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Contractile response of alveolar epithelial cells to biochemical or mechanical stimulation probed by traction microscopy

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Contractile response of alveolar epithelial cells to biochemical or mechanical stimulation probed by traction microscopy

A dissertation by
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in partial fulfilment of the requirements for
the degree of Doctor of Philosophy

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Appendix A Preparation of collagen I coated polyacrylamide gels

A.1 Materials

- Glass-bottomed petri dishes (35 mm) - Mattek, Ashland, MA
- 3-aminopropyltrimethoxysilane – Sigma (28,177-8)
- 10 % Glutaraldehyde diluted in PBS – Sigma (G-6403)
- Plus One Repel-Silane ES – Amersham (17-1332-01)
- 40% Acrylamide solution – BioRad (161-0140)
- 2% BIS solution – BioRad (161-0142)
- 200 nm diam. fluorescent latex beads – Molecular Probes (F-8811)
- 10 % Amonium persulfate diluted in water - BioRad (161-0700).
- TEMED – Sigma (T-9281)
- Sulfo-SANPAH – Pierce-Cultek (22589). 10 mM (5mg/ml) in 1:20 (DMSO:HEPES). Dilute 5 mg of sulfo-SANPAH in 50 ul DMSO and then add 950 ul de HEPES 200 mM.
- Rat tail collagen type I (0.2 mg/ml) in PBS – Upstate, Lake Placid, NY (08-115)

A.2 Coating of the glass-bottomed petri dishes

2.1. Place a drop of 3-aminopropyltrimethoxysilane on the glass part of the petri dish with a plastic Pasteur and smear it.

2.2. Let the drop dry out (about 15 min). The surface of the drop will appear cracked.
2.3. Wash extensively the Petri dishes with distilled water until they seem clean.
2.4. Place a drop of 10% glutharaldehyde on the glass part of the Petri dish and let it sit for 30 min. Cover the Petri dishes to prevent the smell of glutharaldehyde.
2.5. Wash extensively the Petri dishes with distilled water and let them dry out.

The coated Petri dishes can be stored for some days before use.

A.3 Coating of the coverslips with repel silane

3.1. Flush 1 ml of Repel Silance into an absorbing paper.
3.2. Brush a circular 20 mm diam. coverslip with the silane-soaked absorbing paper and let it sit for 5 min.
3.3. Flush 1 ml of distilled water into an absorbing paper.
3.4. Brush the coverslip with the water-soaked absorbing paper.
3.5. Flush 1 ml of 70% alcohol into an absorbing paper.
3.6. Brush the coverslip with the alcohol-soaked absorbing paper.

The coverslips must be coated immediately before preparing the polyacrylamide gels.

A.4 Preparation of polyacrylamide gels

To obtain 5 ml of the 2% acrylamide and 0.1% BIS solution mix:

- 250 μl of 40% acrylamide.
- 750 μl of 2% BIS.
- 4000 μl of distilled water.
- 40 μl of fluorescent latex beads (previously, mix them intensively in a vortex).
- 25 μl of 10% ammonium persulfate.
- 2.5 μl of TEMED.

Once TEMED is added to the mix, gel polymerization will start. Therefore, the two

following steps must be performed immediately after the mix is prepared:

4.1. Place 10 μl of the polyacrylamide solution onto the glass surface of the Petri dish.
4.2. Flatten the drop using the circular coverglass previously coated with Repel Silane.
4.3. Turn the resulting sandwich assembly upside down to allow the fluorescent beads to sediment in the top of the gel.
4.4. Polymerization will finish ~30 min later. A polymerized gel disk can be observed surrounded by unpolymerized liquid polyacrilamide.
4.5. Carefully peel of circular cover glass and store the gel immersed in distilled water at 4ºC until collagen coating.

Gel disks are typically 70-100 μm thick and have a diameter of 8-10 mm. It is desirable to coat the gels with collagen and proceed to cell culture during the same day.

A.5 Conjugation of collagen to the polyacrylamide gels

5.1. Drain off the water from the surface of the gel.
5.2. Place 25 μl of sulfo-SANPAH onto the surface of the gel.
5.3. Expose the surface of the gel to UV light from a germicidal lamp at a distance of ~20 cm for 5 mins. From this point on, sterelized technique must be used.
5.4. Wash the surface of the gel with PBS twice. In all the washing steps, always avoid flushing water directly onto the gels.
5.5. Place 50 μl of 0.2 mg/ml collagen I on the top of the gel and allow it to react at least 2 hours at 4ºC.
5.6. Just before cell culture, wash the gels extensively with PBS.

