Title:

Visualization and quantitative analysis of the actin cytoskeleton upon B cell activation

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Keywords: Lymphocytes, B cells, immunological synapse, actin cytoskeleton, microscopy, total internal reflection microscopy, TIRF, live cell imaging, signalling microclusters, image analysis

Abstract

The formation of the immunological synapse upon B cell activation critically depends on the rearrangement of the submembranous actin cytoskeleton. Polymerization of actin monomers into filaments provides the force required for B cell spreading on the antigen-presenting cell (APC). Interestingly, the actin network also participates in cellular signaling at multiple levels. Fluorescence microscopy plays a critical role in furthering our understanding of the various functions of the cytoskeleton, and has become an important tool in the studies on B cell activation. The actin cytoskeleton can be tracked in live cells with various fluorescent probes binding to actin, or in fixed cells with phalloidin staining. Here, we present the usage of TIRF microscopy and an image analysis workflow for studying the overall density and organization of the actin network upon B cell spreading on antigen-coated glass, a widely used model system for the formation of the immunological synapse.

1 Introduction

The actin cytoskeleton is a pivotal player in lymphocyte activation as it provides the forces responsible for the dramatically changing cellular morphology upon the formation of the immunological synapse (IS) [1–3]. Moreover, the actin cytoskeleton has been implicated in the regulation of cell signaling through the control of membrane protein diffusion and organization [4]. For example, the actin cytoskeleton affects B cell receptor (BCR) signaling by controlling interactions between the BCR and its co-receptors [5–7]. The importance of actin in the immune system is further demonstrated by diseases, such as the Wiscott-Aldrich syndrome (WAS), caused by defected action of an immune cell-specific actin-regulatory protein WASP [8, 9] .

Within the first minutes of the B cell encounter of surface-bound antigens on antigen presenting cells (APC), the cortical actin filament (F-actin) network is destabilized to promote efficient reorganization of the actin architectures [10]. Similarly to T cell IS, B cells form a radially protruding leading edge, composed of lamellipodia and filopodia [1]. This leads to the B cell spreading over the APC, critical for the subsequent gathering and internalization of antigen [2, 3].

The important role of actin in the IS is reflected by the distinct actin structures at different parts of the IS. Outermost lamellipodia consists of dense, highly branched actin meshwork that is followed by more aligned, concentric F-actin in the lamella and, finally, the central area largely void of obvious actin architectures [1]. Traditionally, in fixed cells the actin cytoskeleton has been visualized by staining with fluorescently labeled phalloidin [11]. With its high specificity for F-actin, phalloidin has maintained its position as the gold standard of actin staining. However, cell fixation and permeabilization with detergents can expose the samples for artefacts. Furthermore, the knowledge of the underlying dynamics of the system can only be achieved by visualization of the actin structures in living cells. Coinciding with the escalation of the usage of fluorescence microscopy and the development of fluorescent proteins in the last 10–15 years, also various actin tracers optimized for live cell microscopy have been introduced.

The first marker for live cell imaging of actin was GFP-actin [12]. While this probe is still highly relevant for applications such as FRAP (fluorescence recovery after photobleaching), its impaired competence in F-actin polymerization, potentially leading to incomplete, or biased

representation of the cellular actin network, has raised the need for better markers [13]. Nowadays researchers can choose the most suitable tool for their studies from an array of actin tracers with different strengths and disadvantages. Most of these tracers are based on fusing fluorescent proteins (FP) to actin-binding peptides from different cytoskeletal regulators. When choosing the marker, one should take into account, for example, the estimated coverage of different actin structures, potential influence on actin dynamics as well as the probe kinetics informing about the probe suitability to the application of interest [14]. Currently, one of the most widely used actin tracers is LifeAct, a short actin-binding peptide fused to different FPs [15]. A relatively recent alternative is to use nanobodies, such as Actin-Chromobody (ChromoTek), utilizing actin-directed single chain antibodies, fused to FP. One of the drawbacks of all these tracers is that they have to be transfected or genetically engineered into the cells of interest. To combat this issue, a cell-penetrable actin tracer, SiR-Actin (Spirochrome), was recently brought to markets.

