Journal of Visualized Experiments

Studying Organelle dynamics in B Cells during Immune Synapse Formation

--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE59621R1
Full Title:	Studying Organelle dynamics in B Cells during Immune Synapse Formation
Keywords:	cell polarity; lysosomes; Cytoskeleton; Centrosome; Immune synapse; B lymphocytes
Corresponding Author:	María-Isabel Yuseff Pontificia Universidad Catolica de Chile Santiago, Región Metropolitana CHILE
Corresponding Author's Institution:	Pontificia Universidad Catolica de Chile
Corresponding Author E-Mail:	myuseff@bio.puc.cl
Order of Authors:	Jorge Ibañez
	Danitza Fuentes
	Jonathan Lagos
	Jorge Cancino
	María-Isabel Yuseff
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)

Cover Letter

PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
FACULTAD DE CIENCIAS BIOLÓGICAS

Dr María Isabel Yuseff
Pontificia Universidad Católica de Chile
Department of Cellular and Molecular Biology
Av. B. O'Higgins 340. Santiago, Chile
Phone: 56-2-23541843
E-mail: myuseff@bio.puc.cl

Santiago, March 28th 2019.
To Alisha DSouza, Ph.D.
Senior Review Editor JoVE

Dear Dr. DSouza.

Please find enclosed a revised manuscript by Ibañez et al. entitled *"**Studying Organelle dynamics In B cells during Immune Synapse formation**"*, which we would like you to consider for publication in Journal of Visualized experiments, as a Video Article.

As described in detail in our point-by-point response, we have addressed all of the major and minor points raised by the reviewers and have included the changes in the manuscript and corresponding video. We therefore hope that you will find our revised manuscript now suitable for publication in Journal of Visualized experiments.

Sincerely,

María Isabel Yuseff
Associate Professor
Pontificia Universidad Católica de Chile
Department of Cellular and Molecular Biology
Av. B. O'Higgins 340. Santiago, Chile
Phone: 56-2-23541843

TITLE:

Studying Organelle Dynamics in B Cells during Immune Synapse Formation

AUTHORS & AFFILIATIONS:

Jorge Ibañez-Vega[1]*, Danitza Fuentes[1]*, Jonathan Lagos[1,2], Jorge Cancino[3] and María Isabel Yuseff[1].

[1]Laboratory of Immune Cell Biology, Department of Cellular and Molecular Biology, Pontificia Universidad Católica de Chile. Santiago, Chile.
[2]Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile.
[3]Centro de Investigaciones en Biología Celular y Biomedicina, Facultad de Ciencia, Universidad San Sebastián. Santiago, Chile.

Corresponding Author:

Maria Isabel Yuseff (myuseff@bio.puc.cl)

Email Addresses of Co-authors:

Jorge Ibañez-Vega (jsibanez@uc.cl)
Danitza Fuentes (dnfuentes1@uc.cl)
Jonathan Lagos (jelagos@uc.cl)
Jorge Cancino (jorge.cancino@uss.cl)

KEYWORDS:

Cell polarity, lysosomes, cytoskeleton, centrosome, immune synapse, B lymphocytes, image analysis

SHORT ABSTRACT:

Herein we describe two approaches to characterize cell polarization events in B lymphocytes during the formation of an IS. The first, involves quantification of organelle recruitment and cytoskeleton rearrangements at the synaptic membrane. The second is a biochemical approach, to characterize changes in composition of the centrosome, which undergoes polarization to the immune synapse.

LONG ABSTRACT:

Recognition of surface-tethered antigens, by the B cell receptor (BCR), triggers the formation of an immune synapse (IS), where both signaling, and antigen uptake are coordinated. IS formation involves dynamic actin remodeling accompanied by the polarized recruitment to the synaptic membrane of the centrosome and associated intracellular organelles such as lysosomes and the Golgi apparatus. Initial stages of actin remodeling allow B cells to increase their cell surface and maximize the quantity of antigen-BCR complexes gathered at the synapse. Under certain conditions, such as when antigens are associated to rigid surfaces, this process is coupled to the local recruitment and secretion of lysosomes, which can facilitate antigen extraction. Antigens which have been taken up are internalized into specialized endo-lysosome compartments for processing into peptides, which are loaded onto major histocompatibility complex II (MHC-II)

molecules for further presentation to T helper cells. Therefore, studying organelle dynamics associated with the formation of the IS is crucial to understanding how B cells are activated. In the present review we will discuss both imaging and a biochemical technique used to study changes in intracellular organelle positioning and cytoskeleton rearrangements that are associated with the formation of an IS in B cells.

INTRODUCTION:

B lymphocytes are an essential part of the adaptive immune system responsible for producing antibodies against different threats and invading pathogens. The efficiency of antibody production is determined by the ability of B cells to acquire, process and present antigens encountered either in a soluble or surface-tethered form[1, 2]. Recognition of antigens attached to the surface of a presenting cell, by the BCR, leads to the formation of a close intercellular contact termed IS[3,4]. Within this dynamic platform both BCR-dependent downstream signaling and internalization of antigens into endo-lysosome compartments takes place. Taken-up antigens are processed and assembled onto MHC-II molecules and subsequently presented to T lymphocytes. Productive B-T interactions, termed B-T cell cooperation, allow B lymphocytes to receive the appropriate signals, which promote their differentiation into antibody-producing plasma cells or memory cells[8].

Two mechanisms have been involved in antigen extraction by B cells. The first one relies on the secretion of proteases originating from lysosomes which undergo recruitment and fusion at the synaptic cleft[5,6]. The second one, depends on Myosin IIA-mediated pulling forces that triggers the invagination of antigen containing membranes which are internalized into clathrin-coated pits[7]. The mode of antigen extraction relies on the physical properties of the membrane in which antigens are found. Nevertheless, in both cases, B cells undergo two major remodeling events: actin cytoskeleton reorganization and polarization of organelles to the IS. Actin cytoskeleton remodeling involves an initial spreading stage, where actin-dependent protrusions at the synaptic membrane increase the surface in contact with the antigen. This is followed by a contraction phase, where BCRs coupled with antigens are concentrated at the center of the IS by the concerted action of molecular motors and actin cytoskeleton remodeling[8,9,10,11]. This process is coordinated with the polarization of organelles, which also relies on the remodeling of actin cytoskeleton. For instance, the centrosome becomes uncoupled from the nucleus, by local depolymerization of actin, which allows the repositioning of this organelle to the IS[5,12]. In B cells, repositioning of the centrosome to one cell pole (IS) guides lysosome recruitment to the synaptic membrane, which upon secretion can facilitate the extraction and/or processing of surface-tethered antigens[6]. Lysosomes recruited at the IS are enriched with MHC-II, which favors the formation of peptide-MHC-II complexes in endosomal compartments to be presented to T cells[13]. The Golgi apparatus has also been observed to be closely recruited to the IS[14], suggesting that Golgi-derived vesicles from the secretory pathway could be involved in antigen extraction and/or processing.

Altogether, intracellular organelle and cytoskeleton rearrangements in B cells, during synapse formation are the key steps that allow efficient antigen acquisition and processing required for their further activation. In this work we introduce detailed protocols on how to perform imaging

and biochemical analysis in B cells to study the intracellular remodeling of organelles associated with the formation of the IS. These techniques include: (i) Immunofluorescence and image analysis of B cells activated with antigen-coated beads and on antigen-coated coverslips, which allows visualization and quantification of intracellular components that are mobilized to the IS and (ii) isolation of centrosome-enriched fractions in B cells by ultracentrifugation on sucrose gradients, which allows the identification of proteins associated with the centrosome, which could in turn potentially regulate cell polarity.

PROTOCOL:

NOTE: The following steps were performed using IIA1.6 B cells.

1. B cell activation with antigen-coated beads

1.1. Preparation of antigen-coated beads

1.1.1. To activate B cells, use NH_2-beads covalently coated with antigen (Ag-coated beads), which can be prepared using 50 µL (~20 x 10^6 beads) of 3 µm NH_2-beads with activating (BCR-ligand+) or non-activating (BCR-ligand-) antigens.

1.1.2. For IIA1.6 B cells use anti-IgG-F(ab')$_2$ fragment as BCR-ligand+ and anti-IgM-F(ab')$_2$ or bovine serum albumin (BSA) as BCR-ligand-. To activate primary B cells or IgM^+ B cell line use anti-IgM-F(ab')$_2$ as BCR-ligand+ and BSA as BCR-ligand-.

NOTE: To avoid the binding of ligands to the Fc receptor, one can use F(ab') or F(ab')$_2$ antibodies fragments instead of full-length antibodies.

1.1.3. To proceed with the preparation of Ag-coated beads, place the beads in low protein binding microcentrifuge tubes to maximize the beads recovery during the sample manipulation. Add 1 mL of 1x phosphate-buffered saline (PBS0 to wash beads and centrifuge at 16,000 x *g* for 5 min. Aspirate the supernatant.

1.1.4. Resuspend the beads with 500 µL of 8% glutaraldehyde to activate the NH_2 groups and rotate for 4 h at room temperature (RT).

CAUTION: The glutaraldehyde stock solution should only be used in a chemical fume hood. Follow the instructions on the material safety data sheet (MSDS).

1.1.5. Centrifuge beads at 16,000 x *g* for 5 min, remove the glutaraldehyde and wash the beads three more times with 1 mL of 1x PBS.

CAUTION: The glutaraldehyde solution should be discarded as a hazardous chemical waste.

1.1.6. Resuspend the activated beads in 100 µL of 1x PBS. The sample can be divided into two low protein binding microcentrifuge tubes, 50 µL for the BCR-ligand+ and 50 µL for BCR-ligand-.

1.1.7. To prepare the antigen solution use two 2 mL low protein binding microcentrifuge tubes containing 100 µg/mL of antigen solution in 150 µL of PBS: One tube with BCR-ligand+ and other with BCR-ligand-.

1.1.8. Add 50 µL of activated beads solution to each tube containing 150 µL of antigen solution, vortex and rotate overnight at 4 °C.

1.1.9. Add 500 µL of 10 mg/mL BSA to block the remaining reactive NH_2 groups on the beads and rotate for 1 h at 4 °C.

1.1.10. Centrifuge the beads at 16,000 x *g* for 5 min at 4 °C and remove the supernatant. Wash the beads with cold 1x PBS three more times.

1.1.11. Resuspend the activated beads in 70 µL of 1x PBS.

1.1.12. To determine the final concentration of Ag-coated beads, dilute a small volume of beads in PBS (1:200) and count using a hemocytometer. Then store at 4 °C until the assay.

NOTE: Ag-coated beads should not be stored for more than 1 month.

1.2. Preparation of poly-L-lysine coverslips

1.2.1. Before the B cell activation assay with Ag-coated beads, prepare poly-L-lysine-coated coverslips (PLL-coverslips). Use a 50 mL tube containing 40 mL of 0.01% w/v of PLL solution and add to 12 mm-diameter coverslips. Rotate overnight at RT.

1.2.2. Wash the coverslips with 1x PBS and leave to dry on a 24-well plate lid covered with paraffin film. Proceed with the B cell activation.

1.3. B cell activation

1.3.1. To start B cell activation with beads, first dilute the IIA1.6 B cell line to 1.5 x 10^6 cells/mL in CLICK medium (RPMI-1640 supplemented with 2 mM L-Alanine-L-Glutamine + 55 µM beta-mercaptoethanol + 1 mM pyruvate + 100 U/mL Penicillin + 100 µg/mL streptomycin) + 5% heat-inactivated fetal bovine serum [FBS]).

1.3.2. Combine 100 µL (150,000 cells) of IIA1.6 B cells with Ag-coated beads at 1:1 ratio in 0.6 mL tubes. Mix gently using a vortex and seed onto PLL-coverslips. Incubate for different time points in a cell culture incubator (37 °C / 5% CO_2). The typical activating time points are 0, 30, 60 and 120 min.

NOTE: It is important to mix by vortex instead of pipetting up and down, because this could reduce the number of beads in the sample, due to their accumulation in the plastic tip of the pipette. Use beads coated with BCR-ligand- as a negative control for each assay. We recommend activating the longest incubation time first and then the following time points. Calculate intervals of activation for samples such that they are ready for fixation at the same time.

1.3.2.1. For time 0, place the PLL-coverslips into the 24-well plate lid on ice. Add the cells-Ag-coated bead mixture and incubate for 5 min on ice.

1.3.3. Add 100 µL of cold 1x PBS to each coverslip to stop the activation and continue with the immunofluorescence protocol. See protocol section 3.

2. B cell activation on antigen-coated coverslips

2.1. Preparation of antigen-coated coverslips

2.1.1. Before activation, prepare the antigen solution (1x PBS containing 10 µg/mL BCR-ligand+ and 0.5 µg/mL rat anti-mouse CD45R/B220).

NOTE: Consider preparing the coverslips the day before the assay. B220 improves B cell adhesion, however, the BCR-Ligand+ is sufficient to generate the spreading response.

2.1.2. Place the 12 mm coverslip onto a 24-well plate lid covered with paraffin film, add 40 µL of antigen solution onto each coverslip and incubate at 4 °C overnight. Seal the plate to avoid evaporation of antigen solution.