Appendix B Preparation of collagen type I gels

B.1 Materials

- Flexible-bottomed culture wells (35 mm) – Bioflex, Flexcell International, PA.
- 1 mM HCl
- Rat tail collagen type I (0.2 mg/ml) in PBS – Upstate, Lake Placid, NY (08-115)
- 2× RPMI – Biological Industries, Israel (01-104-5A)
- 0.5 M $NaHCO_3$
- 0.1M NaOH
- 1 M HEPES
- 200 nm diam. fluorescent latex beads – Molecular Probes (F-8811)

To obtain 1 ml of the 260 mM $NaHCO_3$, 50 mM NaOH on 200mM HEPES pH buffer mix:

- 392 μl of NaOH
- 416 μl of $NaHCO_3$
- 192 μl of $NaHCO_3$

B.2 Coating of the flexible-bottomed culture wells

2.1. Pour 500 μl of 0.3 mg/ml collagen I solution diluted with 1 nM HCl per well

2.2. Let it dry out, shaking the remaining liquid from time to time.
2.3. Once the collagen solution has dried out, wash the wells with PBS
2.4. Store at 4ºC until use.

B.3 Preparation of collagen gels

All the materials used in this section must be sterile, and all the protocol steps must be carried out into a sterile hood.

To obtain 1 ml of the 1.45 mg/ml collagen I solution mix (all of them at 4ºC):

- 416 μl of collagen I.
- 500 μl of RPMI 2×.
- 84 μl of the pH buffer.
- 2 μl of fluorescent latex beads (previously, mix them intensively in a vortex).

3.1. To mix the components, use a plastic tube immersed in water at 0ºC.
3.2. Every time a plastic tip is used, cold it with distilled water at 0ºC.
3.3. Mix the resulting collagen solution with a plastic tip, but only a few times.
3.4. Pour 800 μl of the collagen solution in each culture well (the well must be stored at 4ºC until seconds before pouring the collagen solution onto it.).
3.5. Place the well into an incubator at 37ºC and 5% CO_2 for 30 min.
3.6. Fill the well with 2 ml of culture medium at 37ºC and keep it into the incubator.

Cell culture should take place immediately after the collagen gel polymerization finishes. Don't store the collagen gels longer than 6 hours before cell culture.

Appendix C Quantification of F/G-actin ratio

C.1 Materials

- Formaldehyde 37% - Sigma (F-1635)
- Triton X-100 – Sigma (T-8787)
- BSA – Sigma (A9418-50G)
- Saponin – Sigma (S2149)
- Phalloidin-TRITC 1 mg/ml – Sigma (P-1951)
- Alexa 488- conjugated DNase I 5 mg/ml – Molecular Probes (D-12371)
- Mowiol; Calbiochem, La Jolla, CA (475904)

To obtain 1 ml of Phalloidin (1 μg/ml) and DNase I (9 μg/ml) in blocking solution mix:

- 1 μl of Phalloidin
- 1.8 μl DNase I
- 1 ml of BSA 1% and saponin 0.025% in PBS (aliquots can be stored in the freezer)

Caution, DNase I can not be placed in a vortex! Use a belly dancer to softly mix it.

C.2 Actin staining protocol

The cells can be cultured on coverslips, pertri dishes or flexible-bottomed culture wells.

2.1. Wash the cells three times with PBS at 4ºC.
2.2. Fix the cells with 3.7 % formaldehyde for 20 min at room temperature.
2.3. Wash the cells three times with PBS.
2.4. Permeabilize the cells with 0.1% triton X-100 for 3-5 min.
2.5. Wash the cells three times with PBS and remove the remaining PBS
2.6. Add 500 μl of Phalloidin/DNase I buffer per each 35 mm Petri dish.
2.7. Shake them gently on a belly dancer for 40 min.
2.8. Wash the cells three times with PBS.
2.9. The coverslips can now be mounted on a microscopy slide with mounting media or fluorescence image acquisition can already start.

C.3 Image acquisition protocol

3.1. Acquire 5 images per simple, one at the centre and four at the vertices of an imaginary square. Use 10× magnification.
3.2. Choose a field containing a little region without cells. This region will be used to compute background intensity.
3.3. Focus the cells using the F-actin fluorescence filter
3.4. Acquire a fluorescence image of F-actin (0.15 seg)
3.5. Acquire a fluorescence image of G-actin (1 seg) without moving the stage or the Z-focus.
3.6. All other parameters, such as fluorescence lamp intensity or image intensity gain, must be kept constant throughout the experiments.

C.4 F/G actin ratio computation

The software uses the fluorescence image of F-actin to determine the regions with cells and the regions corresponding to background. Determination is performed by automatically finding an optimal threshold intensity value (Fig C.1).

4.1. The background intensity of the F- and G-actin fluorescence images is the median values of the pixels corresponding to the background region.

4.2. The background intensity is subtracted from the intensity of all the pixels corresponding to cell-containing regions.