BCR signaling and the subsequent remodeling of the actin cytoskeleton to form the IS, occur at the close proximity to the plasma membrane. There are many microscopy techniques available to study lymphocyte activation on different scales, each with their own benefits and drawbacks [16]. Among those, the total internal reflection fluorescence (TIRF) microscopy represents a relatively easy-to-use, high resolution method that is well suited for IS studies. In Z-axis, TIRF reaches a resolution of 150–200 nm, which is significantly better than confocal microscopy with typical resolution of ≥500 nm. High Z-resolution in TIRF is achieved by the illumination of the cover glass–sample interface with a laser at high angle to cause total reflection of the incident light [17]. Consequently, the bottom of the sample is illuminated by an exponentially decaying evanescent field, which allows clear visualization of the phenomena at the cell membrane without interfering background fluorescence from other parts of the cell. Very low total exposure to lasers minimizes the phototoxicity and makes TIRF particularly suitable for live-cell imaging. Due to the sensitivity of the laser illumination, however, obtaining equal excitation intensity throughout the field-of-view can be challenging. This problem is effectively solved by ring-TIRF, a modern technique that fast-spins the laser light around the objective thereby creating even TIRF illumination throughout the field of view [18].

In this article we apply TIRF microscopy to the visualization of the actin cytoskeleton upon B cell activation by surface-bound antigens. We include an optimized cost-efficient protocol for transfection of B cell lines and describe the details of sample preparation for TIRF imaging of both fixed and living cells. Finally, we present an efficient image analysis work-flow to quantify cell spreading and actin intensity together with a threshold analysis of high-intensity structures in multiple channels. To provide complementary information about BCR signaling, the methods are compatible with addition of other markers, such as FP-Syk for live cell imaging, or phospho-tyrosine (pTyr) staining in fixed samples, as shown in our examples.

2 Materials

2.1 Cells

1. BJAB human Burkitt lymphoma cell line, and A20 mouse B cell lymphoma line stably expressing the transgenic IgM BCR D1.3 [19] (*see* **Note 1**–**2**).

2. Growth medium for BJAB and A20 cells: RPMI 1640 + 2.05 mM L-glutamine supplemented with 10 % fetal calf serum (FCS), 50 μM β-mercaptoethanol, 4 mM L-glutamine, 10 mM HEPES and 100 U/ml Penicillin-Streptomycin.

2.2 Visualizing actin dynamics in living cells

2.2.1 Transfections

1. AMAXA electroporation (Biosystems).
2. 0.2 cm gap width electroporation cuvettes (Sigma-Aldrich®).
3. Recovery medium: growth medium supplemented with extra 10 % FCS (total final concentration of FCS 20 %) and 1 % DMSO.
4. 2S transfection buffer: 5 mM KCl, 15 mM $MgCl_2$, 15 mM HEPES, 50 mM Sodium Succinate, 180 mM Na_2HPO_4 / NaH_2PO_4 pH 7.2. (*see* **Note 3**).
5. Pasteur pipettes fitting 2 mm gap width cuvette.
6. 6-well plates, cell culture quality.
7. Non-linearized plasmids encoding the markers of interest. For example, a fluorescent actin probe LifeAct-FP, together with a fluorescently tagged protein downstream of BCR signaling, such as Syk (*see* **Note 4**–**5**).