2.1.3. Wash the coverslips with 1x PBS and air dry.

2.2. B cell activation

2.2.1. To start the activation, dilute IIA1.6 B cells to 1.5 x 10^6 cells/mL in CLICK medium + 5% FBS.

2.2.2. Add 100 µL of cells onto an antigen-coated coverslip (Ag-coverslip) and activate for different time points in a cell incubator at 37 °C / 5% CO2. The typical activating time points are 0, 30 and 60 min.

NOTE: As in section 1, we recommend starting with the longest activating time point in order to fix all the samples at the same time.

2.2.3. For time 0, place the 24-well plate lid containing the Ag-coverslip on ice and add the cells. Incubate for 5 min on ice.

2.2.4. Carefully aspirate the media on each Ag-coverslip and then add 100 µL of cold 1x PBS to stop the activation. Continue with the immunofluorescence protocol. See section 3.

3. Immunofluorescence

NOTE: To avoid cross-reactivity of the secondary antibody with the BCR of IIA 1.6 B cells, do not use mouse-derived primary antibodies.

3.1. Remove the 1x PBS and proceed with the fixation of each coverslip.

NOTE: To decide which fixation medium to use, check the antibody/dye data sheet. For example, for actin labeling by phalloidin use paraformaldehyde (PFA) fixation medium, and for the centrosome labeling by pericentrin use methanol fixation.

3.1.1. Add 50 µL of 1x PBS supplemented with 4% PFA and incubate for 10 min at RT.

CAUTION: Formaldehyde is toxic. Please read the MSDS before working with this chemical. PFA solutions should be only made under a chemical fume hood wearing gloves and safety glasses.

3.1.2. Alternatively, add 50 µL of cold methanol and incubate for 20 min.

3.2. Wash three times with 1x PBS.

NOTE: Coverslips can be stored at 4 °C at this point for maximum three days in 1x PBS.

3.3. Remove 1x PBS and add 50 µL of blocking buffer (2% BSA + 0.3 M glycine in 1x PBS) onto each coverslip. Incubate at RT for 10 min.

3.4. Aspirate gently and add 50 µL of permeabilization buffer (PB) (0.2% BSA + 0.05% saponin in 1x PBS). Incubate at RT for 20 min.

3.5. Prepare the antibodies or dyes in PB (use 30 µL per coverslip) and incubate at RT for 1 h or overnight at 4 °C. Refer to the **Table of Materials** for additional details.

3.5.1. To label the microtubule organizing center (MTOC) or centrosome use the following antibodies: anti-γ-Tubulin (1:500), anti-Cep55 (1:500), anti-α-tubulin (1:500), anti-pericentrin (1:1000).

NOTE: For centrosome labeling B cells can be transfected with a centrin-GFP expression plasmid.

3.5.2. For labeling golgi apparatus use anti-Rab6a (1:500).

NOTE: Other antibodies or dyes can also be used.

3.5.3. For labeling lysosomes, use anti-Lamp1 (1:200).

NOTE: Anti-H2-DM and anti-MHC-II also can be used to label antigen processing compartments[15, 16].

3.5.4. For labeling endoplasmic reticulum use anti-Sec61a (1:500).

3.5.5. For actin cytoskeleton consider the following: polymerized actin can be visualized by Phalloidin conjugated to fluorescent dyes.

NOTE: B cells transfected with a LifeAct-GFP/RFP expression plasmid can also be used to label actin.

3.6. Wash the coverslips three times with PBS.

3.7. Dilute the secondary antibody or dyes in PBS (use 30 µL per coverslip) and incubate 1 h at RT.

NOTE: Avoid exposing the samples to direct light to preserve the quality of the fluorescence signal.

3.8. Wash the coverslips three times with PBS.

3.9. Remove the PBS solution from coverslips.

3.10. Add 4 µL of mounting reagent to a microscope slide. Mount the coverslip onto the slide with the cell side facing down. Allow the slides to dry for 30 min at 37 °C or at RT overnight.

NOTE: Consider using an "anti-fade" mounting reagent (see the **Table of Materials**).

3.11. Acquire fluorescence images on a confocal or epifluorescence microscope with a 60x or 100x oil immersion objective. For each acquisition consider the transmitted light or bright field to easily identify B cells interacting with beads.

NOTE: Take three-dimensional (3D) images, covering the entire cell using z-stacks. We recommend taking stacks of 0.5 µm thickness.

4. Image analysis

NOTE: The following algorithms are described for ImageJ software. However, this can be performed using an equivalent software. Also, consider that for all fluorescence intensity measurements we use the integrated fluorescence density ("RawIntDen" in ImageJ), because this parameter considers the total amount of fluorescence in each pixel of the image taking the area into account.

4.1. Analysis of the distribution of organelles in B cells activated with Ag-coated beads

NOTE: To quantify the polarization of cell components to the IS, we define an arbitrary value as a measure of proximity to the IS. The index ranges between -1 (anti-polarized) and 1 (Fully polarized, object on the bead), as was previously presented by Reversat et al.[12].

4.1.1. Estimate the polarity index for the centrosome and Golgi apparatus (**Figure 1B**).

NOTE: This algorithm can be used for organelles that are confined to a one point.

4.1.1.1. First define the bead and cell areas to analyze using the **circle tool** selection to delimit the boundaries of both, and then save them as regions of interest (ROI). See **Figure 1**, **Figure 2**, **Figure 3**, and **Figure 4**.

4.1.1.2. Determine the cell center (**CC**) and the bead center (**BC**) by running **Analyze | Measure** on cell and bead areas respectively. X and Y values obtained from the R**esults** window determine the center coordinates.

4.1.1.3. Manually determine the center of the centrosome or Golgi apparatus (**Organelle**) using the **point tool** selection in ImageJ and run **Analyze | Measure**. X and Y values obtained from the **Results** window determine the coordinates.

4.1.1.4. Then, draw an angle from CC to Organelle (**a**) and CC to BC (**b**) using the angle tool selection and run **Analyze | Measure**. The **angle** value in the **results** window shows the angle (**α**) between both vectors (**a** and **b**).

4.1.1.5. Calculate the polarity index using the following formula:

$$\text{Polarity index} = \cos(\alpha)$$

4.1.2. Estimate the polarity index for lysosomes (**Figure 1F**).

NOTE: We use this algorithm to analyze the polarity of organelles that display a more dispersed distribution, such as lysosomes.

4.1.2.1. Define the bead and cell areas to analyze, using the **circle tool** selection to delimit the boundaries of both, and save them as ROI. Once the bead and cell areas have been determind, set the fluorescence channel and project the image into one z-stack (**Image | Stacks | Z-Project [Sum slices]**), then run **Analyze | Measure** and extract the mass center (MC) coordinates (MX and MY) from the **results** windows.

4.1.2.2. Apply the same algorithm mentioned before changing Organelle for MC. Thus, the angle (**α**) is defined by CC-MC (**a**) and CC-BC (**b**).

4.1.2.3. Calculate the polarity using the following formula:

$$\text{□□□□□□□□□ □□□□□} = \text{□}\frac{\cos(\text{□})}{\text{□}}$$

4.1.3. Estimate lysosome recruitment at the bead (**Figure 2B**).

NOTE: This algorithm is used to quantify the organelle which is in contact with the synaptic interface.

4.1.3.1. Determine the bead and cell areas. Select the fluorescence channel of interest and project the image onto one z-stack (**Image | Stacks | Z-Project [Sum slices]**).

4.1.3.2. Measure the organelle fluorescence in the bead area (**BF**) and at the bead-cell area, as whole cell fluorescence (**WCF**).

4.1.3.3. Calculate the organelle fluorescence percentage at the bead using the following formula:

$$\%\ \text{□□□□ □□□□□□□□□□□□□□} = \frac{\text{□□}}{\text{□□□}} \times 100\%$$

4.1.4. Estimate lysosome recruitment to the synaptic area (**Figure 2B**).

NOTE: This algorithm is used to quantify an organelle adjacent to the IS.

4.1.4.1. Once the bead and cell areas have been determined, determine the angle of the vector between the CC and BC and then rotate the image to achieve a 0° angle.

4.1.4.2. Set the fluorescent channel and project the image into one z-stack (**Image | Stacks | Z-Project [Sum slices]**), eliminate all the fluorescence that falls outside the cell and bead area together (run **edit | clear outside** in ImageJ).

4.1.4.3. Using the **rectangle tool** selection, draw a rectangle adjacent to the bead and measure the synaptic area fluorescence (**SAF**). This rectangle is a quarter of the cell width.

4.1.4.4. Select the entire image and run **Analyze | Measure** to obtain the whole cell fluorescence (**WCF**).

4.1.4.5. Calculate the organelle fluorescence percentage adjacent to the IS using the following formula:

$$\%\ \text{□□□□□□□□□ □□□□□□□□□□□□□□} = \frac{\text{□□□}}{\text{□□□}} \times 100\%$$

4.1.5. Quantify recruitment of cellular components to the centrosome.

NOTE: This algorithm, adapted from Obino et al.[5], is used to quantify the enrichment of organelles at the centrosome area. Briefly, we consider the centrosome area as the domain in

which the centrosome-associated organelle fluorescence remains constant or above 70% in the fluorescence/radius plot. It is essential to set this parameter at resting conditions because this radius could change upon activation.

4.1.5.1. Once bead and cell areas have been determined, define the localization of the centrosome using the **point tool** selection (**Figure 3B**).

4.1.5.2. First, determine the maximum area around the centrosome that is possible to quantify, by drawing a 3 µm-radius circle surrounding the centrosome.

4.1.5.3. Use the ImageJ plugin **Radial Profile**, which measures the fluorescence in concentric circles and displays a fluorescence/radius plot.

4.1.5.4. Identify the maximum radius within which at least 70% of the fluorescence intensity is maintained.

4.1.5.5. Calculate the fluorescence density ratio using the following formula:

$$\text{Ratio de densidad de fluorescencia} = \frac{(\text{Fluorescencia en el área central} / \text{Área central})}{(\text{Área total} - \text{Área central} / \text{Área total} - \text{Área central})}$$

NOTE: The fluorescence density ratio indicates the concentration of an organelle at the centrosome compared to its distribution in the whole cell.

4.2. Analysis of cell spreading and distribution of organelles in B cells activated on Ag-coverslips

4.2.1. Estimate organelle distribution at the IS (**Figure 4B**).

NOTE: This algorithm allows the quantification of the XY distribution of organelles and their concentration at the center of the IS. We define a ratio of fluorescence density between the central and the total synaptic area. The values obtained can vary from negative to positive, indicating a central or a peripheral distribution of the organelle, respectively.

4.2.1.1. Determine the slice in which the cell is in contact with the cover.

NOTE: To delimit the cell boundaries one can label the plasma membrane or actin which is enriched in the periphery of the IS.

4.2.1.2. Change the type of the slice to 8-bit, then binarize (**Process | Binary | Make Binary**) and connect the nearest outsider points **Process | Binary | Outline**. Delimit the boundaries of the cell area (**CA**) using the **polygon tool** selection.

NOTE: This step is useful to increase the contrast of the cell boundaries and make it easier to identify. Also, at this point, it is possible to apply the *Analyze particle* plugin of ImageJ to determine CA automatically. However, other cells in the same field can interfere with the results.

4.2.1.3. Take **CA** parameters (height and width) and delimit a central rounded area, which is separated from the boundaries by a quarter of the height and width values, consider this area as the Cell Center Area (**CCA**).

4.2.1.4. Calculate the fluorescence density distribution at the center of the IS using the following formula:

$$\text{[illegible]} = \frac{\frac{\text{[illegible]}}{\text{[illegible]}}}{\frac{\text{[illegible]}}{\text{[illegible]}}} - 1$$

4.2.2. Measure organelle distribution in Z planes (**Figure 4C**).

NOTE: This analysis determines the general distribution of organelle fluorescence across Z planes of B cells activated onto Ag-coverslips, showing the percentage of fluorescence per Z fraction.

4.2.2.1. To quantify the fluorescence distribution in Z, first determine the plane of the IS where the cell is in contact with the Ag-coverslip and then the plane corresponding to the upper limit of the cell.

4.2.2.2. Draw a line across the cell center.

4.2.2.3. Reslice the image in Z (**Image | Stacks | Reslice**), to obtain an XZ image.

4.2.2.4. Measure the height and divide the XZ image into 10 consecutive rectangles of the same height (Z fraction) from the bottom (IS interface) to the top (upper side of the cell) and quantify the fluorescence signal in each one.

4.2.2.5. Normalize the fluorescence intensity of each Z fraction by the sum of the total fluorescence of the 10 fractions.

4.2.2.6. Plot the percentage of fluorescence intensity per Z fraction of the cell.

5. Isolation of centrosome-enriched fraction from resting and activated B cells

NOTE: Keep all solutions at 4 °C during the experiment to avoid protein degradation. This protocol was adapted from previous work[17, 18].

5.1. Activate 2 x 10^7 B cells with Ag-coated beads in 2 mL of CLICK medium + 2% heat-inactivated FBS (ratio 1:1). Consider non-activated B cells as resting B cells.

5.2. Add cytochalasin D (2 µM) and nocodazole (0.2 µM) and incubate for 1 h at 37 °C.

NOTE: These drugs are used to gently detach the centrosome from the nucleus, by depolymerizing actin cytoskeleton and microtubules, respectively, to avoid nuclear contamination.

