.

Reviewer #2

Major Concerns

1. The author should give a relation with the pattern size and the cell scale for other cell lines.

   Answer: Lines 367-370 of the corrected manuscript give examples of suitable pattern sizes for other cell lines.

2. What is the distribution of cell number on single pattern?

   Answer: The cells are seeded out in concentrations such that occupancy of less than 70% of adhesion sites is achieved. In this case we only find cell number occupancies 0, 1 or 2 in accordance with a Poisson distribution. See also Röttgermann *et al.*, **Soft Matter**, 2014,**10**, 2397-2404 (Reference 18 of the manuscript).

3. Lines 139-161: any guidance on how to optimize the seeing procedure for a specific type of cells?

   Answer: Lines 375-388 of the corrected manuscript give some advice on how to adapt the protocol to other conditions, e.g. other cell types.

4. What is the speed of perfusion? Does the shear stress affect the attachment of cells? How to optimize this parameter (criterion)?

   Answer: The liquid exchange is performed manually. For this reason the generated flow rate and the resulting shear stress cannot be controlled precisely. The liquid exchange has to be done very carefully. We have not observed any cell detachment or morphological changes due to the liquid exchange. We do not expect any long term effects of the two short periods of liquid exchange.

5. I do not think thresholding is a suitable method for the segmentation. For example, there are two cells attached on the patter on the letter bottom corner in Figure 4. But they are segmented into one mask. This will lead to false results in fluorescence analysis. The authors may want to try those segmentation methods that are widely used like active contour, watershed and machine learning method.

   Answer: Active contour and watershed require the cell position as input and, thus, cannot be used as primary segmentation method. Since our goal is automated cell recognition, the input should not be provided interactively by the user but by another segmentation method. Due to the heterogeneous appearance of cells in phase-contrast images, we do not expect that active contour or watershed could outperform the current thresholding algorithm.

   It is planned, however, to detect cell contours and multiple occupancies with machine learning in future versions of PyAMA. In the mean time, the pre-selection may contain some false positives, which need to be deselected manually. Lines 432-434 of the corrected mansucript clarify this.

   To avoid confusing the reader, we have edited Figure 4 to not include a double occupancy.

Minor Concerns

1. There are a few typos that need further type-editing.

   Answer: Various typos have been corrected in the corrected manuscript.

Reviewer #3

Comments

1. Is the presented methodology adaptable to other, smaller cell types such as prokaryotes?

   Answer: The methodology has been developed for studies on eukaryotes and cannot be easily adapted to bacteria. Bacteria are too small and will not array as single cells on pattern, even if smaller patterns could be made.

2. Is there any control of viability for the cells incubated in the microarrays (growth, dyes indicating metabolic activity)?

   Answer: Controls can (and should) be run in parallel or in separate experiments. We have measured cell division times and viability in the past. We included notes regarding control experiments in the revised text.

3. What kinds of tubing and syringes were used? Please indicate and be as precise and extensive with you description of the used materials.

   Answer: No special syringes are needed but we have added the used type of syringe to the material list. The used tubing and luer connectors are listed in the material list and a reference with details for the perfusion system fabrication is added to the protocol section.

Quality guidelines

1. *Are there any other potential applications for the method/protocol the authors could discuss?*
   → Is the presented method also adaptable to establish and track microbial cultures (bacteria, yeasts) or even distinguish between different types of cells that a cultivated in co-culture? Please shortly discuss this aspect.

   Answer: The methodology has been developed for studies on eukaryotes and cannot be easily adapted to bacteria. However, different types of eukaryote cells can be easily studied. Also experiments in co-culture are feasible. A prominent example are cell-killing assays, where the adhesion sites are filled with target cells and killer cells are added (see for example: Chatzopoulou, E.I., C.C. Roskopf, F. Sekhavati, T.A. Braciak, N.C. Fenn, K.-P. Hopfner, F.S. Oduncu, G.H. Fey, and J.O. Rädler. 2016. Chip-based platform for dynamic analysis of NK cell cytolysis mediated by a triplebody. Analyst. 141: 2284–2295.). We added these applications to the discussion.

2. *Are appropriate controls suggested?*
   → As stated before, it would be very important to check for cell viability or their general physiological status to exclude that gene expression heterogeneities originate from the incubation environment.

   Answer: As described in lines 437-440 of the corrected manuscript, single-cell arrays present a uniform microenvironment to all cells, thus excluding an impact of the cell surroundings on heterogeneities in cell behaviour.

3. *Are all the critical steps highlighted?*
   → No, this could be improved. Please highlight which steps of the protocol are particularly important for successful, and more importantly, meaningful experiments and results.

   Answer: The protocol section has been revised to clarify critical steps.

4. *Is there any additional information that would be useful to include?*
   → Yes, please specify the machines and devices that have been used in detail.

   Answer: The table of used materials has been revised and completed.

Conclusion

1. Control experiments are needed to exclude effects of the cultivation environment on cell physiology.

   Answer: We recommend viability testing when using new cell lines for the first time. A corresponding sentence is inserted into the corrected manuscript.

Reviewer #4

1. The authors should mention novel ways of picking up label-free kinetic signals from single-cells to put their solution into a wider context. See for example: Sztilkovics et al. Scientific Reports volume 10, Article number: 61 (2020). Please give a fair comparison, the advantages/disadvantages of employing fluorescence should be discussed.