2.2.2 Microscopy

1. 35 mm glass bottom dishes (MatTek Corporation, Massachusetts, USA) (*see* **Note 6)**.
2. Anti-BCR antibodies for BCR signaling –mediated cell spreading: anti-human IgM for BJAB, and anti-mouse IgM for A20 D1.3 (*see* **Note 7**).
3. Fibronectin for non-BCR mediated cell attachment (*see* **Note 8**–**9**).
4. Imaging buffer: 0.5 mM $CaCl_2$, 2mM $MgCl_2$, 1 g/l D-glucose, 0.5 % FCS in PBS.
5. An inverted fluorescent microscope with a TIRF module to acquire the images. Many vendors provide suitable systems. We have used either Zeiss Axio Observer Z1 with the setup briefly described below, or alternatively, DeltaVision OMX v4 (GE Healthcare Sciences) system with similar specifications, including ring TIRF, multiple scientific CMOS cameras and lasers.
   a) EMCCD camera (Hamamatsu, ImagEM, Model C9100-13).
   b) Objective (Zeiss Alpha Plan-Apochromat 63x/1.46 NA, Oil Korr TIRF).
   c) Incubator (XLmulti S1 DARK LS environmental chamber).
   d) Multi laser module (laser lines 488, 561 and 640 nm).
   e) Emission filters (Zeiss Filter set 77 HE GFP+mRFP+Alexa 633).
   f) Vibration isolation table (Newport, Integrity 3 VCS Table System).

g) Image acquisition software (Zeiss ZEN Blue ver.2).

2.3 Visualizing the actin cytoskeleton in fixed cells

1. Silicone gaskets (MultiWell Chamber coverslip, Grace Bio-Labs, Oregon, USA) and TIRF compatible coverslips fitting the size of the silicone gasket (for example: 24 mm x 40 mm, 0.17 mm thickness)
2. Anti-BCR antibodies or fibronectin for functionalizing the glass (*see* 2.2.2 and **Notes 7, 9**).
3. Fixation buffer: 4 % PFA in PBS
4. Permeabilization buffer: 0.1 % Triton-X in PBS
5. Blocking buffer: 10 mg/ml BSA in PBS
6. Antibodies and phalloidin
    a. Alexa Fluor® 555 Phalloidin (150 U/ml, Life Technologies, Oregon, USA). Prior to use, dilute 1:50–1:200 in blocking buffer.
    b. Anti-pTyr Antibody (1 mg/ml, clone 4G10, Merck Millipore, Massachusetts, USA). Prior to use, dilute 1:500 in blocking buffer.
    c. FITC Rat Anti-mouse IgG2b (0.5 mg/ml, clone R12-3, BD Sciences, California, USA). Prior to use, dilute 1:600 in blocking buffer.
7. PBS

3 Methods

3.1 Visualizing actin dynamics in living cells

3.1.1 Transfections

1. Use 4 ml of recovery medium per well in 6-well plates. Warm up the recovery medium in an incubator (+37°C, 5 % CO_2) (*see* **Note 10**).
2. Centrifuge 4x10^6 cells down very gently (*see* **Note 11**).
3. Gently re-suspend the cells in 180 µl of 2S transfection buffer containing up to 4 µg of non-linearized plasmid DNA (*see* **Note 12**). Place briefly on ice.
4. Transfer the cells with plasmid DNA to an ice cold cuvette pre-cooled on ice (*see* **Note 13**).
5. Transfect the cells using Amaxa nucleofector: for BJABs use program X-001 and for A20 cells, use program X-005.
6. Immediately after nucleofector pulse, fill the cuvette with warm recovery medium and transfer the cells into 6-well plates containing warm recovery media. Rinse the cuvette with recovery medium to collect the remaining cells.
7. Place the cells to the incubator to recover for 24 hours (Figure 1) (*see* **Note 14**).

3.1.2 Preparing the microscope slides

1. Functionalize the MatTek dishes as below. Keep in PBS until adding the cells. Do not let the dishes get dry.
    a. Anti-IgM coating for BCR-mediated spreading: Use anti-IgM in the final concentration of 10 µg/ml in PBS. Incubate 40 min at +37ºC. Rinse with PBS. (*see* **Note 7**).
    b. Fibronectin coating for adhering resting cells: Use fibronectin in the final concentration of 4 µg/ml in PBS. Incubate 30–40 min at RT or +37ºC. Rinse couple of times with PBS.

3.1.3 Live cell imaging

1. Equilibrate the microscope by pre-heating it up to +37ºC *(see* **Note 15**).
2. Place the functionalized MatTek dish on TIRF objective to pre-warm for 5 min (*see* **Note 16**).
3. Carefully replace the PBS on the MatTek dish with 100 000–500 000 cells in 100 µl of imaging buffer (*see* **Note 17**). Note the time.
4. Focus on cells, verify optimal TIRF illumination, and start imaging (Figure 2) (*see* **Note 18**).

