Supporting information

Integrating Modeling and Microscopy Reveals the Role of Nascent Focal Adhesions in Cell Migration

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Drug treatment. Concentrations and usage times of drugs, selected for each experiment.

Experiment	Concentration used before the experiment	Incubation time	Drug maintenance during the experiment	Concentration used during the experiment	Incubation time
Focal adhesion organization	1 µM	45 min	No	-	-
Focal adhesion dynamics	1 µM	45 min	Yes	200 nM	-
Cytoskeletal dynamics	1 µM	15 min	Yes	200 nM	Overday
Cell migration	1 µM	45 min	Yes	200 nM	Overnight

Table 1 - Concentrations and incubation time used for Cytochalasin D drug.

Experiment	Concentration used before the experiment	Incubation time	Drug maintenance during the experiment	Concentration used during the experiment	Incubation time
Focal adhesion organization	50 nM	30 min	No	-	-
Focal adhesion dynamics	50 nM	30 min	No	-	-
Cytoskeletal dynamics	50 nM	30 min	No	-	-
Cell migration	50 nM	30 min	Yes	10 nM	Overnight

Table 2 - Concentrations and incubation time used for Jasplakinolide drug.

Immunofluorescence assay. Cells were fixed under a chemical fume; upon medium removal and washing in PBS, each sample was incubated for 15'at RT with 300 µl of 4% paraformaldehyde. Then, cells were washed three times in PBS and rinsed with 0.1M Glycine (EuroClone, Italy) to reduce sample autofluorescence. Samples were permeabilized with 0,25% Triton X-100 (Triton X-100, Sigma Aldrich, Germany) in PBS for 10 minutes. Cells were incubated with a blocking solution made of PBS Tween 0,1% (Tween 20, Sigma Aldrich, Germany) + FBS 2% for 4 hours at RT, and then

treated to recognize the focal adhesions, with a primary rabbit to vinculin antibody (Abcam, United Kingdom) diluted 1:400 in the blocking solution, that was left inside the samples at 4°C overnight.
After the overnight incubation the samples were rinsed three times with a PBS Tween 0,1% solution, to wash out any excess of unbounded antibodies, and then incubated 45' with a solution composed by PBS Tween 0,1% + FBS 2% and containing the secondary antibodies anti rabbit Alexa Fluor 488 (dilution 1:1000, Abcam, United Kingdom) and the phalloidin TRITC marker (1:100, Sigma Aldrich, Germany).
A last set of three rinsing with PBS Tween 0,1% was performed, prior to the incubation for 10 minutes of the samples with Hoecst 33342 (Thermofisher, United States) diluted with a ratio of 1:1000 in PBS, to allow the visualization of nuclei inside the samples. Cells were mounted with Mowiol-DABCO solution before the image acquisition.

DNA transfection. The chosen amount of pDNA for transfection (500ng per sample) was selected from JetPRIME ® protocol guidelines, corresponding to 0.36 μL of vinculin-eGFP plasmid per a single Lab-Tek well.
The volume of pDNA were added to 25 μL of JetPRIME ® buffer, vortexed for 10 seconds to allow complete mixture of components and subsequently spun down; after this, 1.12 μL of JetPRIME ® reagent were added to the pDNA-buffer solution and the resulting product was newly vortexed and spun down as before, and then left to incubate at room temperature for 10 minutes. After this time, the transfection mix was added to the well containing the seeded cells in 300 μL of fresh medium. The same process was repeated for each well that underwent transfection and the samples were left to incubate for 24 hours in the incubator at a temperature of 37 °C and CO2 at 5%.

In-vivo actin and nuclear labelling. SiR-Actin (Spirochrome, Switzerland) at a stock concentration of 50 nM and Verapamil (Spirochrome, Switzerland) at a stock concentration of 10 mM were diluted with a ratio respectively of 1:2000 and 1:1000 in fresh DMEM, and cells were incubated for 45'. After this incubation, the sample was rinsed with PBS and incubated for 10' with a solution of DMEM containing Hoechst 33342 (1:1000 dilution), to enable nuclear staining within the biological sample. The solution was then replaced with standard phenol red-free culture medium, and the experiments were carried out.

Migration analysis. Using a custom-made Python code, the results from the previous analysis of tracks were exploited to recover several significant parameters in migration. Amongst these parameters the single displacement between consecutive time frames (dp), the mean velocity between consecutive frames ($v = dp/dt$, with $dt = 15$ minutes), the overall path covered at different timepoints, calculated as the sum of all the dp up to the selected time point, and final displacement, calculated as the vectorial distance between the nucleus of the cell between the last and first temporal frame, were computed.