4.3. The sum of corrected pixel intensities was computed for each F-actin and G-actin image.

4.4. The F/G-actin ratio corresponds to the ratio between the two sums.

$$F/G = \frac{\sum\left(F - \overline{F}_{back}\right)}{\sum\left(G - \overline{G}_{back}\right)}$$

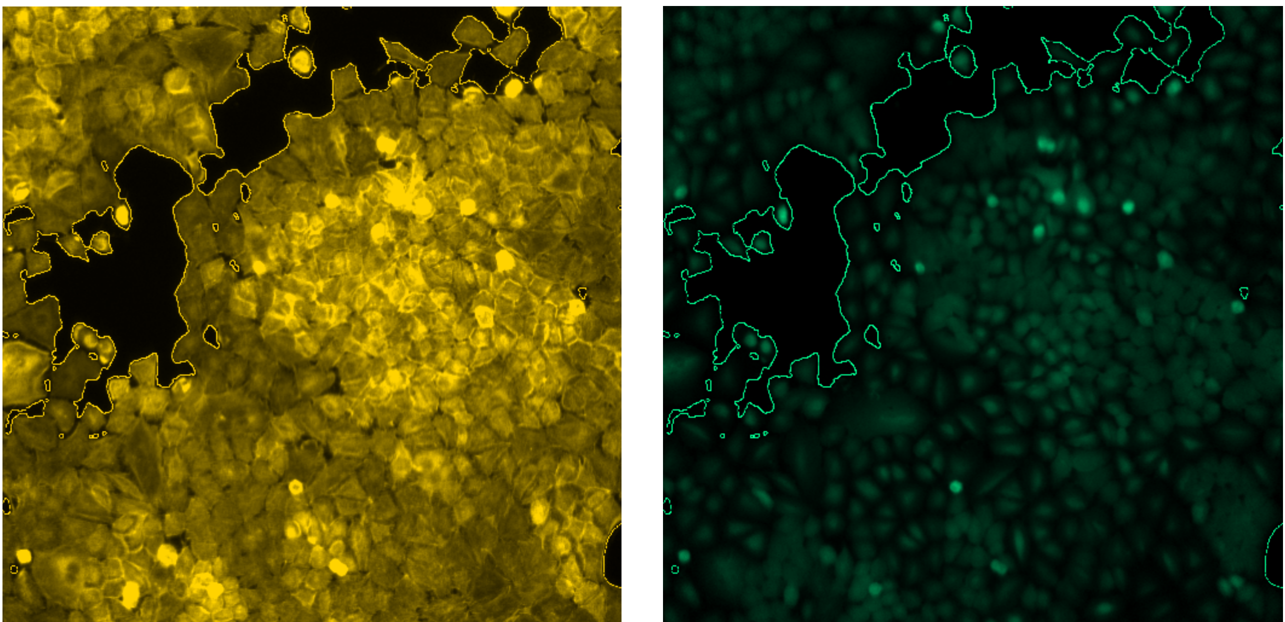
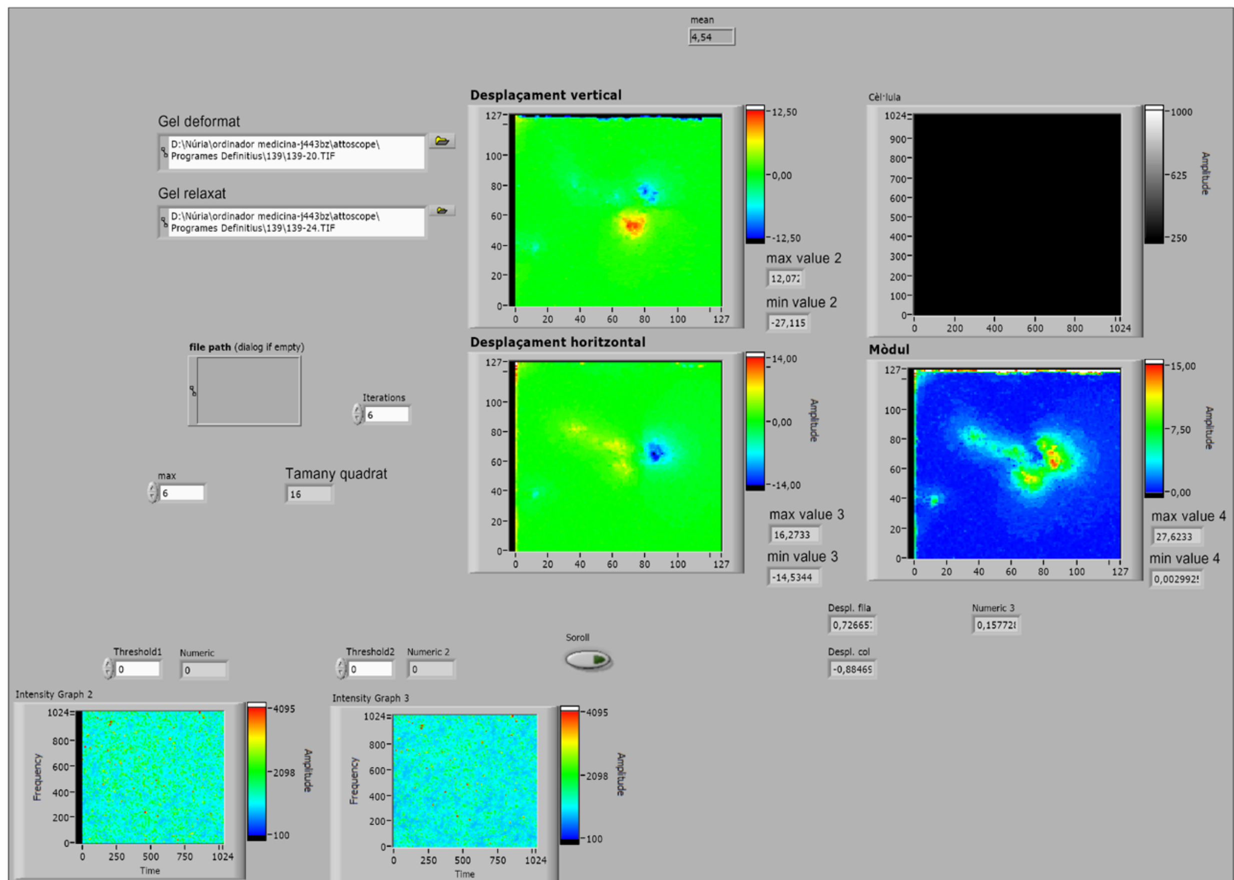