3.2 Visualizing the actin cytoskeleton in fixed cells

Below, we provide a protocol for immunofluorescence staining, adjusted for TIRF imaging, which uses reusable 8-well silicone gaskets as an economical alternative to performing the staining on MatTek dishes with considerably higher reagent volume requirements.

3.2.1 Preparing the immunofluorescence samples for TIRF imaging

1. Place a silicone gasket on a cleaned (*see* **Note 19**) long TIRF quality coverslip (*see* **Note 20**) by gently pressing it on the glass (*see* **Note 21**).
2. Functionalize the slides as in 3.1.2 using the volume of 30 µl per well.
3. Apply 50 000–100 000 cells per well in 30 µl of imaging buffer (see **Note 17b**).
4. Activate the cells for the time of interest in the incubator at +37ºC (*see* **Note 22**).
5. Fix cells for 10–15 min at the room temperature (RT). Rinse once with PBS (*see* **Note 23**).
6. Permeabilize the cells for 5 min, RT. Rinse once with PBS.
7. Block the cells for 1 h, RT.
8. Stain for F-actin and pTyr:

a. Apply fluorescently labeled phalloidin and anti-pTyr antibody to the cells (*see* **Note 24**). Incubate for 1 h, RT.
b. Rinse well (3 times) with PBS.
c. Apply the secondary antibody against anti-pTyr to the cells. Incubate for 1 h, RT.
d. Rinse well (3 times) with PBS and overfill the wells with PBS.

9. Cover the gasket with a clean (*see* **Note 19**) microscope slide to seal it, and to provide support for handling (Figure 3) (*see* **Note 25**).

3.2.2 TIRF imaging of fixed samples

As in 3.1.3.4.

3.3 Data analysis

3.3.1 Image analysis

Here we provide an example how to use the open source software Image J to quantitatively analyze the actin cytoskeleton, together with markers such as pTyr, during B cell activation. The same work-flow can be applied for analysis of both fixed or live cells. The surface area of B cells is quantified based on actin intensity. In addition, through local thresholding, we analyze actin-rich areas as well as pTyr enriched puncta, indicating signaling microclusters. We present a series of steps, applicable in various image analysis software, required to obtain such quantitative information. These steps can be combined in a script for semi-automatized batch analysis, such as the script we have made available, together with informative sample images, at our website (http://mattilalab.utu.fi/). For more detailed information about actin dynamics and retrograde flow live cell imaging together with specialized analysis methods is required. For example, a method for analyzing the actin retrograde flow in immune synapse through time-lapse kymographs is shown in [20].

1. Import raw images to Image J (Bioformats plugin).
2. Select the regions of individual cells on the actin channel and make three duplicates of each selection (Fig 4 A1, D1) (*see* **Note 26**).
3. Restore the selections on pTyr channel and make two duplicates.
4. In order to measure the total surface contact area, perform automatic thresholding (Huang method) on one duplicate on actin channel. On the binary image, fill holes in order to get a single area.

5. Create automatic selection based on thresholded binary image (Fig 4 A2, D2).
6. Restore the thresholded selection on non-thresholded duplicate of the same cell on actin channel, to create the region-of-interest (ROI) to be measured (Fig 4 A3, D3).
7. Select the parameters of choice for measurement, such as area, perimeter and various intensity parameters.
8. Measure within the ROI and save the measurements.
9. In order to measure only the regions enriched in actin, perform local thresholding (Bernsen method) on actin channel on second original duplicate, to create a local thresholded image (Fig 4 B2 and E2). Specify the thresholding radius based on the minimum size of the structures you are interested in (*see* **Note 27**).
10. Repeat the creation of ROI, now based on local thresholding (Fig 4 B3, E3), as in steps 5–8.
11. Measure the pTyr puncta, by repeating steps 9–10 for pTyr channel duplicates, using adjusted radius for local thresholding (Fig 4 C1–3, F1–3).
12. Autoutomatize the steps from 3–11 in Image J, by recording the steps in the macro recorder and copy and generalize the used commands in Image J Scripting console.
13. Import the results to a data analysis program, like MS Excel or GraphPad Prism, for graphical presentation and calculation of average, standard deviation and statistical significance (Fig 5).