5.3. Wash each sample with 5 mL of cold 1x TBS (50 mM Tris-HCl, pH 7.6, 150 mM NaCl) and then with 1 mL of 0.1x TBS supplemented with 8% sucrose.

5.4. Resuspend the cells with 150 µL of centrosome lysis buffer (1 mM HEPES, pH 7.2, 0.5% NP-40, 0.5 mM $MgCl_2$, 0.1% beta-Mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) or protease inhibitor cocktail) and pipette up and down until viscosity decreases, which indicates cell lysis is identified in the sample. Put the sample in a 1.5 mL tube.

5.5. Centrifuge at 10,000 x *g* for 10 min at 4 °C, to separate the organelles from the nucleus.

5.6. Carefully recover the supernatant and place it on top of a 1.5 mL tube with 300 µL of gradient buffer (GB) (10 mM PIPES pH 7.2, 0.1% Triton X-100, 0.1% beta-mercaptoethanol) containing 60% sucrose.

5.7. Centrifuge at 10,000 x *g* for 30 min at 4 °C to concentrate the centrosomes in the 60% sucrose fraction.

5.8. Meanwhile, prepare a discontinuous gradient in 2 mL ultracentrifuge tubes by overlaying 450 µL of GB + 70% sucrose with 270 µL of GB + 50% sucrose and then 270 µL of GB + 40% sucrose.

5.9. After the first centrifugation (centrosome-concentrated fraction), discard the upper fraction (less dense portion) until reaching the interface and vortex the remaining sample in the tube. Then, overlay on top of the discontinuous gradient previously prepared with the centrosome enriched sample.

NOTE: Be careful not to disrupt the gradient, all pipetting procedures must be carefully performed.

5.10. Centrifuge at 40,000 x *g* for 1 h at 4 °C with minimal acceleration and with the centrifuge brake set to off, to avoid disrupting the gradient.

5.11. Collect 12 fractions of 100 µL into separate tubes beginning from the top.

5.12. Identify the centrosome enriched fractions by immunoblot using γ-tubulin as the centrosome marker.

NOTE: We usually find centrosome-enriched extracts between fractions 6 and 8.

REPRESENTATIVE RESULTS:

The present article shows how B cells can be activated using immobilized antigen on beads or coverslips to induce the formation of an IS. We provide information on how to identify and quantify the polarization of different organelles by immunofluorescence and how to characterize proteins that undergo dynamic changes in their association to the centrosome, which polarizes to the IS, using a biochemical approach.

The imaging of B cells by immunofluorescence allows us to follow the dynamics of organelles such as the centrosome, Golgi apparatus and lysosomes, which are recruited to the IS upon B cell activation. One can obtain quantitative parameters to measure the polarization of these organelles to the IS and compare them under different conditions. As we show in **Figure 1A**, the Centrosome and Golgi Apparatus are recruited to the IS upon B cell activation. Recruitment was not observed in B cells stimulated with BCR-ligand- indicating that BCR engagement is required to mobilize the centrosome and Golgi apparatus to the IS (**Figure 1A**). To obtain a polarity index for each organelle, as a measurement of its proximity to the IS, we considered three features: the distance between the organelle and the center of the cell, the distance between the bead center and the cell center, and the angle between these two vectors (**Figure 1B**). This index ranges between -1 (anti-polarized) and 1 (fully polarized, object on the bead). **Figures 1C** and **1D** show graphs where polarity indexes of each organelle are plotted versus time of activation. In agreement with immunofluorescence staining, both the centrosome and Golgi apparatus, display more positive polarity indexes upon longer time points of activation, reflecting how they are progressively recruited to the IS. This algorithm is applicable for organelles confined to one point. Additionally, we quantified the polarization of organelles that display a dispersed distribution, such as lysosomes (**Figure 1E**), by applying the same algorithm mentioned before, but changing the point coordinate by the mass center coordinates of lysosomes (**Figure 1F**). We can observe that the polarity indexes of lysosome pools reach more positive values upon activation, which indicates that the lysosomes are being mobilized towards the synapse upon B cell activation (**Figure 1G**).

We also defined two algorithms to determine organelles that are in contact with the synaptic interface (bead) and the amount of organelles at the proximity of the synapse, but not necessarily in contact. As we show in **Figure 2**, we quantified lysosomes contacting the synaptic interface (**Figure 2A**), by dividing the LAMP-1 signal at the bead area by the total fluorescence in the cell plus the bead (**Figure 2B**). It is important to consider that the diameter of the bead area determines the ranges of the percentages obtained. For example, in the quantification displayed (**Figure 2B**), we draw a circle with a 3 µm diameter to measure the fluorescence at the bead, which is a restrictive parameter considering the bead size (3 µm). Using this parameter, the results show that lysosomes are progressively recruited to the synaptic interface upon B cell activation, reaching 10–15% of the total mass (**Figure 2C**). Another approach shown is the quantification of lysosomes to the synaptic area. **Figure 2D** shows that lysosomes progressively accumulate up to 25% of their lysosomes at the IS. Overall, by performing both types of analysis one can evaluate the polarization and docking of organelles at the IS.

During B cell activation, changes in the pool of centrosome-associated proteins have been documented[5]. For instance, one of these proteins that changes their association at the Centrosome during B cell activation is actin. In this case actin becomes depleted within this region, which allows the centrosome to become detached from the nucleus, promoting its polarization to the IS[5]. Here we present the quantification of actin at the Centrosome and how it is depleted upon B cell activation (**Figure 3**). To define the centrosome area used to quantify associated actin, we measured the fluorescence intensity of this label in concentric circles surrounding the centrosome. The radius was defined based on the point where at least 70% of the fluorescence intensity of the label is maintained (**Figure 3B).** Using this approach one can quantify the decrease in actin density at the centrosome upon B cell activation, as shown in **Figure 3C**.

To obtain greater resolution in the distribution of organelles at the IS interface, we activated B cells on antigen-coated coverslips, labeling actin, Golgi apparatus and endoplasmic reticulum (**Figure 4A**). The distribution of organelles at the IS can be performed by diving the synaptic area in a central and peripheral area, applying the criteria shown in **Figure 4B**. This was based on a previous work showing that BCRs gather within this central area, which most likely corresponds to the central supramolecular activation complex (cSMAC)[10,19]. **Figure 4A** shows that organelles recruited to the IS, such as the Golgi apparatus and the endoplasmic reticulum display opposite distributions. Indeed, their distribution indexes correlate with their immunofluorescence staining shown in **Figure 4C**. The Golgi apparatus shows a positive value, which means that it is concentrated at the center of the IS while the endoplasmic reticulum shows negative values, which means it is mainly localized at the peripheral region of the IS. Additionally, it is possible to take the values of the synaptic area previously determined, to measure the spreading area during B cell activation, as it is shown in **Figures 4D** and **4E**. These results show an increase in the spreading area upon B cell activation, as previously described[10,20].

As in the bead assay, we can determine the distribution of organelles toward the immune synapse by taking the XZ images of B cells activated onto antigen-coated coverslips and measuring the organelle fluorescence across the Z dimension (**Figure 4F**). The fluorescence intensity of actin in the Z plane was measured, as it is represented in the XZ plane in **Figure 4F**, where a progressive enrichment of actin at the synaptic interface (fractions 1 and 2) can be observed (**Figure 4G**).

In addition to the B cell imaging analysis, we performed the isolation of centrosome-enriched fractions to quantify centrosome-associated proteins (**Figure 5A**). This approach can be important to complement the study of IS formation in B cells, given that the centrosome polarizes to the synaptic membrane and has been shown to coordinate lysosome trafficking involved in antigen extraction and presentation[21]. This method was previously described using large amounts of cells (1 x 10^9 cells)[17, 18] but we have standardized a simplified version, which uses a reduced amount of cells and can be performed using lower-volume ultracentrifuge-tubes (2-3 mL). As we show in **Figure 5B**, we analyzed different cell fractions by immunoblotting and determined which ones correspond to centrosome-rich fractions, by gamma tubulin staining.

Here we show an example of proteins that undergo changes in their accumulation at the centrosome, such as Actin and Arp2, which have been previously reported[5].

FIGURE AND TABLE LEGENDS:

Figure 1: **Polarization of organelles towards the IS**. (**A**) Immunofluorescence staining of the Golgi apparatus (Rab6a) and microtubules (α-tubulin) in IIA1.6 B cells incubated with BCR-ligand+ (0, 30, 60 and 120 min) or BCR-ligand- beads (120 min). Arrowheads indicate the centrosome. BF = Bright field. Scale bar = 3 μm. (**B**) Scheme depicting how to calculate the polarity index of organelles (centrosome or Golgi apparatus) toward the IS. a = Distance between the center of the cell (CC) to the organelle. b = Distance from the CC and to the bead center (BC). (**C**, **D**) Representative dot plots showing polarity indexes of organelles at different time points of activation. Each dot represents one cell. (**E**) Immunofluorescence staining of Lysosomes (Lamp1) in B cells incubated with BCR-ligand+ (0, 30, 60 and 120 min) or BCR-ligand- beads (120 min). BF = Bright field. Scale bar = 3 μm. (**F**) Scheme representing how to calculate the polarity index of Lysosomes. a = Distance between CC to the mass center (MC). b = Distance from CC to BC. (**G**) Representative dot plots showing polarity indexes of lysosomes at different time points of activation. Each dot represents one cell. 2-way ANOVA with Sidak's post-test was performed. Means with SEM are shown.

Figure 2: **Accumulation of lysosomes at the IS**. (**A**) Immunofluorescence staining of lysosomes (Lamp1) in B cells incubated with BCR-ligand+ (0, 30, 60 and 120 min) or BCR-ligand-beads (120 min). BF = bright field. Scale bar = 3 μm. (**B**) Scheme depicting how to calculate the accumulation of organelles such as lysosomes at the bead and the synaptic area. Fluorescence of the whole cell (WCF), bead fluorescence (BF) and the synaptic area fluorescence (SAF) are indicated. (**C**, **D**) Representative bar graphs for the accumulation of lysosomes at the bead and the synaptic area. N = 1. 20 > Cells. 2-way ANOVA with Sidak's post-test was performed. Means with SEM are shown.

Figure 3: **Quantification of actin at the centrosome during IS formation.** (**A**) Immunofluorescence staining of actin (Phalloidin) and microtubules (α-Tubulin) in B cells incubated with BCR-ligand+ (0 and 60 min) or BCR-ligand- beads (0 and 60 min). Arrow heads indicate the centrosome. Scale bar = 3 μm. (**B**) Scheme depicting how to calculate the recruitment or depletion of Centrosome associated components. The centrosome area is calculated by using a radius (X μm) that maintain at least 70% of the maximum fluorescence. (**C**) Representative dot plots showing actin at the centrosome under activating (BCR-ligand+) and non-activating (BCR-ligand-) conditions. N = 1. 15 > Cells. 2-way ANOVA with Sidak's post-test was performed. Means with SEM are shown.

Figure 4: **Distribution of cellular components at the synaptic interface.** (**A**) Immunofluorescence staining of actin (Phalloidin), Endoplasmic Reticulum (Sec61a), Golgi apparatus (transfected with a KDELR-DN-GFP a Negative Dominant of KDEL Receptor, which localizes in the Golgi). B cells were seeded onto coverslips coated with a BCR-ligand+ for 60 min. BF = bright field. Scale bar = 5 μm. (**B**) Scheme depicting how to determine the spreading area of the cell and the recruitment of organelles at the center of the IS. In the scheme, CA indicates the cell area delimited by cortical

actin and CCA indicates the center cell area. (**C**) Golgi apparatus and endoplasmic reticulum distribution at the IS, represented by a bar graph where a value >0 indicates an enrichment in the center of the IS and < 0 indicates a peripheral distribution. N = 1. 20 > Cells. Means with SEM are shown. (**D**) Representative images of B cells labelled for actin (Phalloidin) activated onto Ag-coated coverslips at different time points (0, 30 and 60 min), showing the XZ and XY plane. (**E**) Scheme depicting how to determine the distribution of the signal of interest in the Z plane, and the plot per Z fraction. The rectangle indicates the IS interface. (**F**) Graph showing the spreading area of B cells in E. (**G**) Distribution of actin across the Z plane represented by a line plot of the percentage of fluorescence distribution versus each Z fraction. The rectangle represents the IS interface. N = 1. 25 > Cells. Means with SEM are shown.

Figure 5: **Centrosome enriched fractions of B cells.** (**A**) Workflow of how to obtain centrosome-enriched fractions by sucrose gradient ultracentrifugation. (**B**) Immunoblot of fractions obtained from resting and activated B cells (60 min). Centrosome-rich fractions are detected by labeling with γ-tubulin and are indicated within the dashed rectangle (fraction 6 to 8). Centrosome associated proteins (Actin and Arp2) are shown in centrosome-rich fractions in activating and resting conditions.

DISCUSSION:

We describe a comprehensive method to study how B lymphocytes re-organize their intracellular architecture to promote the formation of an IS. This study includes the use of imaging techniques to quantify the intracellular distribution of organelles, such as centrosome, Golgi apparatus and lysosomes during B cell activation, and how they polarize to the IS. Additionally, we describe a biochemical approach to study changes in the centrosome composition upon B cell activation.