   Answer: The LISCA method was developed to extend the use of fluorescence markers for automated analysis of time courses of fluorescence signals from single cells. There are many other techniques that allow single-cell readouts. The referee mentions the elegant approach by Sztilkovics *et al.* measuring adhesion forces and adhesion energies of single cells. However, these mechanical measurements are difficult to compare to fluorescence readout. The applications of Sztilkovics *et al.* and the LISCA approach are almost orthogonal, and a comparison of such completely different methods would rather confuse the reader. However, we mention in the discussion now the possibility of using Fluid_FM, e.g. for subsequent analysis.

2. At the end of the manuscript I miss at least 2-3 sentences about the possibility of picking up the cells for further investigations. Computer controlled micropipette, FluidFM BOT could be mentioned and discussed. These are both highly relevant techniques to pick up the interesting cells from the planar array. (See the above paper and references therein.)

   Answer: The idea of picking cells up at the end of time-lapse studies for further analysis, e.g. single-cell sequences, is very ambitious but well justified. We included this aspect in the discussion and mention the possibility of applying FluidFM Bot.

Reviewer #5

Major Concerns

1. The protocol is unclear. Many steps of the protocol are incomplete or not well described.

   Answer: The protocol has been revised and clarified in the corrected manuscript.

2. The english is also bad in many sentences, what makes it even harder.

   Answer: Language issues have been addressed in the corrected manuscript.

3. It is unclear whether the contents of this manuscript are included in other publications by the same authors.

   Answer: Lines 45–48 of the corrected manuscript indicate publications in which the µPIPP patterning is described.

Minor Concerns

1. Line 85. ml must be mL ; it is not clear the meaning of PLL(20kDa)-g[3.5] ; the composition of the PLL-PEG solution is not clearly described.

   Answer: The unit “ml” has been corrected into “mL”. “PLL(20 kDa)-g[3.5]-PEG(2 kDa)” is the official name provided by the manufacturer.

2. Line 94. Should say: "For the fabrication of PDMS stamps, mix the PDMS monomer with ..."

Answer: The corresponding protocol section has been revised. The suggested amendment is not necessary any more.

3. Line 99. The whole point 3.1 is unclear.

   Answer: The point 3.1 has been clarified in the corrected manuscript.

4. Line 107. Why is it talking about channels? What protection foil?

   Answer: These questions have been clarified in the corrected manuscript.

5. In order to understand the protocol, it is critical to have details on the equipments used. However some details are lacking, for example, what plasma cleaner ir used?

   Answer: The missing entries in the Table of Used Materials have been completed, including details on the plasma cleaner.

6. Line 115. ...plasma cleaner under a biosafety cabinet..." must by "...in the safety cabinet..."

   Answer: The preposition has been corrected in the corrected manuscript.

7. Lines 115 - 118. The English is bad, the text is hard to understand.

   Answer: The protocol step has been clarified in the corrected manuscript.

8. Line 119 - 121. The English is bad, the text is hard to understand.

   Answer: The protocol step has been clarified in the corrected manuscript.

9. Line 122. Still, I did not understand what the foild is....

   Answer: The protocol step has been clarified in the corrected manuscript.

10. Line 122. What is a six channels sticky slide?

    Answer: Sticky slides are established microscopy slides that allow manufacturing custom microstructured surfaces. Details on properties and usage of the six-channel sticky slides used in this protocol can be obtained from the table of used materials and from the manufacturer's website. A more detailed definition would go beyond the scope of the article.

11. Line 170. It is unclear which perfusion system they are using. The table of materials is unclear.

    Answer: The perfusion system is in-house fabricated using the listed material. We inserted this information in the protocol section with a reference where the assembly of the tube system is described in more detail.

12. Line 176. They do not explain how to keep the sterile conditions, what is highly relevant for the experiments.

    Answer: The connection of the tubes to the slide has to be done in a biosafety cabinet. We added this information to the protocol section.

13. Line 191. "...pre-warmed heating chamber..." To what temperature?

    Answer: The protocol step has been clarified in the corrected manuscript.

14. Line 228. 300 uL seems like a very large volume... The channels dimensions are not described. What volume may hold each channel?

    Answer: The volume of 300 µL was chosen to ensure a total volume exchange of the liquid in the channel. The channel volume is 30 µL, the Luer reservoirs have an additional volume of 120 µL, and there is the extra dead volume of the tubing.

15. For what is used the NIS element software?

    Answer: NIS Elements is provided by the manufacturer of the microscope (Eclipse Ti-E) and is used for controlling the microscope, acquiring microscopy images and exporting the images to the TIFF format. Depending on his microscope, a researcher using LISCA may or may not use NIS Elements for microscopy.

16. Lines 426 - 429. It is unclear what the authors mean in this paragraph.

    Answer: The paragraph has been clarified in the corrected manuscript.

17. Line 489. FACS must be defined.

    Answer: The term “FACS” has been replaced by “flow cytometry” in the corrected manuscript.

18. Woschée, D. et al. Single Cell Fluorescence Acquisition on Micropatterned Surfaces (to be published). Seems to be the same publication.

    Answer: This reference refers to another manuscript in preparation, which is concerned with technical aspects of various image analysis algorithms and their performance. To avoid confusion, we removed this citation.

19. I also miss a clear scheme describing the fabrication of the Single-Cell Arrays

    Answer: A scheme of the microarray fabrication by µPIPP is added as new Figure 3.

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Author of this Springer Nature content	yes
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Lead author	Anita Reiser
Title of targeted journal	JoVE
Publisher	MyJoVE Corp
Expected publication date	Nov 2020
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