Notes

1. The cells should be cultured in complete growth medium. Keep the cells in their logarithmic growth phase for the day of the experiment or transfection.

2. While A20 is more common research tool, BJABs allow better visualization of the actin cytoskeleton, while they spread with broader lamellipodia. BJABs are available through *Thermo Fisher Scientific* or *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH* and A20 through *Thermo Fisher Scientific* or ATCC ®, for example.

3. The 2S transfection buffer composition is published in [21], which we adjusted by a small modification in the concentration of Na_2HPO_4 / NaH_2PO_4 (180 mM). Preparation: weigh all the components into a 100 ml flask. Add water up to 80–90 ml. Adjust pH with NaOH to 7.2. Complete with water up to 100 ml, sterile filter (0.2 pore size) and freeze in aliquots.

4. As an example, we used here GFP-Syk and Ruby-LifeAct. Plasmids encoding for various actin probes in different colors can be bought from Addgene (Cambridge, USA), as well as constructs for BCR signaling proteins.

5. In our experience, GFP–anti-actin nanobody / Actin-Chromobody behaves quite comparably to FP-LifeAct in B cells and shows similar transfection efficiencies. In our brief experience, SiR-Actin, the cell penetrable actin probe, does not reach to comparable coverage of the B cell actin cytoskeleton.

6. MatTek dishes can be purchased in two different qualities. TIRF quality with lower variability in cover slip thickness is recommended for TIRF imaging for best performance.

7. Choose the correct anti-BCR depending on the receptor expressed in your cells. We use here anti-human IgM for BJAB, and anti-mouse IgM for A20 D1.3.

8. Aliquot fibronectin in the concentration of 1 mg/ml and avoid re-freezing the aliquots.

9. Alternatively, poly-L-lysine and antibodies against irrelevant, or largely non-signaling receptors (for example, 1 µg/ml of anti-MHCII M5/114 (ATCC TIB120) for mouse primary B cells as in [6]) can be used. However, it is important to consider that all these reagents are likely to affect the cells in some way and the concentrations should be optimized for each cell type.

10. Make sure that the recovery medium is warm when adding the cells (use a heat block under the plate, for example). The temperature of the recovery medium will effect on the transfection efficiency and cell viability.

11. Cell viability is increased by gentle handling. For example, centrifugation should be performed with minimum rpm and time (we routinely use 18.0 G for 5 min). To reduce shear stress, use bigger, 1 ml tips when possible.

12. We normally use total of 4 µg of plasmid DNA for BJAB and 3 µg (or 4 µg in the case of double of transfection) for A20 cells. The total volume of DNA should be kept minimal, not more than 10 µl, to ensure successful electroporation pulse.

13. Pipette cells carefully along the cuvette wall to avoid any air bubbles. Make sure, that the cuvette is dry from outside.

14. The expression is typically visible already within few hours of nucleofection and can last for 2–3 days. Longer recovery time increases the viability of the cells, however, reduces the percentage of cells expressing the ectopic marker.

15. Make sure that you are adequately trained for the use of the TIRF microscope and familiar with the procedures how to adjust the laser angle and verify the optimum TIRF illumination for your samples.

16. Try to place the dish as straight as possible on the objective, as any tilt will affect the TIRF angle. Also, the more straight your dish is, the less focus correction is required when moving around the dish.

17. A) You can pre-warm the cells briefly beforehand to minimize focal shifts due to the temperature changes. B) If you wish to do image analysis for the area of spreading on anti-BCR, adjust the cell number down to avoid close contacts between the cells.

18. By switching among fluorescent channels check for possible chromatic aberration difference between the channels and apply focus correction to compensate it.

19. To enable cleaning of the cover slip and thereby more efficient coating, we recommend the removal of the silicone gasket from its original cover slip. Clean the cover slip, for example, with isopropanol and ethanol.