Immunofluorescence analysis. The gray-scale raw images of vinculin structures (Supplementary Figure 2A-ii) were filtered to ensure the removal of any background signal represented by soluble vinculin in the cytosol: to do so the gray-scale image was divided by a duplicate image, which was processed and smoothened with a Gaussian Blur filter of large radius ($\sigma \geq 50$ pixel units); the radius of the filter was specifically selected according to the digital zoom of the current image, by considering the ratio between the maximum size of elements to detect during segmentation, which was of 10 μm independently on the applied magnification, and the pixel length of the selected zoom. Subsequently, adhesions were emphasized in the resulting image by an automatic enhancement of brightness and contrast and further sharpened by means of the application of a Laplacian operator, with a smoothing scale of 1.0. Lastly, the image containing the remaining features was binarized using Otsu's thresholding method, that allows for an automatic recognition of the threshold value. Once the segmented image was recovered (Supplementary Figure 2A-iii), morphological information regarding adhesions was obtained by analyzing the image using an open-source code extracted from (1). In this process only adhesions with a size greater than 0.1 μm^2 in size were analyzed (Supplementary Figure 2A-iv), to ensure the removal of any possible background noise generated by the segmentation process; once the analysis was complete, retrieved objects with a size greater than 10 μm^2 were manually excluded from any further investigation, as considered clusters of several smaller focal adhesions rather than mature adhesions. Adhesions were analyzed making a distinction between the center of the cell and the periphery, which was traced defining a region from the leading edge with a length equal to 10% of the distance between the center of the cell and the leading edge itself (Supplementary Figure 2A-iv).

Sensing focal adhesion kinetics analysis. Prior to the segmentation of the adhesion structures, each cell was isolated from its background by manual selection of the cell borders; this process was done for each of the frames, accounting for

the possible morphological changes that occurred to the cell in each subsequent timeframe, as a consequence of cell protrusion or retraction in time.
To detect the direction of cell migration and the regions of cell protrusion and retraction, a preliminary evaluation was done by manually creating an outline of the cell at the initial acquisition time and comparing it with the position of the cell at the last available timeframe (30') to define the overall direction of cell migration that allow the identification of front and rear inside the cell body with associated regions of interest.
Segmentation of the shortened timelapse images was performed employing the same pipeline already discussed in the section "Morphological analysis of focal adhesion structures" for fixed image of immunoassayed cells.
After this process, a second timelapse image containing only segmented nascent adhesions or focal complexes was generated, by employing the "Analyze Particles" feature on the first segmented image and selecting only those elements that yielded a pixel size between 3 and 6 (corresponding to a size in μm^2 between 0.1 and 0.25) or between 7 and 24 (corresponding to a size in μm^2 between 0.25 and 1).
Lastly, to compute quantitative measurements, the density and variability of nascent adhesions were computed according to the following formulas for each of the three ROIs and for each timeframe:

$$density = \frac{\#\ of\ NAs/FCs\ present\ in\ the\ selected\ timeframe}{area\ in\ \mu m^2 of\ the\ selected\ ROI} \qquad \text{(Eq 1)}$$

$$variability = dev.st\left(\frac{\#\ of\ NAs/FCs\ present\ in\ the\ selected\ timeframe}{area\ in\ \mu m^2 of\ the\ selected\ ROI}\right) \qquad \text{(Eq 2)}$$

For every region a single density and variability value was generated by computing the mean of the single values obtained in all 60 timeframes.

Fluorescence recovery after photobleaching analysis. Fluorescence intensity data obtained by the experiment was processed in Excel, in order to obtain for each dataset a value of normalized fluorescence intensity ($I(t)_{NORM}$), according to a double normalization process (2):

$$I(t)_{NORM} = \frac{R_0}{S_0} \times \frac{I(t)_S - I(t)_B}{I(t)_R - I(t)_B} \qquad \text{(Eq 3)}$$

where R_0 and S_0 are the mean intensities prior to bleaching of, respectively, the reference and stimulation ROIs, whereas $I(t)_S$, $I(t)_B$ and $I(t)_R$ are the intensities of the ROIs in time as returned by the measurement software.
When fitting normalized experimental data, in cases on diffusion uncoupled FRAP, it is possible to employ either a single or double (or eventually higher) exponential depending on the type of reaction. In this thesis work, assuming that the dominant reaction is a sliding of the fibers due to the binding/unbinding of actin oligomers to the fibers, the chosen model was a single exponential, meaning that the data was fitted to a curve with the following equation:

$$y(t) = y_m - (y_m - y_0)e^{-kt} \qquad \text{(Eq 4)}$$

To compute the recovery time of the protein of interest in the FRAP experiment it is necessary to consider a characteristic time of the curve that is $t_{1/2}$, that is the time at which the recovery curve arrives at a fluorescence intensity that is half of the fluorescence intensity value reached at the plateau; this time, also referred to as
half-life or turnover time of the protein, is obtained as:

$$t_{1/2} = \frac{-\ln(0.5)}{k} \qquad \text{(Eq 5)}$$

Lastly mobile the mobile fraction (M_f) is computed as:

$$M_f = \frac{y_m - y_0}{1 - y_0} \qquad \text{(Eq 6)}\ .$$

Bibliography

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