Fig C.1 Fluorescence images of a A549 cell culture where F-actin (left) and G-actin (right) has been stained. The contour has been optimally established by using the F-actin image. The pixels outside the contour are used to determine the background intensity.

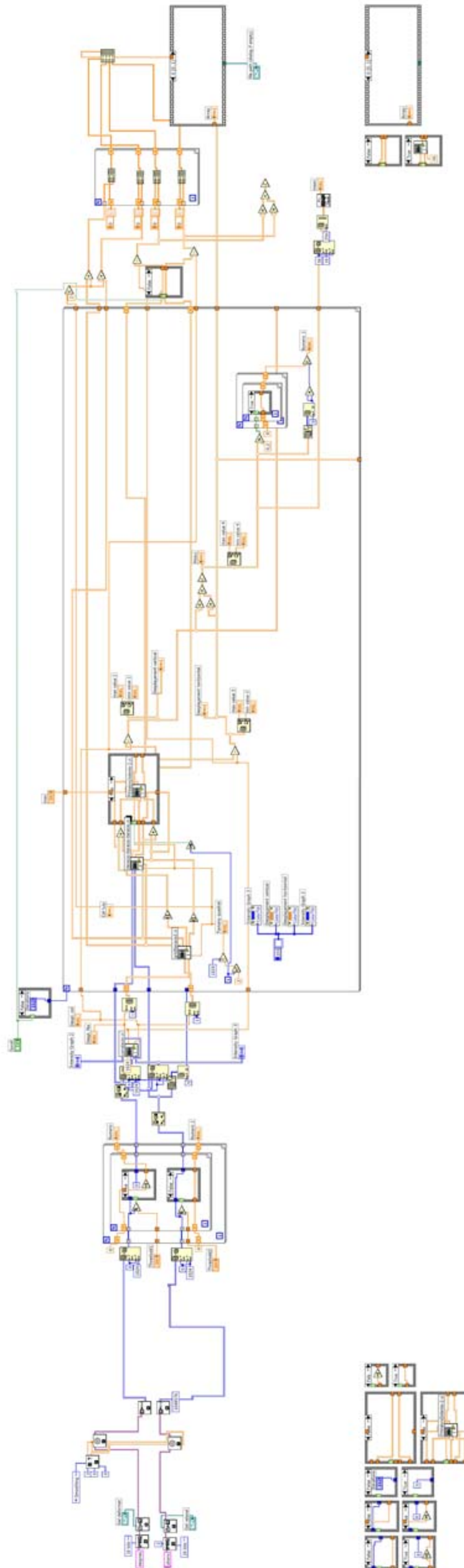
Appendix D Program codes

D.1 Computation of the displacement field

D.1.1 Front panel

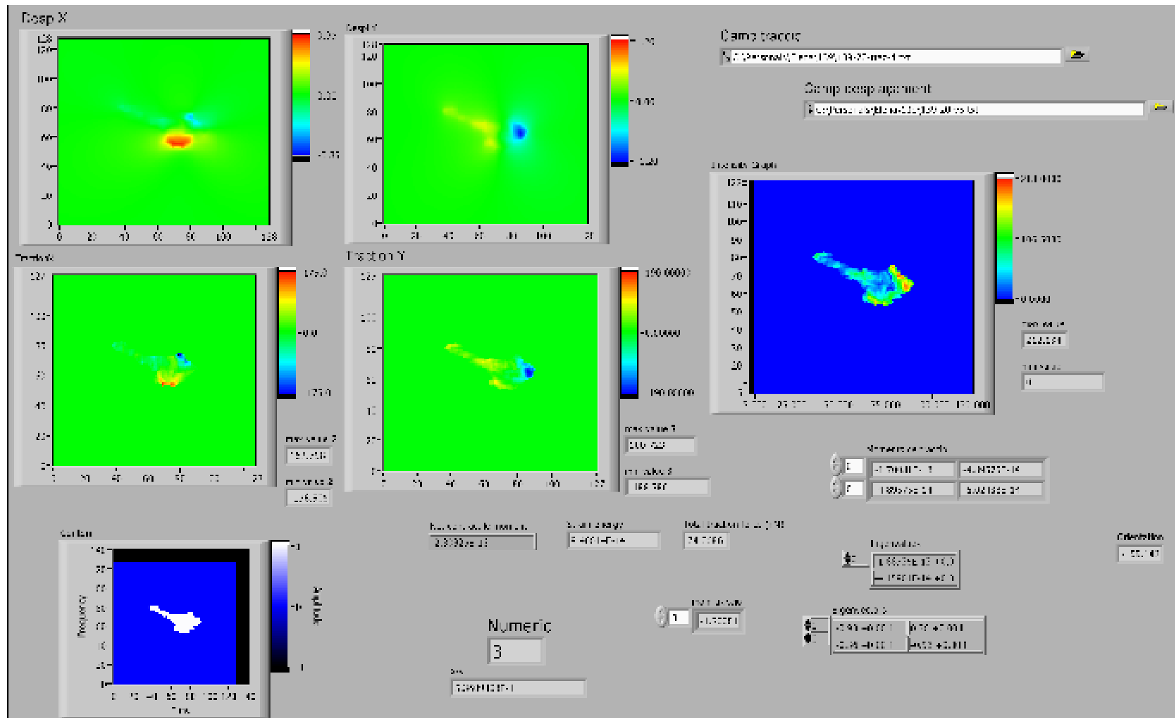


D.1.2 Block diagram

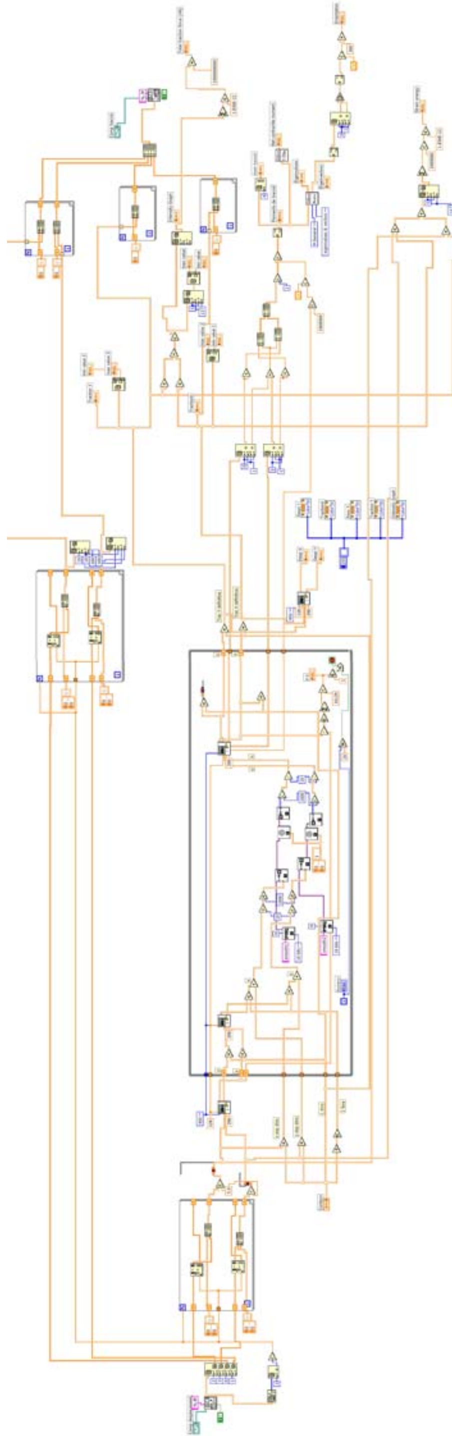


D.2 Computation of the traction field

D.2.1 Front panel

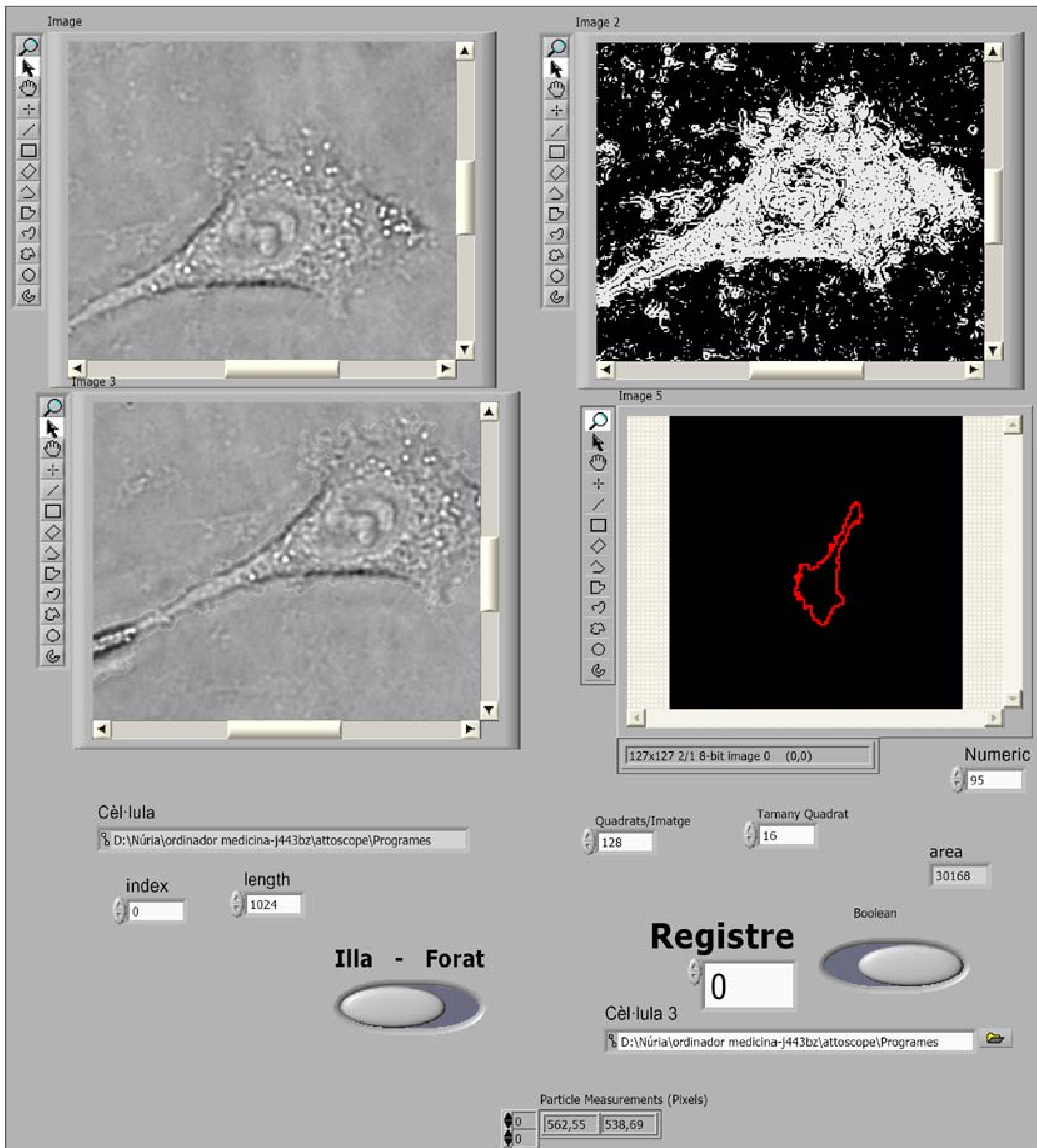


D.2.2 Block diagram

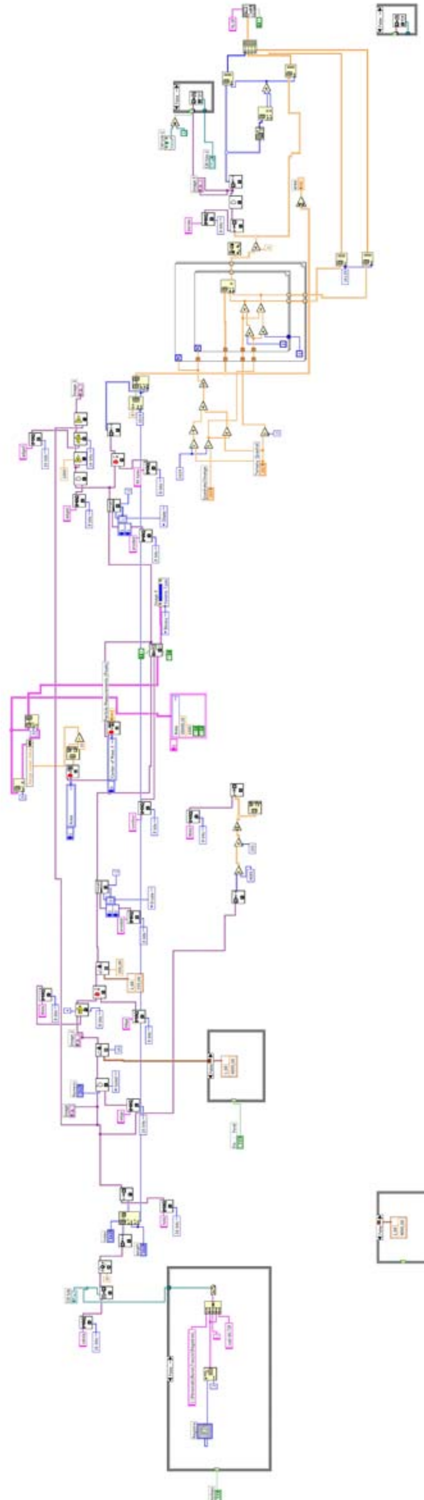


D.3 Computation of the cell contour