20. You can remove the dust on the silicone gasket by pressing it with tape. For easier handling, you can trim the silicone gasket with scissors to fit it on 4 cm long coverslip.

21. Try to avoid having bubbles between the glass and silicone to ensure the gasket will not leak.

22. For longer time points usage of an incubator is recommended. However, for shorter time points, such as 5–15 minutes, a heat block or even incubation in room temperature, for possible practical reasons, can be sufficient.

23. Throughout the staining procedure, be gentle when aspirating and pipetting to avoid cell detachment.

24. Phalloidin is good to be added in the first staining step, as it also stabilizes the F-actin.

25. Prior to applying the microscope slide, overfill the wells with PBS with the help of surface tension to generate a drop higher than the gasket. Press a microscope slide on top of the gasket starting from one side; by this you will remove the excess of PBS and avoid air bubbles. Press the slide on the gasket for a moment and dry with a tissue from the edges to ensure it is attached firmly.

26. When analyzing live samples, one must take into account the added variation due to possible variation in the expression levels.

27. The thresholding radius (in pixels) depends on the samples and the resolution of the images. The optimal value should be empirically determined.

28. Figure 2 image and images used for the quantitative analysis were acquired with Zeiss Axio Observer TIRF microscope and ZEN 2 software. The example images in Figure 4 and 5 were acquired using DeltaVision OMX v4 (GE Healthcare Sciences) microscope using ring TIRF mode. Figure 4 image was deconvoluted with DeltaVision softWoRx software.

Acknowledgements

Imaging was performed at the Cell Imaging Core, Turku Centre for Biotechnology, University of Turku and Åbo Akademi University. We thank Markku Saari and Jouko Sandholm from the Cell Imaging Core for their help with the microscopes, and the whole Mattila lab for their critical comments to the manuscript. Turku University Hospital (TYKS) is acknowledged for the support in basic laboratory materials. This work was supported by the Academy of Finland (grant ID: 25700, 296684 and 307313 for PKM, and 286712 for VŠ), University of Turku Graduate School (UTUGS) (for MV), VSSHP research support (for PKM), as well as Sigrid Juselius (for PKM), Jane and Aatos Erkko (for PKM), Magnus Ehrnrooth (for PKM and VŠ) and Finnish Cultural (for VŠ) foundations.

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Figure legends:

Figure 1. Transfection efficiencies in A20 D1.3 and BJAB B cell lines, using 2S transfection buffer [21] and Amaxa nucleofector programs X-005 and X-001, respectively (A). 24h after the transfection, 86% of A20 D1.3 (B), and 31% of BJAB (C) expressed LifeAct-GFP.

Figure 2. Time-lapse image series of a BJAB cell on anti-human IgM coated glass, imaged by TIRF micrsocopy. (A) LifeAct-Ruby as a marker for the actin cytoskeleton. (B) Syk-GFP as a marker for BCR signaling. The time since contact in seconds is shown in the upper right corner of (B) (*see* **Note 28)**. Scale bar, 10 µm.

Figure 3. Assembly of the cover slip, silicone gasket and the microscope slide for TIRF microscopy of fixed samples. The setup allows for low reagent consumption as compared to the usage of MatTek dishes, for example. In the final step, starting from one side, the microscope slide is gently pressed on slightly overfilled sample wells to squeeze any air and excessive fluid out (*see* **Note 25**).

Figure 4. BJAB cells were let to spread for 20 min at 37ºC on cover slips coated with 10 µg/ml anti-human IgM (A) or 4 µg/ml Fibronectin (B). The cells were fixed and stained with phalloidin AlexaFluor-555 (A1, B1) and anti-pTyr antibodies followed by secondary antibodies labeled with AlexaFluor-488 (A2, B2). The images were deconvoluted (*see* **Note 28).** Scale bar, 10 µm.