To promote the formation of an IS in B cells, we used immobilized antigens (antigen-coated beads) instead of soluble immune-complexes because the former triggers the establishment of a discrete contact site where cytoskeleton rearrangements and organelle polarization can easily be studied. A critical aspect in imaging B cell activation is the preparation of the samples. All image acquisitions should ideally obtain a 1:1 cell : bead ratio, to quantify the polarization of organelles to a unique site of antigen contact. However, in some cases B cells can capture two or more beads, thereby making it difficult to choose a single site of antigen encounter. To avoid the formation of bead aggregates, it is important to vortex antigen-conjugated beads thoroughly before adding them to the cells. Additionally, for immunofluorescence experiments, we recommend excluding from quantification cells that capture more than one bead, in order to avoid misinterpretations during image analysis. Another critical step in the preparation of samples is the fixation/permeabilization condition. For instance, we recommend fixing cells with methanol to optimize microtubule staining and 4% PFA for phalloidin staining. When simultaneously labeling actin cytoskeleton and microtubules, an alternative can be to label the actin pool by transfecting cells with a LifeAct-GFP/RFP expression plasmid in combination with microtubule staining. Image acquisition used to quantify the polarization of the Centrosome and other intracellular organelles can be performed with an epifluorescence microscope. However, to obtain an accurate quantification at different z-planes, for instance in the spreading assay, confocal microscopy is required.

For the centrosome isolation protocol described here, a critical step is obtaining an appropriate amount of samples to be able to quantify proteins by immunoblot. Given that B cells have a large nucleus and their cytoplasm is less than 30% of their cellular volume, obtaining higher yields of cytoplasmic proteins can be challenging. For this reason, we recommend using approximately 20 x 10^6 B cells for each activation time point for centrosome isolation.

A significant limitation of these methods is that activation with antigen-conjugated beads does not include the complexity of the environment in which surface-tethered antigens are presented to B cells in vivo. This limitation can be addressed by supplementing the culture or beads with other co-stimulatory factors, such as cytokines and components of the extracellular matrix. However, it is crucial to use the appropriate controls in these cases in order to correctly interpret the results.

The significance of the methods presented in this work relies on the integrated approach used to study the B cell immune synapse: (1) the polarization of organelles and their distribution and (2) the changes in protein composition at the centrosome. Due to the large number of cells required to perform a significative analysis and the large amount of data generated in this process, the different quantification algorithms presented in this work facilitate the analysis of cell imaging. Moreover, the algorithms used for cell polarization are easy to automatize by MACROS functions in ImageJ, which is a useful tool to decrease repetitive tasks and save time.

These experimental procedures can be extrapolated to other types of cells that establish polarized phenotypes in order to accomplish a certain cellular function. Additionally, these protocols can be optimized by including other assays, for instance, the activity of the associated proteins such as kinases or proteases at the Centrosome-enriched fractions. Overall, these studies can provide mechanistic insight into cell signaling and molecular pathways that regulate the establishment of cell polarity.

AKNOWLEDGEMENTS:
M.-I.Y. is supported by a research grant from FONDECYT #1180900. J.I., D.F. and J.L. were supported by fellowships from the Comisión Nacional de Ciencia y Tecnología. We thank to David Osorio from Pontificia Universidad Católica de Chile for the video recording and editing.

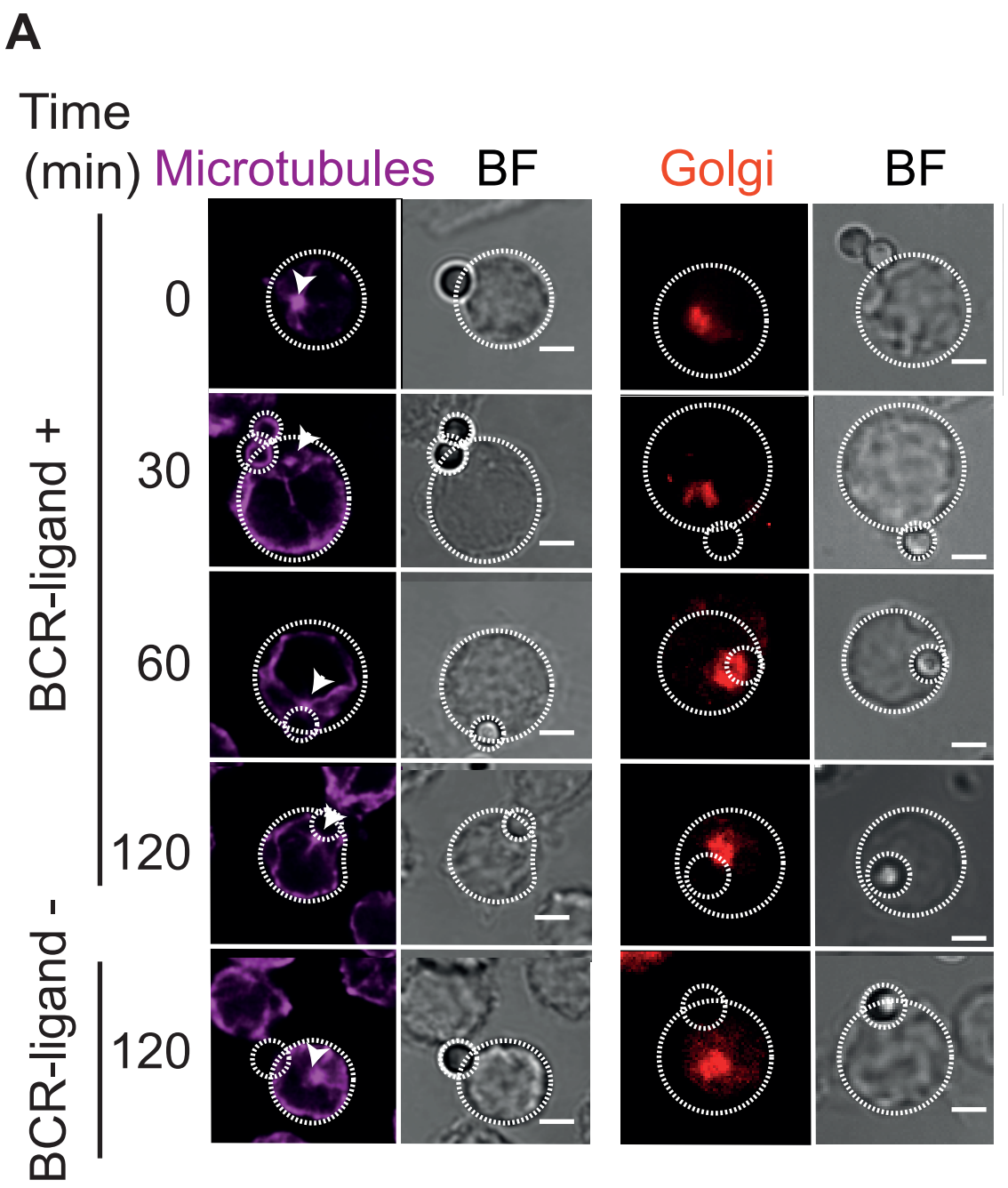
DISCLOSURES:
The authors have nothing to disclose.

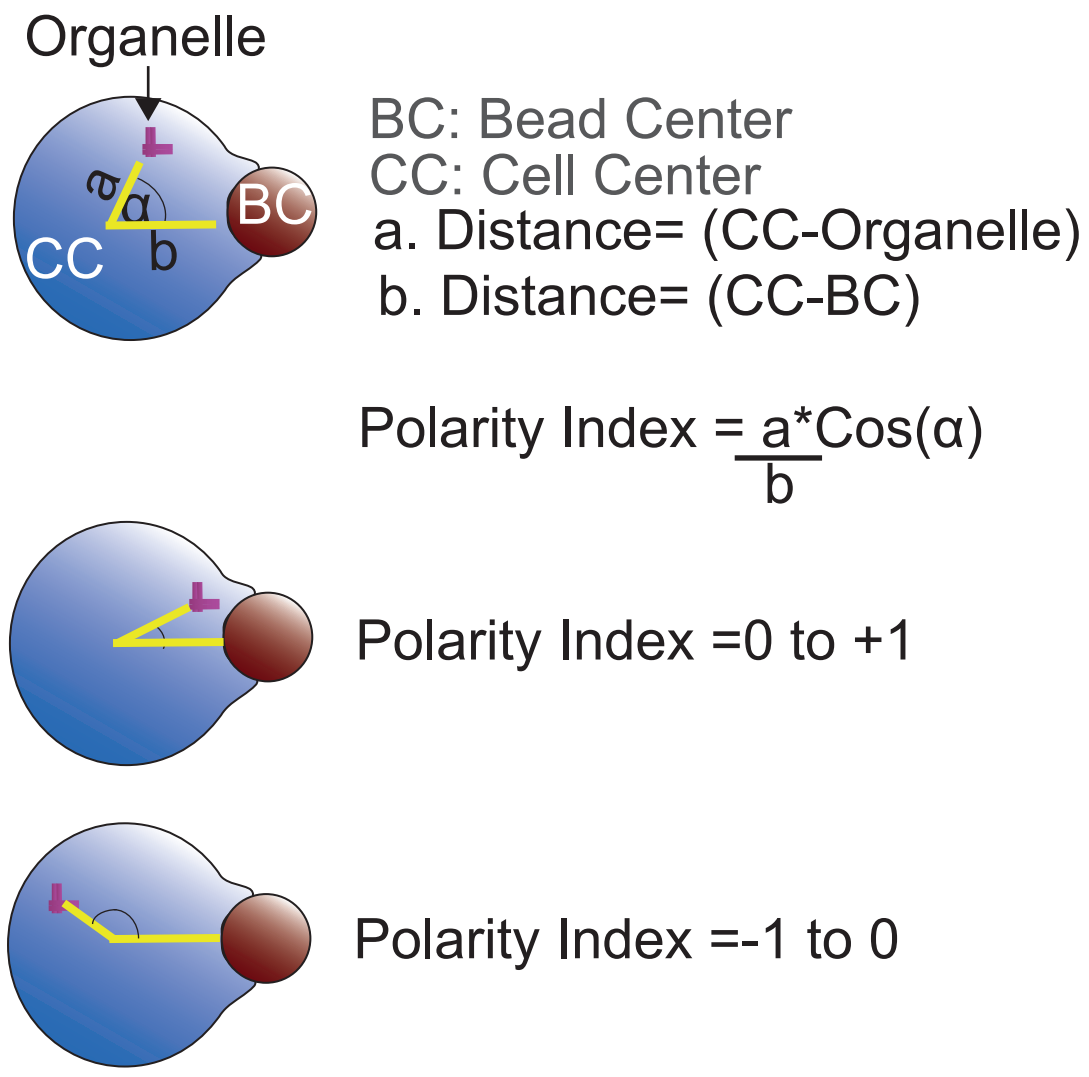
REFERENCES:
1. Carrasco, Y.R., Batista, F.D. B Cells Acquire Particulate Antigen in a Macrophage-Rich Area at the Boundary between the Follicle and the Subcapsular Sinus of the Lymph Node. *Immunity*. **27** (1), 160–171, doi: 10.1016/j.immuni.2007.06.007 (2007).
2. Batista, F.D., Harwood, N.E. The who, how and where of antigen presentation to B cells. *Nature Reviews Immunology*. **9** (1), 15–27, doi: 10.1038/nri2454 (2009).
3. Grakoui, A., et al. The immunological synapse: a molecular machine controlling T cell