D.3.1 Front panel

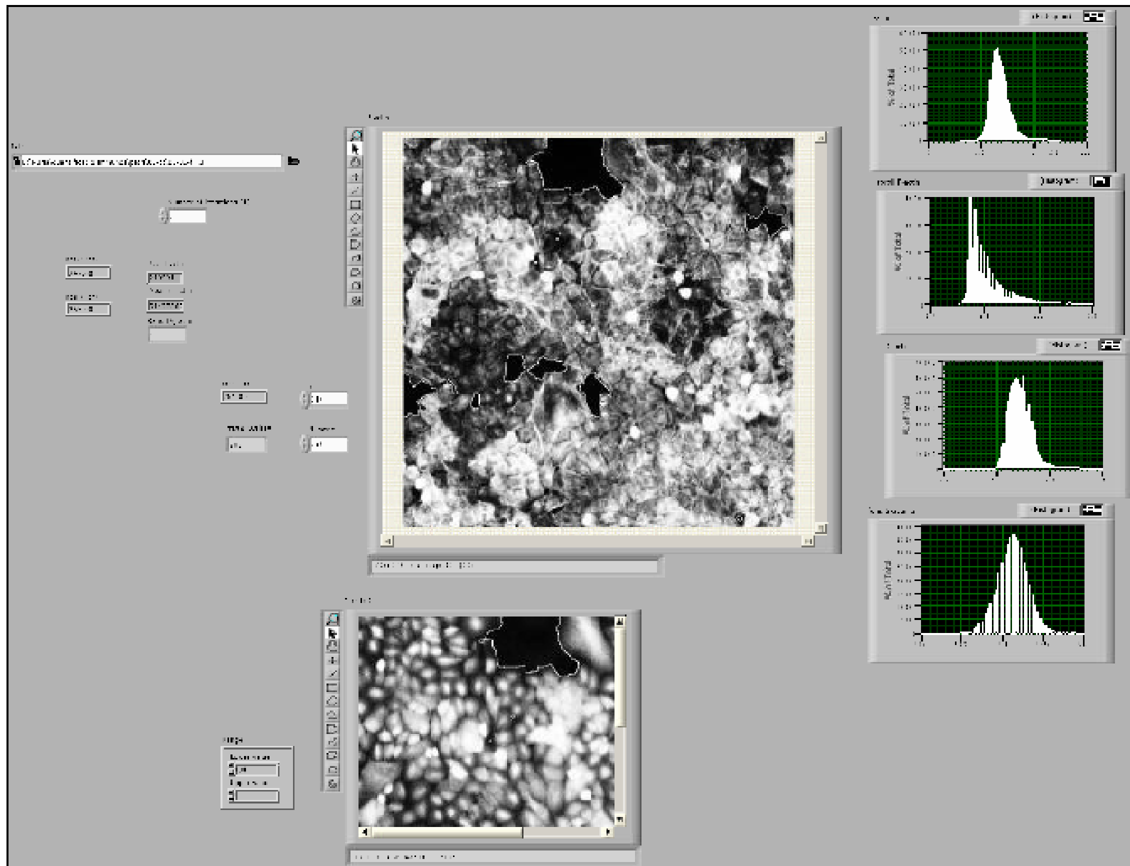


D.3.2 Block diagram

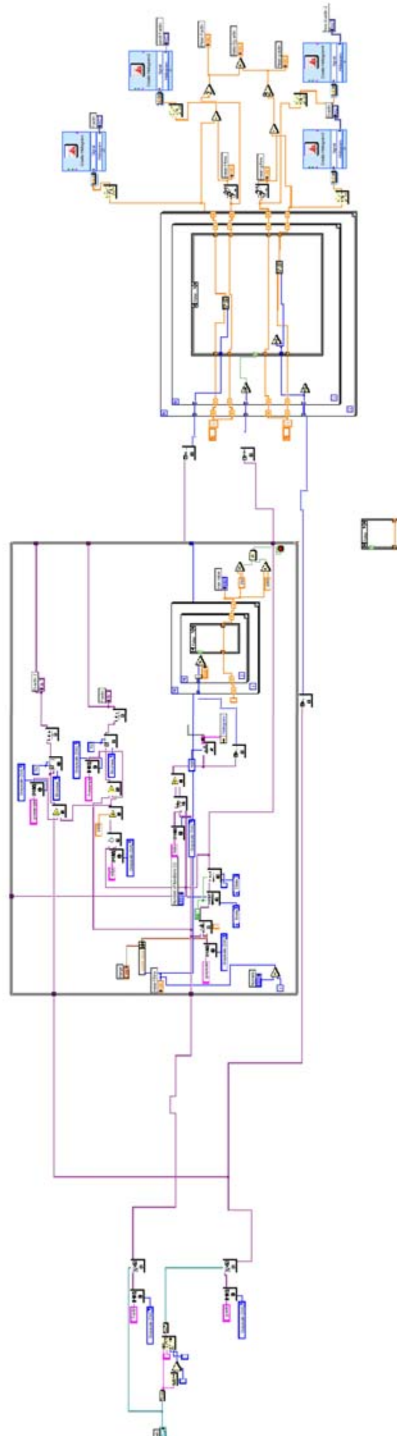


D.4 Computation of F/G-actin fluorescence ratio

D.4.1 Front panel



D.4.2 Block diagram



Appendix E Publications and congress contributions

E.1 Publications

1. Roca-Cusachs P., I. Almendros, R. Sunyer, N. Gavara, R. Farré, and D. Navajas. Rheology of passive and adhesion-activated neutrophils probed by Atomic Force Microscopy. Biophys J. 91 (9): 3508-3518, Nov. 2006

2. Gavara N., R. Sunyer, P. Roca-Cusachs, R. Farré, M. Rotger, D. Navajas. Thrombin-induced contraction in alveolar epithelial cells probed by traction microscopy. J Appl Physiol 101: 512–520, Aug. 2006.