Figure 5. Image analysis work-flow. BJAB cells were let to spread on cover slips coated with 10 µg/ml anti-human IgM (A–C) or 4 µg/ml Fibronectin (D–F) and prepared for imaging as in Fig 4. The cells were stained with phalloidin (A, B, D, E) and anti-pTyr antibodies (C, F). Columns on the left (A1–F1) show the selection of individual cells in actin and pTyr channels. The middle columns (A2–F2) show automatic thresholding of whole cell perimeter (A2, D2) or local structures (B2, C2, E2, F2) into binary image. The columns on the right (A3–F3) show the ROIs, obtained from the binary image in the

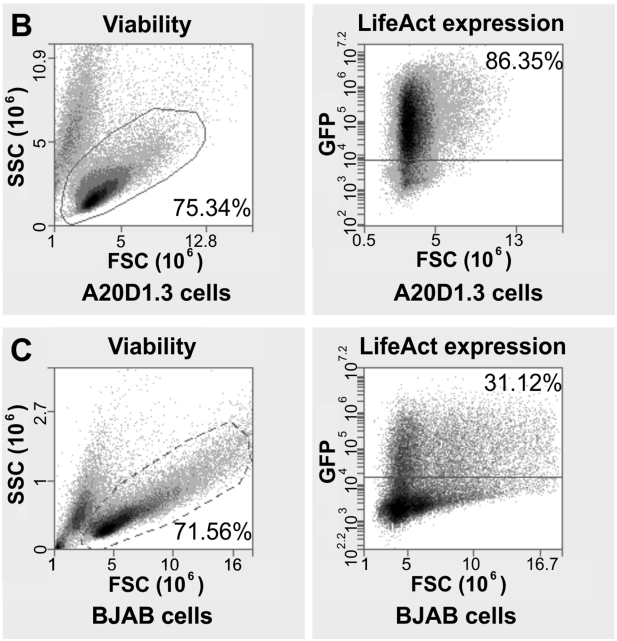
middle column, on the original image for the measurements of parameters of choice (area, intensity etc). Scale bar, 10 µm.

Figure 6. Measurements obtained from an experiment illustrated in Figure 5. (A) Whole cell area as thresholded on actin image, as shown in Figure 5 A2 and D2. (B) Total area of the actin-rich regions obtained via local thresholding, as shown in Figure 5 B2 and E2. (C) Median intensity of the actin-rich regions, as shown in Figure 5 B3 and E3. (D) Total area of the pTyr-rich regions obtained via local thresholding, as shown in Figure 5 C2 and F2. (E) Number of pTyr-rich regions per cell as shown in Figure 5 C2 and F2. (F) Median intensity of the pTyr-rich regions, as shown in Figure 5 C3 and F3. The data was obtained using a script allowing for batch analysis using Image J, freely available at (http://mattilalab.utu.fi/). Each dot represents one cell and the black line median value. 120 cells on anti-IgM coted cover slips and 150 cells on Fibronectin coated cover slips were analyzed.

Sustar *et al.* 2017
Figure 1.

A

Cells (Amaxa program)	**Viability**	**Expression** (LifeAct)
A20 D1.3 (X-001)	75.34%	86.35%
BJAB (X-005)	75.56%	31.12%



Sustar *et al.* 2017
Figure 2.