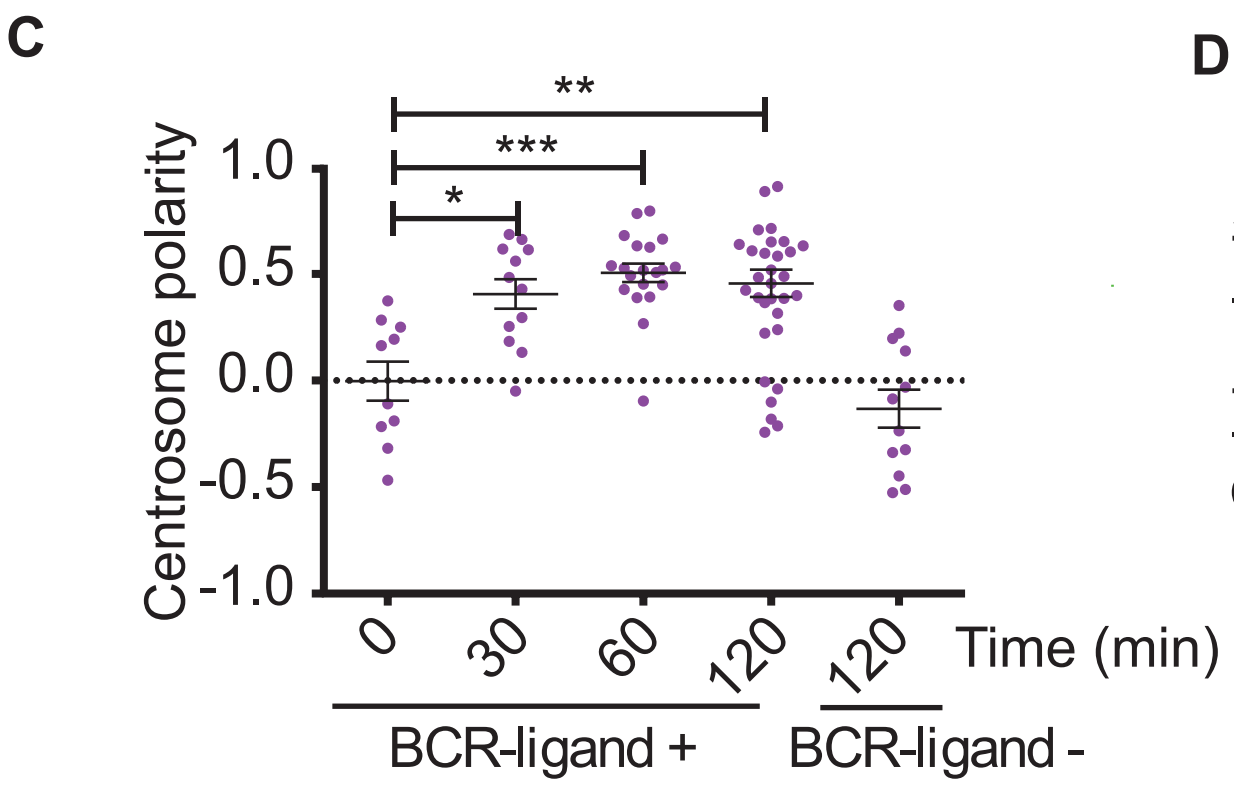
activation. *Science (New York, N.Y.)*. **285** (5425), 221–7, at <http://www.ncbi.nlm.nih.gov/pubmed/10398592> (1999).
4. Batista, F.D., Iber, D., Neuberger, M.S. B cells acquire antigen from target cells after synapse formation. *Nature*. **411** (6836), 489–94, doi: 10.1038/35078099 (2001).
5. Obino, D., et al. Actin nucleation at the centrosome controls lymphocyte polarity. *Nature Communications*. **7**, doi: 10.1038/ncomms10969 (2016).
6. Yuseff, M.I. et al. Polarized Secretion of Lysosomes at the B Cell Synapse Couples Antigen Extraction to Processing and Presentation. *Immunity*. **35** (3), 361–374, doi: 10.1016/j.immuni.2011.07.008 (2011).
7. Spillane, K.M., Tolar, P. Mechanics of antigen extraction in the B cell synapse. *Molecular Immunology*. **101** (March), 319–328, doi: 10.1016/j.molimm.2018.07.018 (2018).
8. Schnyder, T. et al. B Cell Receptor-Mediated Antigen Gathering Requires Ubiquitin Ligase Cbl and Adaptors Grb2 and Dok-3 to Recruit Dynein to the Signaling Microcluster. *Immunity*. doi: 10.1016/j.immuni.2011.06.001 (2011).
9. Wang, J.C. ,et al. The Rap1 – cofilin-1 pathway coordinates actin reorganization and MTOC polarization at the B cell immune synapse. 1094–1109, doi: 10.1242/jcs.191858 (2017).
10. Fleire, S.J., Goldman, J.P., Carrasco, Y.R., Weber, M., Bray, D., Batista, F.D. B cell ligand discrimination through a spreading and contraction response. *Science*. **312** (5774), 738–741, doi: 10.1126/science.1123940 (2006).
11. Vascotto, F., et al. The actin-based motor protein myosin II regulates MHC class II trafficking and BCR-driven antigen presentation. *Journal of Cell Biology*. **176** (7), 1007–1019, doi: 10.1083/jcb.200611147 (2007).
12. Reversat, A., et al. Polarity protein Par3 controls B-cell receptor dynamics and antigen extraction at the immune synapse. *Molecular biology of the cell*. **26** (7), 1273–85, doi: 10.1091/mbc.E14-09-1373 (2015).
13. Lankar, D., Vincent-Schneider, H., Briken, V., Yokozeki, T., Raposo, G., Bonnerot, C. Dynamics of Major Histocompatibility Complex Class II Compartments during B Cell Receptor–mediated Cell Activation. *The Journal of Experimental Medicine*. **195** (4), 461–472, doi: 10.1084/jem.20011543 (2002).
14. Duchez, S., Rodrigues, M., Bertrand, F., Valitutti, S. Reciprocal Polarization of T and B Cells at the Immunological Synapse. *The Journal of Immunology*. **187** (9), 4571–4580, doi: 10.4049/jimmunol.1100600 (2011).
15. Pérez-Montesinos, G., López-Ortega, O., Piedra-Reyes, J., Bonifaz, L.C., Moreno, J. Dynamic changes in the intracellular association of selected rab small GTPases with MHC class II and DM during dendritic cell maturation. *Frontiers in Immunology*. **8** (MAR), 1–15, doi: 10.3389/fimmu.2017.00340 (2017).
16. Delphine Le Roux,* Danielle Lankar,* Maria-Isabel Yuseff,* Fulvia Vascotto, T.Y., Gabrielle Faure-Andre´, E.M., Nicolas Glaichenhaus,‡ Be´ne´dicte Manoury,* Christian Bonnerot, § and Ana-Maria Lennon-Dume´nil* Syk-dependent Actin Dynamics Regulate Endocytic Trafficking and Processing of Antigens Internalized through the B-Cell Receptor. *Molecular biology of the cell*. **18** (September), 3451–362, doi: 10.1091/mbc.E06 (2007).
17. Reber, S. Chapter 8 Isolation of Centrosomes from Cultured Cells. **777** (2), 107–116, doi: 10.1007/978-1-61779-252-6.
18. Gogendeau, D., Guichard, P., Tassin, A.M. *Purification of centrosomes from mammalian*

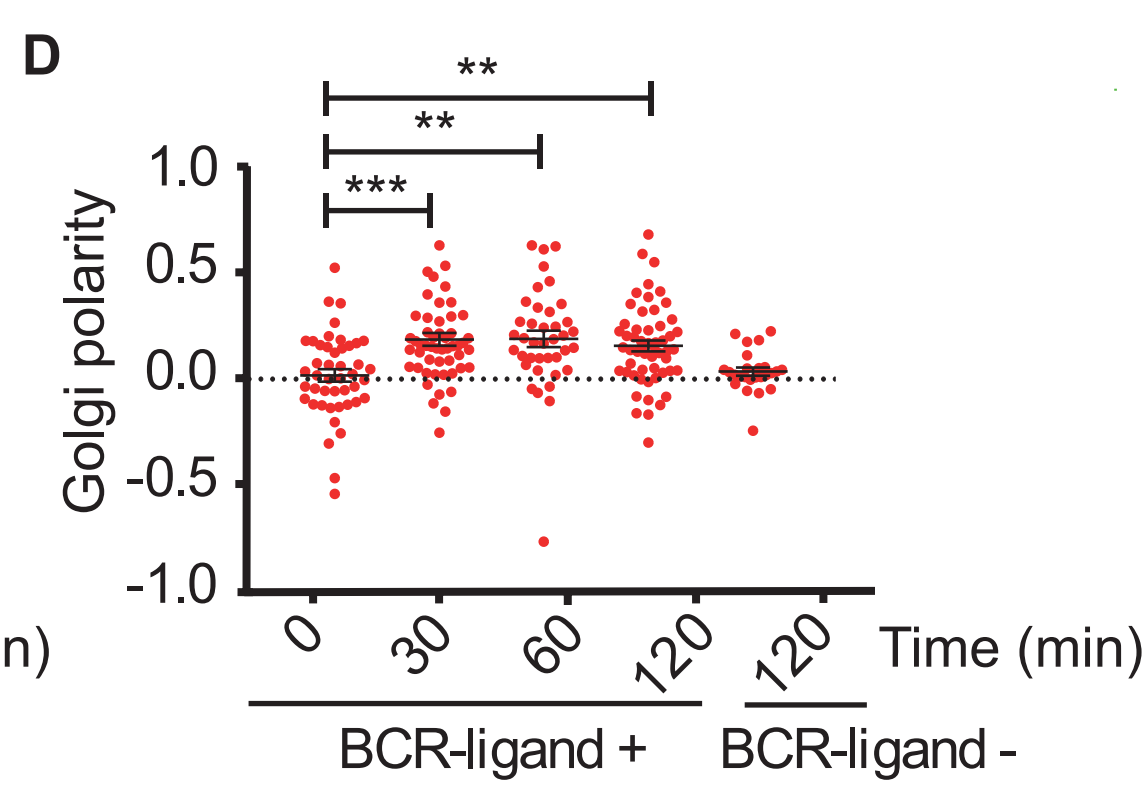
cell lines. *Methods in Cell Biology*. **129**, doi: 10.1016/bs.mcb.2015.03.004. Elsevier. (2015).
19. Obino, D. et al. Vamp-7–dependent secretion at the immune synapse regulates antigen extraction and presentation in B-lymphocytes. *Molecular Biology of the Cell*. **28** (7), 890–897, doi: 10.1091/mbc.E16-10-0722 (2017).
20. Harwood, N.E., Batista, F.D. Early Events in B Cell Activation BCR: B cell receptor. *Annual Review of Immunology*. doi: 10.1146/annurev-immunol-030409-101216 (2010).
21. Yuseff, M.I., Lennon-Duménil, A.M. B cells use conserved polarity cues to regulate their antigen processing and presentation functions. *Frontiers in Immunology*. **6** (MAY), 1–7, doi: 10.3389/fimmu.2015.00251 (2015).

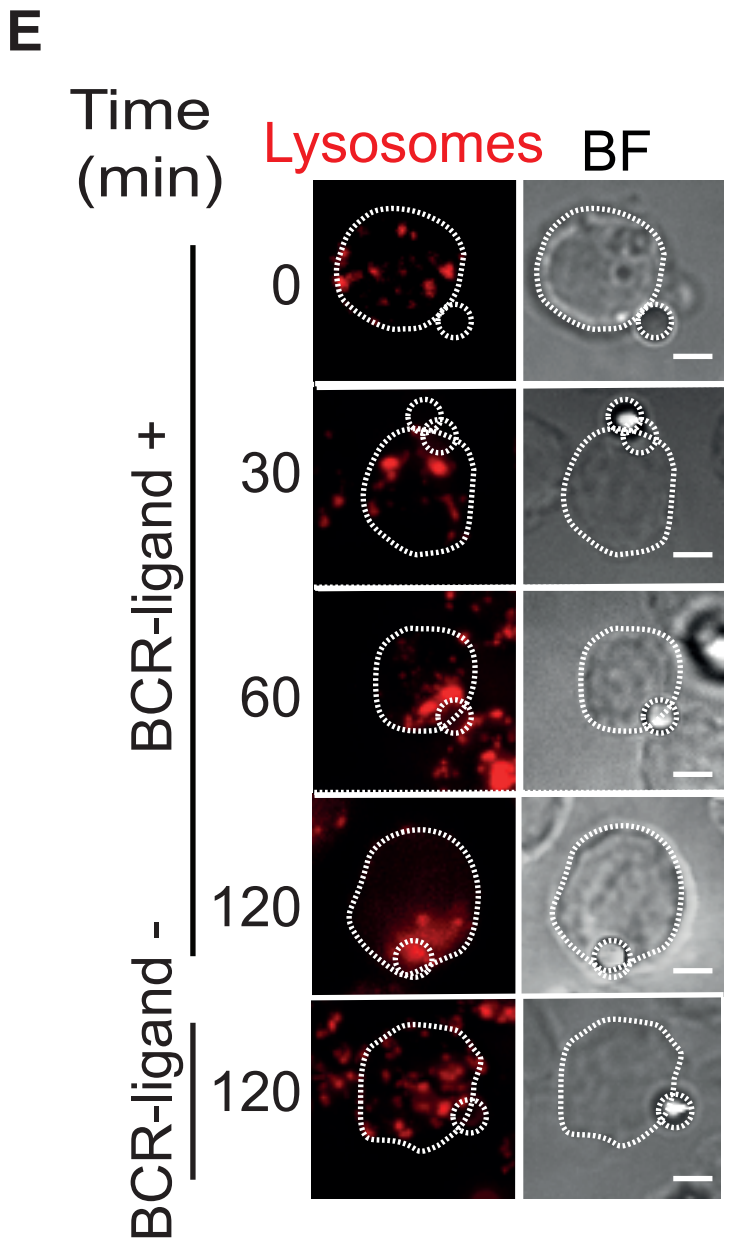
Figure 1.
Ibañez et al.