3. Trepat X., F. Puig, N. Gavara, J.J. Fredberg, R. Farre, D. Navajas. Effect of stretch on the structural integrity and micromechanics of human alveolar epithelial cell monolayers exposed to thrombin. Am J Physiol Lung Cell Mol Physiol 290:L1104-10, Jun. 2006.

4. Rico F., P. Roca-Cusachs, N. Gavara, R. Farré, M. Rotger, and D. Navajas. Probing Mechanical Properties of Living Cells by Atomic Force Microscopy with Blunted Pyramidal Cantilever Tips. Phys Rev E 72 (2) 021914, Aug. 2005

E.2 Congress contributions

1. Farré R., F. Puig, N. Gavara, R. Sunyer, D. Navajas. Cell stiffening and contraction

induced by thrombin in alveolar epithelial cells treated with dexamethasone. 16th European Respiratory Society Annual Congress, Munich, Germany, September, 2-6, 2006

2. Gavara N., Puig F., R.Sunyer, R.Farré, D. Navajas. Effects of dexamethasone in contraction of alveolar epithelial cells. 5th World Congress of Biomechanics, Munich, Germany, August 31 –September 4, 2006.

3. Puig F., N. Gavara, R. Sunyer, D. Navajas, R. Farré. Dexamethasone induces stiffening of alveolar epithelial cells. 5th World Congress of Biomechanics, Munich, Germany. 31 August 31 – September 4, 2006.

4. Gavara N. Thrombin induces contraction of alveolar epithelial cells. (Oral contribution). I Jornada de Biofísica. Barcelona, Spain. June 27, 2005.

5. Trepat X., F. Puig, N. Gavara, R. Farré, D. Navajas. Combined effect of thrombin and stretching on the mechanical properties of alveolar epithelial cells. International Conference American Thoracic Society, San Diego, CA. May, 2005

6. Gavara N., F. Rico, R. Sunyer, P. Roca-Cusachs, D. Navajas. Implementation of a traction microscope to measure contraction of cultured adherent cells. (Oral contribution). IV Jornades de Recerca en Enginyeria Biomèdica, Barcelona, Spain. June 8-10, 2004

7. Rico F., L. Buscemi, N. Gavara, M. Rotger, R. Farré, and D. Navajas, Contact Model for Probing Cell Mechanics by Atomic Force Microscopy with Pyramidal Tips. 1st NanoSpain Workshop, San Sebastián, Spain. March 10-12, 2004.

This is my verse,

so far.

To be continued …