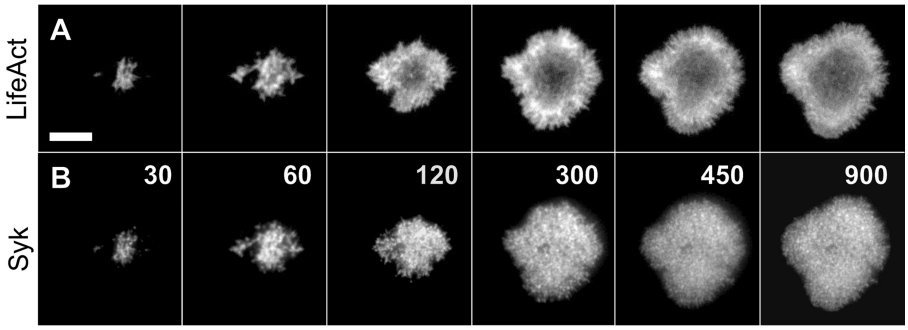


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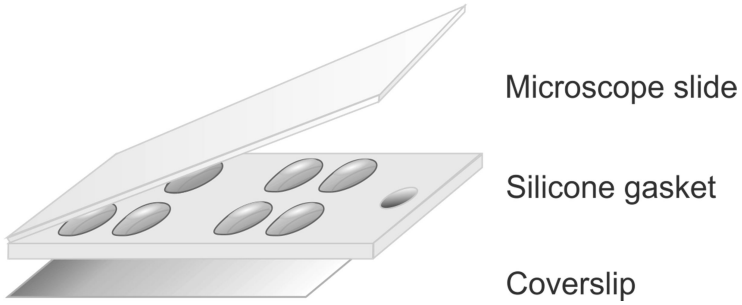
Figure 3.



Sustar et al, 2017
Figure 4

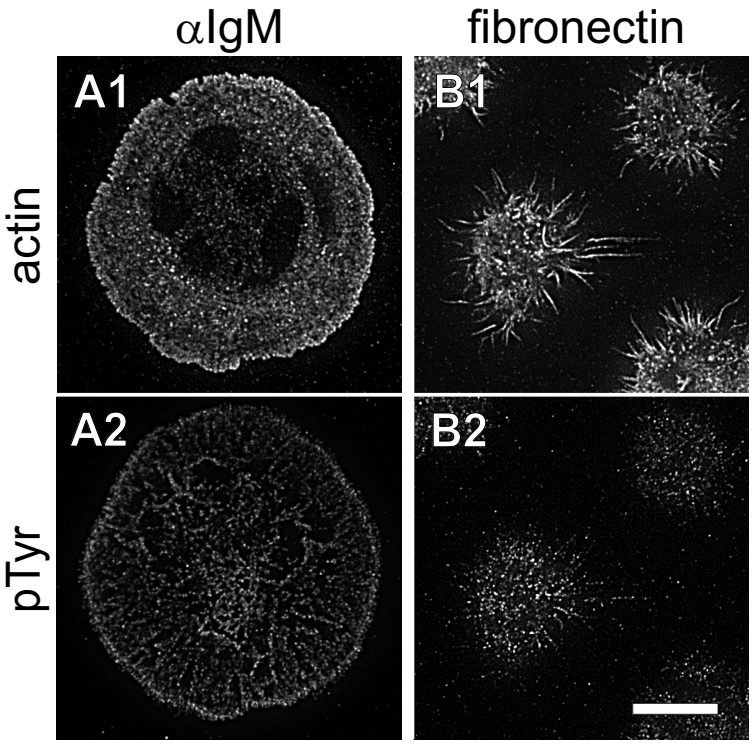


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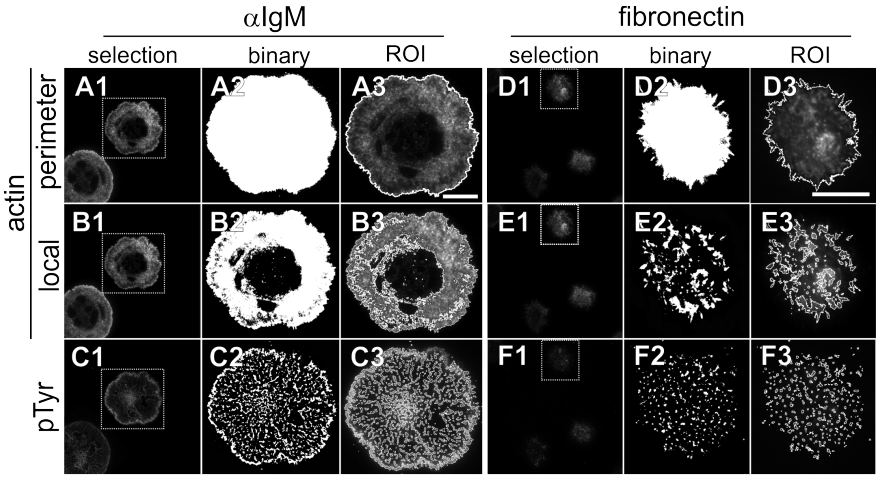
Figure 5.



Sustar *et al.* 2017
Figure 6.