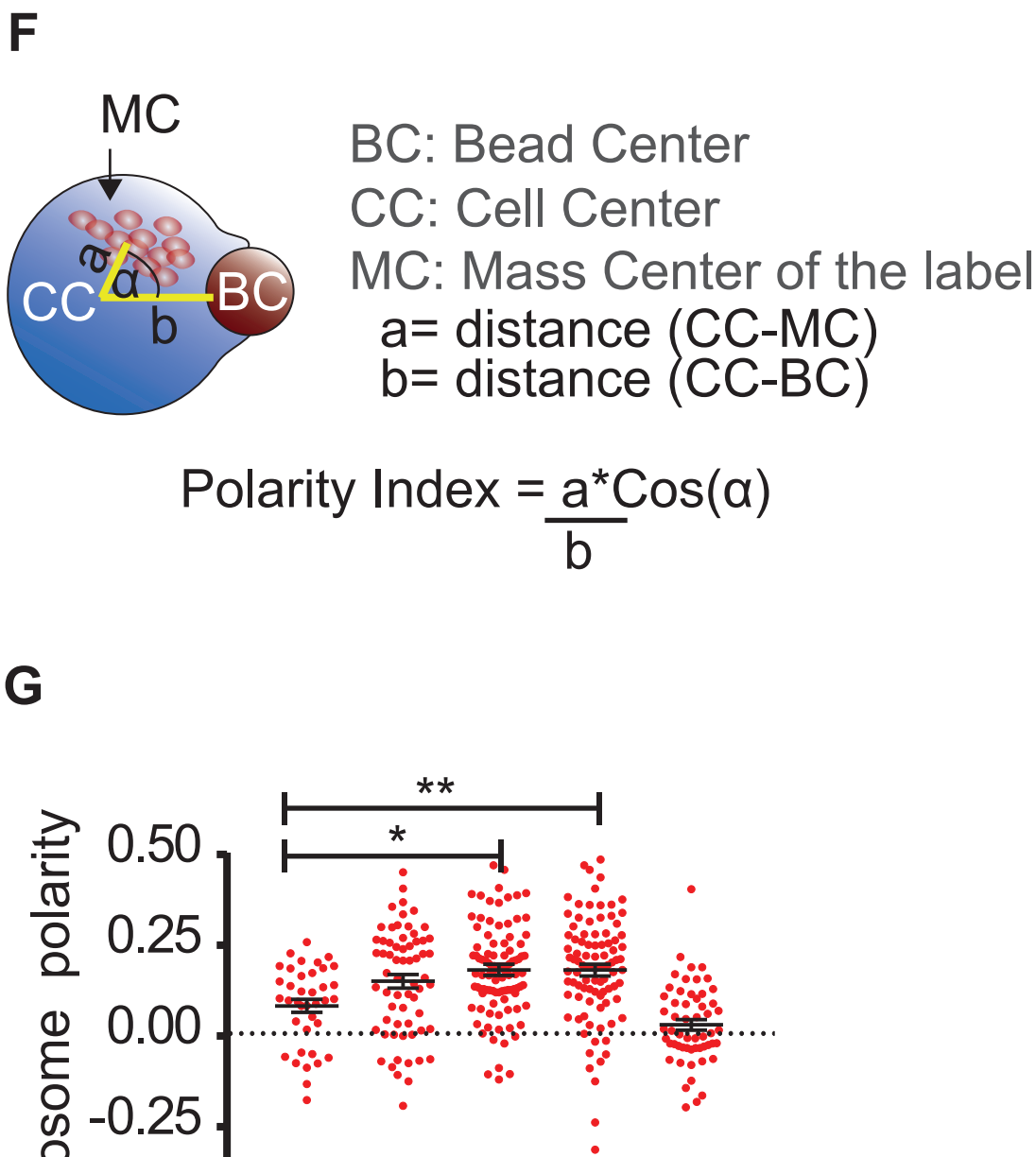


Figure 2.
Ibañez et al.

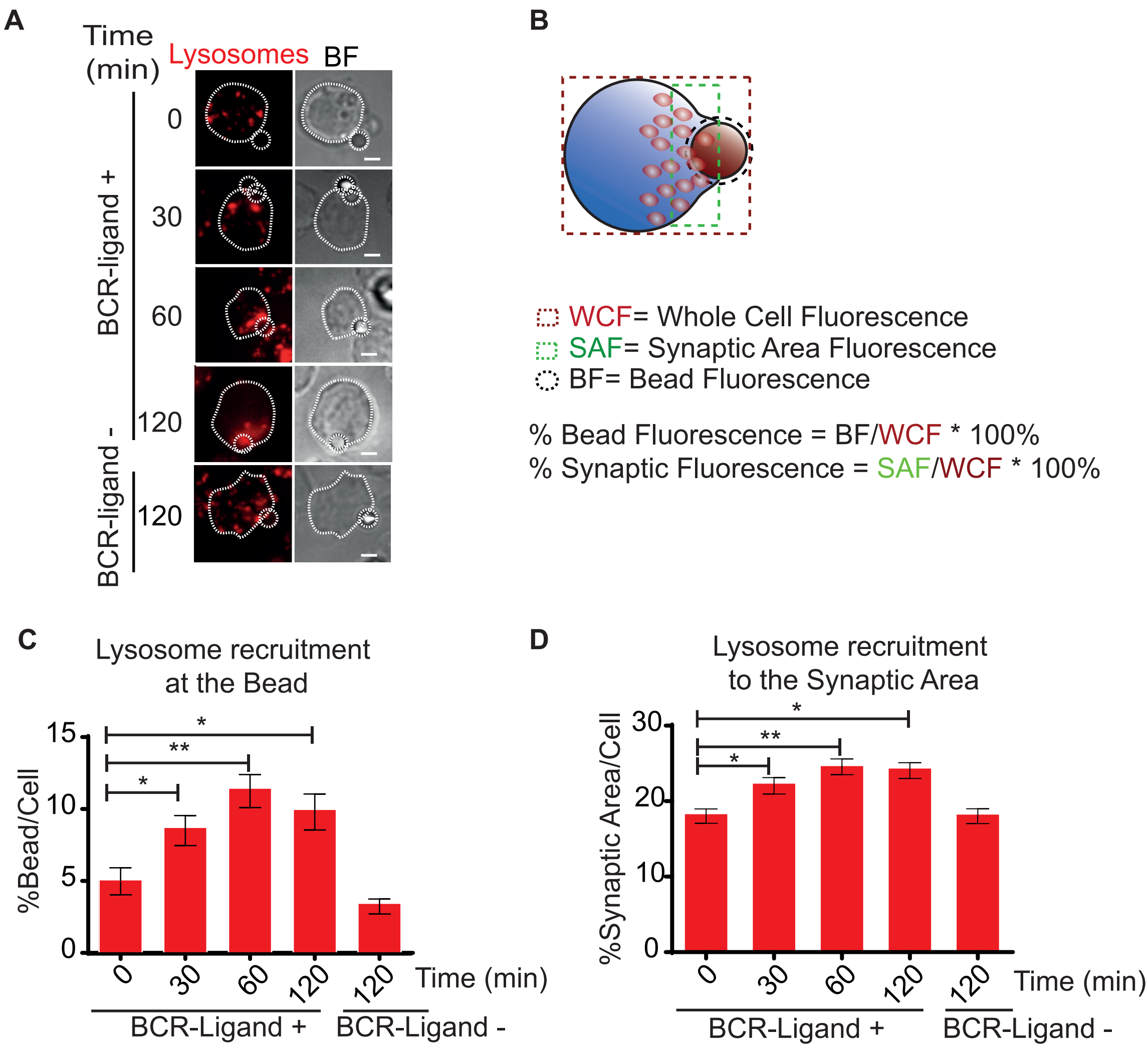
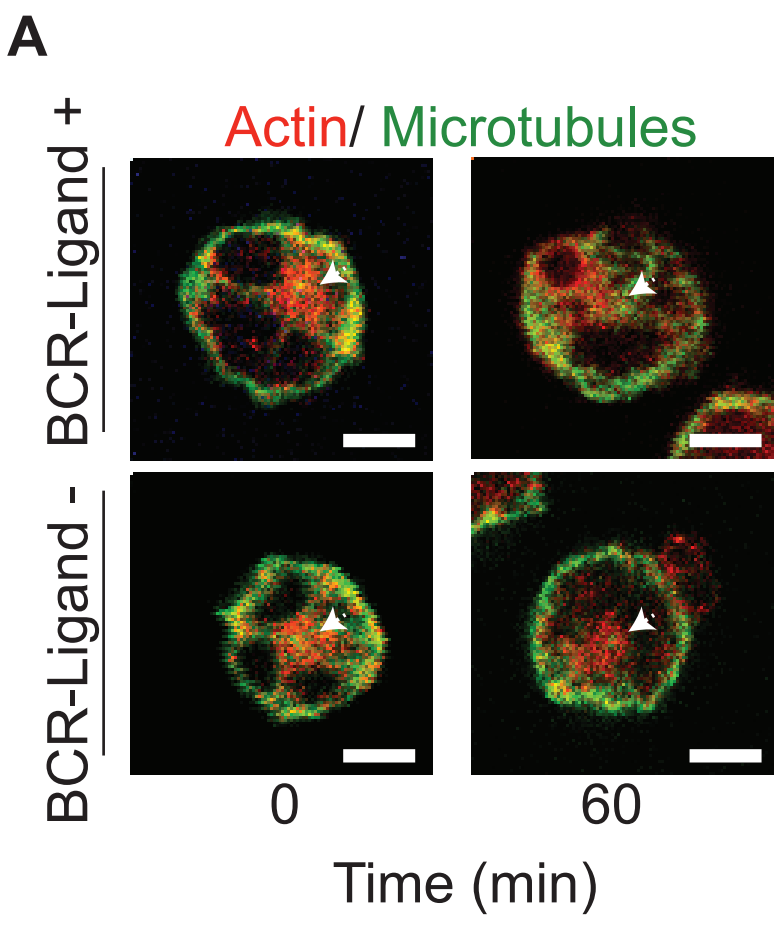
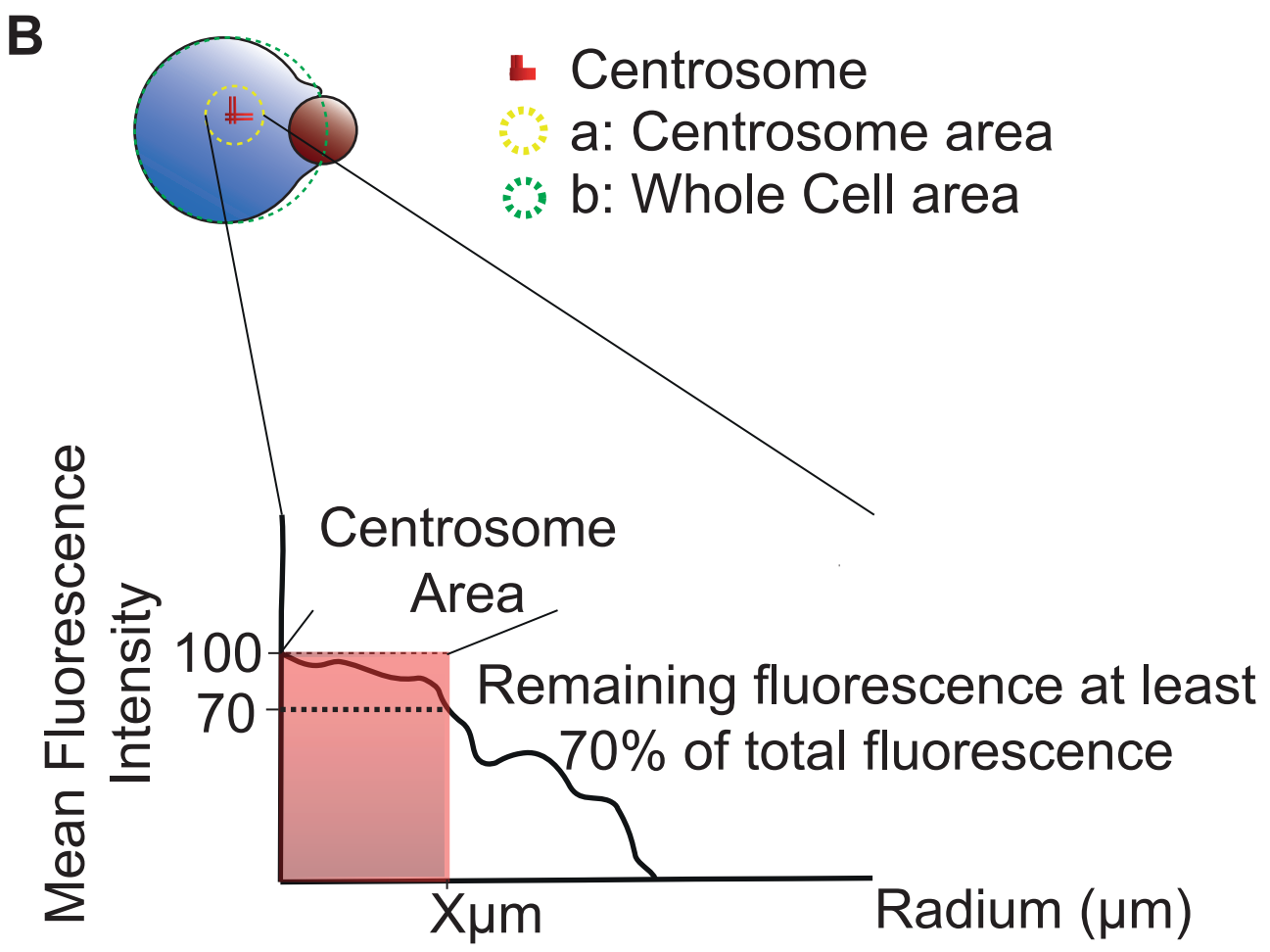


Figure 3.
Ibañez et al.





$$\text{Fluorescence density ratio (Centrosome /Cell)} = \frac{\dfrac{\text{Centrosome Fluorescence}}{\text{Centrosome Area}}}{\dfrac{\text{Cell Fluorescence}}{\text{Cell Area}}}$$

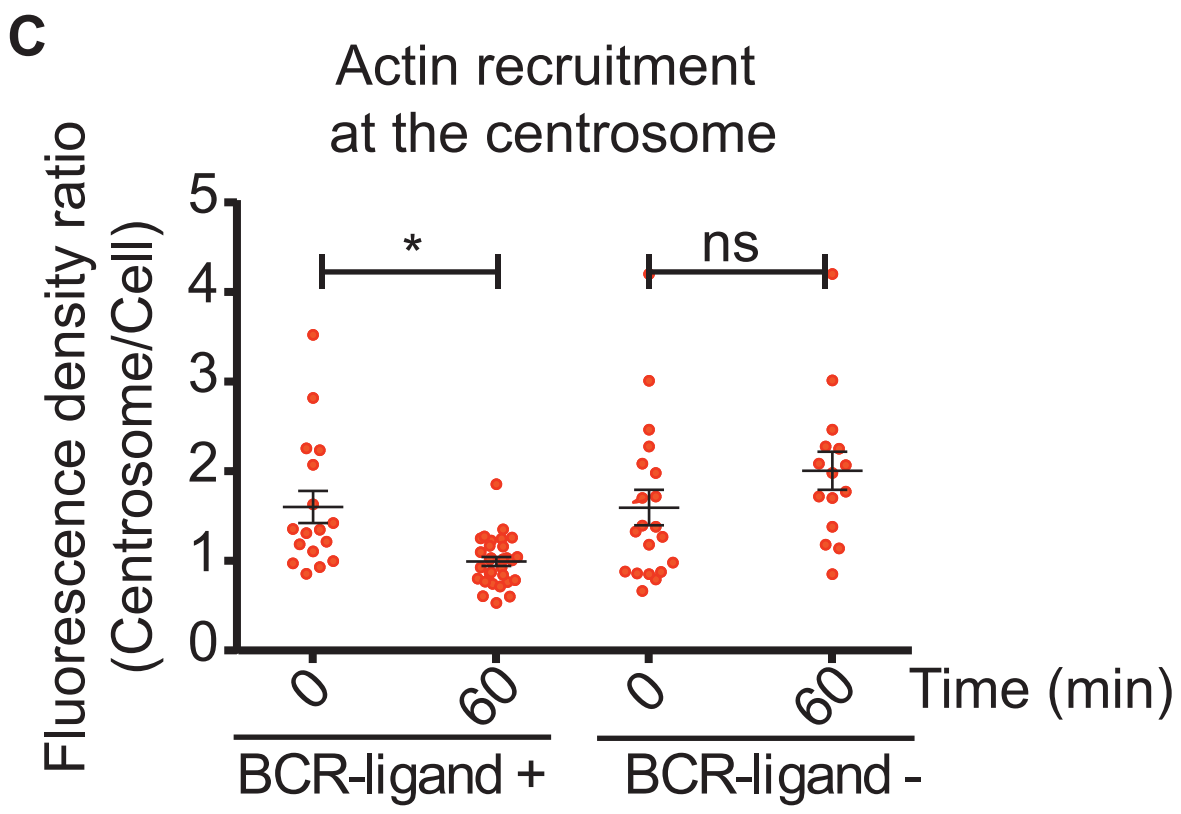


Figure 4.
Ibañez et al.

Figure 6.
Ibañez et al.

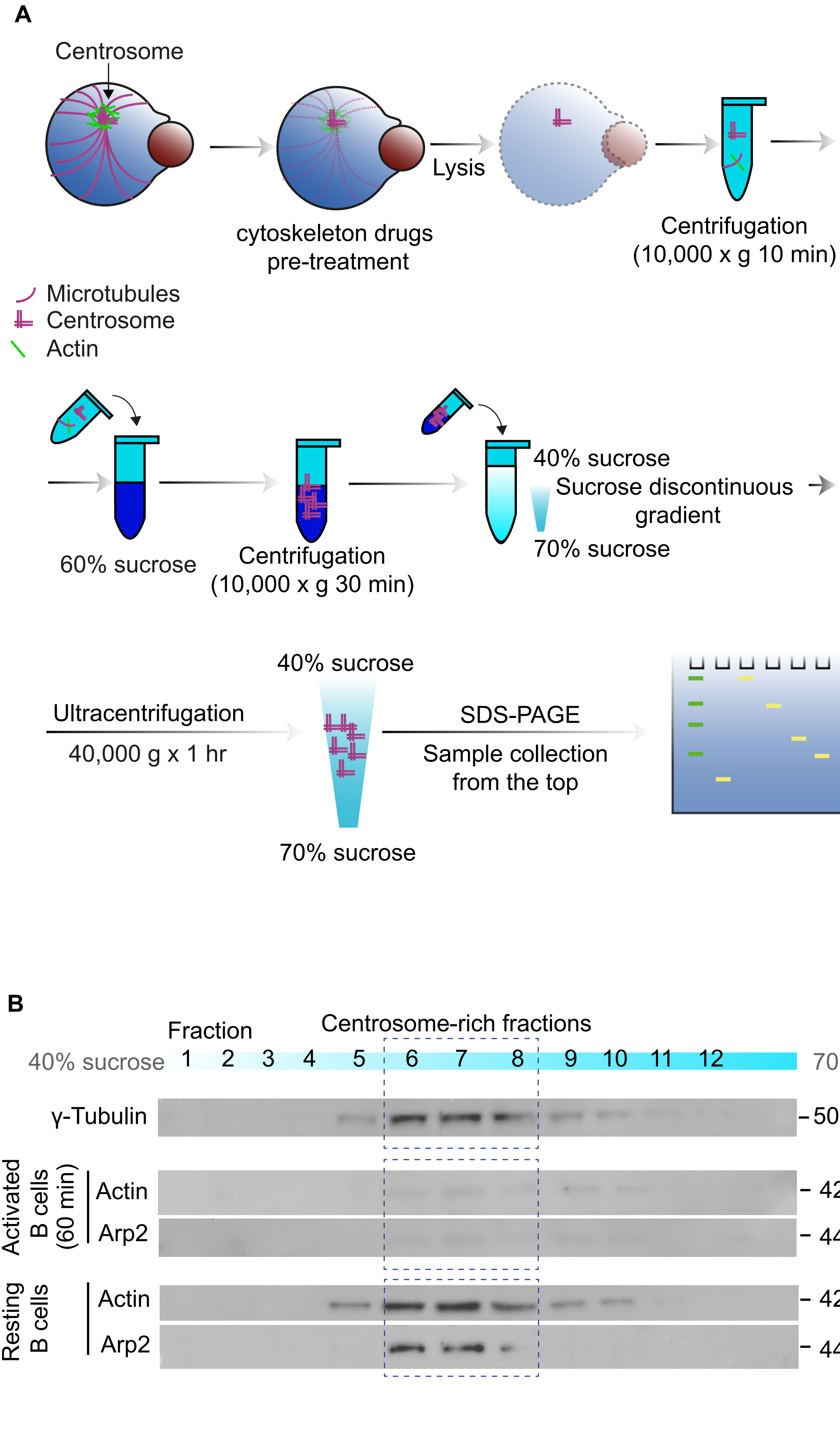


Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
IIA1.6 (A20 variant) mouse B-lymphom	ATCC	TIB-208	Murine B-cell lymphoma of Balb/c origin that expresses an IgG-containing
100% methanol	Fisher Scient	A412-4	
10-mm diameter cover glasses thickne	Marienfield-	111500	
2-mercaptoethanol	Thermo Fish	21985023	
Alexa 488 fluor- donkey ant-rabbit IgG	LifeTech	A21206	1:500 dilution recomended but should be optimized
Alexa Fluor 546 goat anti-Rabbit IgG	Thermo Fish	A-11071	1:500 dilution recomended but should be optimized
Alexa Fluor 647-conjugated phalloidin	Thermo Fish	A21238	1:500 dilution recomended but should be optimized
Amaxa Nucleofection kit V	Lonza	VCA-1003	Follow the manufacturer's directions for mixing the transfection reagents
Amaxa Nucleofector model 2b	Lonza	AAB-1001	Program L-013 used
Amino- Dynabeads	ThermoFishe	14307D	
Anti-pericentrin	Abcam	ab4448	1:200 dilution recomended but should be optimized
Anti-rab6	Abcam	ab95954	1:200 dilution recomended but should be optimized
Anti-sec61	Abcam	ab15575	1:200 dilution recomended but should be optimized
BSA	Winkler	BM-0150	
$CaCl_2$	Winkler	CA-0520	
Culture plate T25	BD	353014	
Fiji Software	Fiji col.		
Fluoromount G	Electron Mic	17984-25	
Glutamine	Thermo Fish	35050061	
Glutaraldehyde	Sigma	G7651	
Glycine	Winkler	BM-0820	
Goat-anti-mouse IgG antibody	Jackson Imm	315-005-003	IIA1.6 positive ligand
Goat-anti-mouse IgM antibody	Thermo Fish	31186	IIA1.6 negative ligand
HyClone Fetal bovine serum	Thermo Fish	SH30071.03	Heat inactivate at 56 °C for 30 min
KCl	Winkler	PO-1260	
Leica SP8 TCS microscope	Leica		
NaCl	Winkler	SO-1455	
Nikon Eclipse Ti-E epifluorescence mic	Nikon		
Parafilm	M	P1150-2	
Paraformaldehyde	Merck	30525-89-4	Dilute to 4% with PBS in a safety cabinet, use at the moment
Penicillin-Streptomycin	Thermo Fish	15140122	Liquid
Polybead Amino Microspheres 3.00µm	Polyscience	17145-5	
Poly-L-Lysine	Sigma	P8920	Dilute with sterile water

Rabbit anti- alpha tubulin antibody	Abcam	ab6160		1:1000 dilution recommended but should be optimized
Rabbit anti mouse lamp1 antibody	Cell signaling		3243	1:200 dilution recomended but should be optimized
Rabbit anti-cep55	Abcam	ab170414		1:500 dilution recomended but should be optimized
Rabbit Anti-gamma Tubulin antibody	Abcam	ab16504		1:1000 for Western Blot
RPMI-1640	Biological In	01-104-1A		
Saponin	Merck		558255	
Sodium pyruvate	Thermo Fish		11360070	
Sucrose	Winkler	SA-1390		
Triton X-100	Merck	9036-19-5		
Tube 50 ml	Corning		353043	

BCR on its surface without FcγIIR

with the DNA

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Studying Organelle dynamics in B cells during Immune Synapse Formation.
Author(s):	Jorge Ibañez, Danitza Fuentes, Jonathan Lagos, Jorge Cancino and Maria Isabel Yuseff.

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Maria Isabel Yuseff
Department:	Department of Cellular and Molecular Biology
Institution:	Pontificia Universidad Catolica de Chile
Title:	Studying Organelle dynamics in B cells during Immune synapse formation
Signature:	[signature]
Date:	27/12/18

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Point-by-point response to reviewers:

Dear reviewers,

We thank you for your constructive feedback, which has greatly improved the quality of our publication. All of the major points raised have been addressed both in the written and video sections and have now been included in the new version of our manuscript, as discussed below:

Reviewer #1:

1. **Manuscript Summary:**

The manuscript describes interesting methods and nicely combines various variants of them by providing protocols with different options for microscopy-based analysis and biochemical analysis. The image analysis part is very interesting and important. However, the writing of the protocols should be substantially improved.

2. **Major Concerns:**

The protocols section is difficult to follow. Below just some example comments that should give an idea how to improve the section:

- Authors should state why different steps are done. Starting from lack of stating which cells are used and what should be taken into account in choosing cells (and ligand).

  ANSWER: We agree with the reviewer and have now improved the protocol section, indicating a brief explanation of each step. Also, in this new version, we indicate at the beginning of each protocol that we use IIA1.6 mouse B cells (Page 3, section 1.1.1 of protocols).

- It is not always obvious, for example, which sample is taken in the next step and what is preparation for further needs. Especially the preparation of the gradient ultracentrifugation is difficult to follow. Could also the image be improved for this?

  ANSWER: We improved the workflow of the Centrosome isolation procedure and the explanation of the protocol, adding a brief explanation for each step (Page 10, section 5 of protocols)

- How is the poly-L-lysine coating performed? Concentration, time of incubation?

ANSWER: We added a new protocol section to clearly explain how Poly-L-Lysine coating is performed, specifying the concentration of Poly-L-Lysine, time and temperature of incubation (page 5, section 1.2).

- How long and on which temperature are the activated cells let to adhere the poly-L-lysine cover slips?

ANSWER: We apologize for not explaining this part of the procedure and now state, in the new version of the manuscript, that we seed B cells onto the Poly-L-Lysine covers after mixing them with Antigen-coated beads. The temperature and time of the incubation is also explained in this new version. (page 5, section 1.3)

- How are immunofluorescence samples fixed? 50ul of 4%PFA on top of 100 PBS or replacing? Methanol on top of existing PBS PFA, or replacing, or only on separate samples?

ANSWER: We have clarified this issue in the appropriate protocol section. We replace cold-1X PBS previously added to each cover with fixation solution. PFA and Methanol are frequently used for fixation in our system. Additionally, we highlight that for Actin and centrosome labelling we recommend using PFA and methanol, respectively (Page 6, section 3.1).

- A bit more explanation of the methods is required.

ANSWER: We improved the explanation of all methodologies

- For instance, in the synaptic membrane assay, why is the synaptic membrane preserved, but all other membranes lost? What else is preserved? There is a lot of total Syk also in the synaptic membrane fraction of non-activated cells, how is this explained? Is Syk bound to membrane or cytoplasmic?

ANSWER: Given the length of our manuscript, we have now eliminated this section on order to have space to explain imaging and centrosome isolation procedure.

- What is in the other fractions of the sucrose gradient? What else can be looked for at the same time?

ANSWER: Given that there is a previous centrifugation step (centrifugation of proteins in 60% sucrose) most of cytosolic components are discarded. Therefore, when we assess the discontinuous gradient centrifugation (Between 40% and 70%), only microtubules or proteins associated to microtubules are enriched in these fractions, with a peak in the centrosome-enriched fraction. Proteomics analysis of these centrosome fractions has been previously published (Obino et al. 2015), where centrosome proteins, motor proteins, as well as actin regulators have been described. Additionally, we do not discard the possibility that organelles with the same density could be associated to centrosome fractions. However, we believe that this could be beneficial, given that the centrosome polarizes to the IS. Thus, comparing the composition of centrosome fractions from activated and resting B cells could provide valuable insight on how vesicle trafficking is regulated at this level.

- More emphasis should be put on clear description of the image analysis parts. The protocol text typically only refers to one panel (2B, 3B, 4B) of in each figure. How about the other steps? It would be informative to give example cells with example values and also to provide a cartoon that shows the extreme cases. Now it is not clear in many cases what would be the extreme values and how would that scenario look. What would be the polarity index for a fully polarized marker? And why, for instance, Lysosome recruitment quantifications give such low values (bead/cell max 12%, synaptic area/cell max 25%) if Lysosomes are so strongly polarized as shown by the images? From the text "Actin recruitment at the Centrosome" one would expect the ratio of Actin at the Centrosome/ cell value to go up. Should it rather be "displacement of Actin from the Centrosome"? goes down upon activation, although in the images it looks rather higher

  ANSWER: We substantially improved this section in order to clarify the methods and procedures of image quantification (Text and Video). Also, we included the graph depicting the “extreme cases” where organelles are fully polarized (at the bead) or anti-polarized and what polarity index values mean. The quantification of lysosome recruitment at the bead is relatively low, because we use a circle with a 3μm diameter (same size as the bead used for B cell activation) to delimit the area of lysosome recruitment to the bead. This restrictive area allows us to quantify the Lysosomes that are closely docked at the surface of the bead. For this reason, we also established a second area termed “synaptic area”, which is a rectangular area adjacent to immune synapse, that allows us to quantify the recruitment of organelles that are near to the IS but not necessarily docked at the IS. Thus, the bead area is used to quantify organelles at the immune synapse and the synaptic area is used for organelles adjacent but not necessary at the immune synapse. We believe that both methods are relatively easy to apply and are helpful to characterize organelle recruitment within the proximity of the synapse (Page 7, section 4).

We changed the title “Actin recruitment at the Centrosome” for “Displacement of Actin from the Centrosome”. Also, we improved the image quality using a confocal microscope (Figure 3A).

- Figure 4D: the text states that the values >1 mean enrichment and <1 exclusion. Yet, in the figure both Golgi and ER show values <1, and rather 0 is marked as a division point. This should be revised and, again, example schematic cells with extreme behaviors and corresponding values would be helpful.
  Figure 4F doesn't exist.

  ANSWER: We corrected the values and added a schematic example of extreme values in the organelle distribution at the synapse. Also, we eliminated the reference to Figure 4F.

- Figure 5B. Why is BCR used as loading control? Should it also not be enriched to the synaptic membrane? The loading control should be something that does not get enriched at the synapse.

  ANSWER: We have decided to eliminate this section in order to focus on imaging analysis and cell fractionation.

- Figure 6A. It is not clear at all how the gradient is prepared and how the sample is loaded to the gradient. Could the preparation of the sample for the gradient separation be better illustrated?

  ANSWER: We improved the workflow of the Centrosome isolation procedure (Figure 5A).

3. Minor Concerns:

Regarding the discussion:

- Soluble immune complexes are not supposed to form an immunological synapse, which by definition, requires a cell-cell interface (and can be mimicked with big beads or antigen-coated planar surfaces).
- Why is imaging of intracellular organelles by epifluorescence microscopy as "high resolution"?

  ANSWER: We have deleted this error from the text.

- Figure 3A. What means: "the bottom square indicates the magnification of the Centrosome"?
  Figure 4A what means: "vector-GFP"?

  ANSWER: We changed the FIGURE 3A, and we pointed out the Centrosome using an arrow head. For Figure 4A Vector-GFP means KDELR-DN (Dominant negative)-GFP. We changed this label. (Page 14, Figure 4)

Reviewer #2:

1. **Manuscript Summary:**

The mechanism by which B cells form an immune synapse (IS) with antigen-presenting cells (APC) is a timely and important topic. The polarization of cellular structures (centrosome, lysosomes) towards the B cell IS optimizes the ability of B cells to acquire antigens from APCs and then direct BCR-antigen complexes to antigen processing- and MHC loading-compartments. This is a critical step that allows B cells to present antigens to T cells and obtain T cell-derived second signals that are required for their activation. In several outstanding publications Dr. Yuseff has used quantitative image analysis to characterize the polarization of the cellular components towards the B cell IS and identify critical regulators of this process. There is much more to be learned about how B cell polarity is established. Hence, a detailed protocol and visual description of the relevant experimental approaches will be a valuable contribution to the field.

2. **Major Concerns:**

The microscopy approaches and associated methods for quantitative analysis are of considerable interest and are described reasonably well. However, the biochemical techniques described should probably be removed. Without further validation, it is not clear that the freeze-thaw lysis of cells that are bound to anti-Ig-coated beads allows selective isolation of the bead-associated synaptic membrane, as shown in Figure 5. Also, in the absence of an immunoprecipitation step, the density gradient fractionation approach (Figure 6) is not specific for centrosome-associated proteins.

ANSWER: Isolation of synaptic membranes is a technique used in other cell types such as neurons and T Cells to study changes in the composition of the synaptic interface. Although, we believe that isolation of synaptic membranes can be used to obtain quantitative data of molecules recruited to the synaptic membrane of B cells, we have decided not to include this section and focus on imaging analysis and characterization of centrosome-rich fractions.

Regarding the second point, a previous proteomic analysis of centrosome-rich fractions has confirmed that indeed this procedure can be used to enrich the centrosome and centrosome associated proteins (Obino et al. 2015). Additionally, we agree that the density gradient fractionation of the centrosome is not as specific as an immunoprecipitation assay. However, the objective of this approach is to characterize dynamic changes in centrosome composition during the formation of an IS in B cells. Given that this organelle becomes translocated to the IS and regulates lysosome recruitment, we believe that this analysis could provide mechanistic insight on how both membrane trafficking and cytoskeleton dynamics are regulated during this process.

I have indicated on the PDF places where more experimental details or clarifications are needed, and I have made suggestions for improving the writing. I summarize below some of the major points that the authors should address.

3. **Major points:**

- Page 2. In the introduction, the authors should acknowledge that work by Tolar's group indicates that lysosome exocytosis at the IS occurs mainly when myosin-based forces on the BCR are insufficient to extract antigen from a surface. However, centrosome and lysosome polarization towards the IS occurs regardless of whether lysosome exocytosis is involved in antigen extraction, so this does not detract from the utility of the experimental approaches being described in this manuscript.

  ANSWER: We have acknowledged the work of Tolar's group in the introduction to explicit the two known mechanisms that have been suggested to control antigen extraction, the mechanical (Force) and chemical (Lysosome secretion) extraction. Additionally, we use a system of high stiffness (bead and coverslips coated with antigen), thus we are favoring the secretion of lysosomes over the mechanical antigen extraction. (page 2)

- Page 4. The authors indicate in section 1.1 that anti-IgG is used as the specific antigen and anti-IgM is the irrelevant control antigen. This would be valid only for IgG+ B cell lines (the type of cells the authors use is described only in the table of key reagents). The authors should make this protocol more general to include primary B cells and both IgM- and IgG-expressing B cell lines, and then describe the appropriate anti-isotype reagents to be used in each case.

  ANSWER: We agree with the reviewer that we should make this protocol more general considering the isotype of the BCR of B cells. To this end, we introduce notes along each step of the protocol indicating the specification of using IgG or IgM antibodies to activate B cells (Page 4 section 1.1.1).

- Page 6. The authors should be note that methanol fixation is optimal for staining microtubules, but it destroys the phalloidin-binding site on actin. PFA fixation is better for actin staining.

  ANSWER: We corrected this observation in the manuscript and we now specify that PFA is better for actin staining (page 6, section 3.1).

- Page 7, step 12. The authors should indicate here that confocal z-stacks need to be acquired for the cell spreading assay and quantification of organelle polarization towards anti-Ig-coated coverslips.

ANSWER: We agree that to evaluate the distribution of lysosomes throughout the cell in the spreading assay, a confocal z-stack acquisition is required. We now indicated on the manuscript that z-stack is needed to acquire images in confocal microscopy for B cells activated on anti-Ig-coated coverslips (Page 7 section 3.11).

- Page 8. It is not clear what the "mass center" is. This should be defined. As shown in Fig. 1F, does this mean the center of a group of labeled organelles such as lysosomes? If that's the case, how does this differ from what is in sections 1.1 or 1.3?

  ANSWER: We agree with the reviewer and now provide a definition for mass center. A mass center is a mean of the coordinates of the label. This allows us to work with organelles that have a dispersed localization in the cell. This concept does not apply to the centrosome or the Golgi Apparatus because these organelles can be easily identificated with a point and are localized in a confined space (Page 7, section 4.1.1). Section 4.1.3 and 4.1.4 shows how to quantify the fluorescence of organelles at the bead area and the synaptic area, which correlates with the synaptic membrane (interface) and the adjacent area, respectively. It is possible that organelles polarize in some grade to the bead, but do not completely dock at the immune synapse. Thus, both approaches, bead recruitment (4.1.3) and synaptic area recruitment (4.1.4), provide complementary information.

- Page 8, step 1.1.3. By including the cosine in these calculations does this overestimate the extent of polarization? If the angle is large, the organelle can be in the half of the cell nearest the bead but still quite far from the contact point with the bead.

  ANSWER: The cosine of the angle between the vector from the organelle to the center of the cell and the vector from the bead center and the cell center, allows us to generate a projected version of the organelle vector to the bead vector. Also, the result of Cosine always will be between 1 and -1, depending on the angle between vectors. Thus, when the angle is between 90° to 270° will give negative values which indicates a non-polarized phenotype, due to the value of cosine. And angles between 90° to 0° or 270° to 0 will give positive values, which indicate a polarized phenotype. So, the cosine in these calculation does not overestimate the polarization values.

- Page 9, using immunofluorescence to assess the recruitment of components to the centrosome. Wouldn't it be better to specifically label the centrosome by staining for pericentrin, instead of using tubulin?

  ANSWER: We agree that staining Pericentrin will show a clearer staining of the centrosome. However, tubulin also can be used taking into consideration that the centrosome is represented by the brightest point in the labeling and where all the microtubules intersect, as was previously

showed (Obino et al. 2015). This plasticity of labelling is important when the antibodies used for centrosome labelling are made in the same host as the organelle labelling antibodies.

- Page 11, Figure 5. Without further analysis, e.g. immunofluorescence, it is hard to know what portion of the cell membrane is being precipitated by the anti-Ig-coated beads. Does the freeze-thawing leave a cell membrane ghost such that the whole cell membrane is being precipitated? Figure 5A suggests that it is only the region of the membrane that was closest to the bead, plus some of the bead-proximal cytoplasm, organelles, and perhaps cytoskeletal structures. How was this determined and validated? In Figure 5 there is no enrichment of the BCR or Syk between 0 min and 30 min (is 0 min the control beads with the irrelevant Ag?). Why wouldn't there be an enrichment of the BCR at the bead contact site? I recommend removing the section on this approach as its ability to identify proteins that are enriched at the site of bead contact needs to be validated.

  ANSWER: As it was previously discussed, we excluded this methodology from the manuscript.

- Page 12, Figure 6. These authors need to be careful not to over interpret data that are obtained using this density gradient centrifugation approach. There are likely many types of macromolecular complexes in the same density fractions as the centrosome. The presence or absence of a protein in this fraction cannot be equated with its association with the centrosome. This is best determined by microscopy. I recommend removing the section on this approach.

  ANSWER: We thank the reviewer to assess this issue. However, we disagree in removing this section, given that previously published results (Obino et al. 2015) have shown a proteomic analysis of centrosome fractions isolated from B cells using the same procedure. The objective of this approach is to characterize dynamic changes in centrosome composition during the formation of an IS in B cells and not to prove their direct interaction with the centrosome. Given that this organelle becomes translocated to the IS and regulates lysosome recruitment, we believe that this analysis could provide mechanistic insight on how both membrane trafficking and cytoskeleton dynamics are regulated during this process. Additionally, we complemented the actin depletion at the centrosome findings with imaging analysis (Figure 3A).

4. **Minor Concerns:** Please see additional queries that I marked on the PDF.