Imaging of vimentin intermediate filaments organization in epithelial cells

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Abstract
The extracellular environment has a major influence on cell polarity and thus cell orientation. In the same fashion the localization of the cell nucleus is precisely regulated. One of the key players in the positioning of the nucleus might be cytoplasmic intermediate filaments (IF). Studying their exact function is crucial for the understanding how cell motility is regulated, especially in tumor cells which show high IF levels. Here, we show our methodology to study how cytoplasmic intermediate filaments are distributed in micropatterned cells as well as their interaction with the nucleus upon its deformation.

1. Introduction

Introduction Eukaryotic cells are governed by a cytoskeleton that is mainly believed to provide structural support for the cell, as well as enabling intracellular transport. The polymeric frame is composed of actin microfilaments (MF), tubulin microtubules (MT) and IFs such as vimentin or keratin. The cytoskeletal system is also implicated in cell adhesion as well as in many regulatory signaling pathways [1].

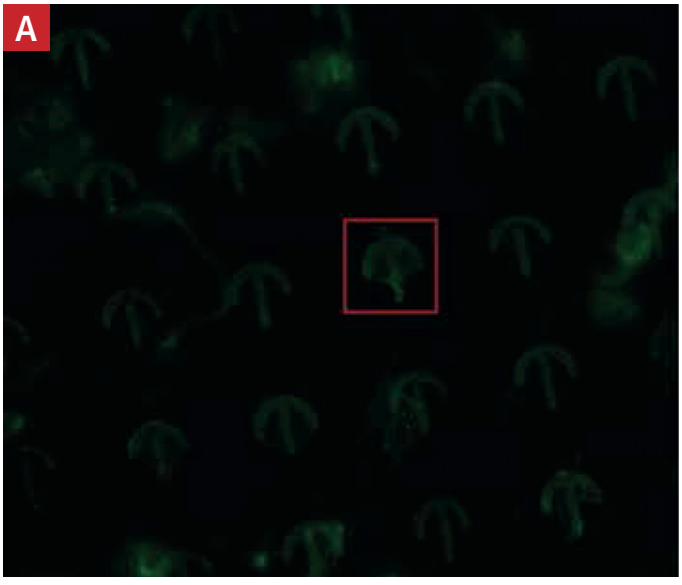
Part of the definition of cell polarity relies on the position of the nucleus, in stationary as well as in migrating cells [2]. This positioning has been shown to be related to actin microfilaments through the Linkage of Nucleoskeleton and Cytoskeleton (LINC) complex.

Other studies have investigated the role of micro-tubules in nucleus displacement, both in a microtubuleorganizing center (MTOC) dependent or independent manner [3]. Additionally, there are clues pointing to intermediate filaments as a third party involved in the positioning of the nucleus (as well as other organelles), but its mechanism is not yet fully understood [4].

Studying the regulatory effects of IFs in cell motility is essential, especially for understanding tumor cells which show a dramatic increase of IF concentration in their cytoplasm [5].

In this study we investigate the role of vimentin IF organization in epithelial cells (retinal pigment epithelia RPE1) on a micropatterned surface by imaging the fluorescently labelled cell components actin, vimentin and the nucleus.

Using a very sensitive camera allowed for the detection of very weak fluorescent signals, opened up the possibility of investigating the impact of cell shape and polarity on the organization of the cytoskeleton. The camera's large field of view also allowed for the detection of great number of cells in a relatively short time.



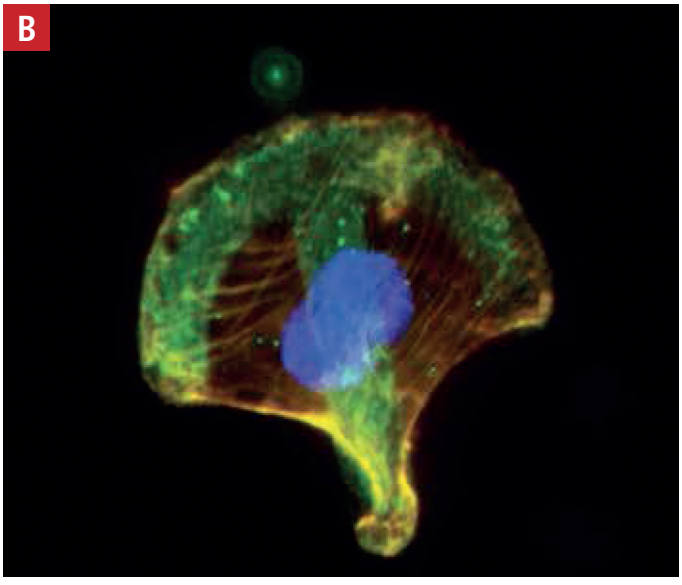


Figure 1: **A Mosaic of nine images showing a big field of micro-patterns, an advantage of the large chip of the ORCAFlash 4.0 V2.**

B Zoom on a single cell.
Green: AlexaFluor 648 tagged fibronectin. Red: Tritc- Phalloidin. Actin Blue: Nucleus. Please note that there is a bleedthrough of the actin signal into the signal of the patterns (scale bar represents 100 µm on image A. and 15 µm on image B.)

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2. Materials and Methods

Micropatterning

Glass coverslips, 25 mm in diameter, were coated with poly-L-lysine functionalized with poly-ethylene glycol chains (PII-g-PEG) at a concentration of 0.1 mg/ml.

This coating was removed at precise locations with a defined shape by illumination with 180 nm UV light through a quartz-chrome photo mask. The resulting "holes" were then filled with fibronectin by letting the protein adsorb on the glass for one hour. For a detailed protocol see [2].

Typically, four different shapes of patterns were used: non-polarized Hand circular shapes as well as polarized crossbow and pacman shapes. The diameter of these patterns is 40 µm. The crossbow and pacman patterns do simulate the shape of a migrating cell with the rounded edge mimicking a lamellipodium at the front of a migrating cell.

Cell Culture

RPE1 cells overexpressing fluorescent vimentin (labeled with a GFP tag) were cultured in Dulbecco's Modified Eagle Medium – Nutrient Mixture F12 (DMEM F12) supplemented with 10 % fetal calf serum, 1 % glutamine and 1 % penicillin/streptavidin antibiotics. Typically 70,000 cells were seeded onto the micro-patterns. Cells were allowed to spread on the micro-patterns overnight.

Cell Fixation

Cells were fixed in paraformaldehyde (4 % in PBS) for 10 minutes. After carefully rinsing the coverslips in PBS, the cell membrane was permeabilised by incubating with 0.5 % Triton X-100 for 10 minutes. Actin was then labeled with fluorescent phalloidin (TRITC) at a concentration of 1µg/ml for 30 minutes. Finally, the coverslips were mounted on a glass slide with a Moewiol mounting solution containing 1 µg/ml Hoechst staining.

Imaging

Fluorescent images were recorded using an ORCA-Flash 4.0 V2 sCMOS camera (Hamamatsu Photonics) mounted to a microscope (Eclipse Ti, Nikon). The camera had a small pixel size of 6.5µm and a total resolution of 2048x2048 pixels, providing a large spatial resolution.

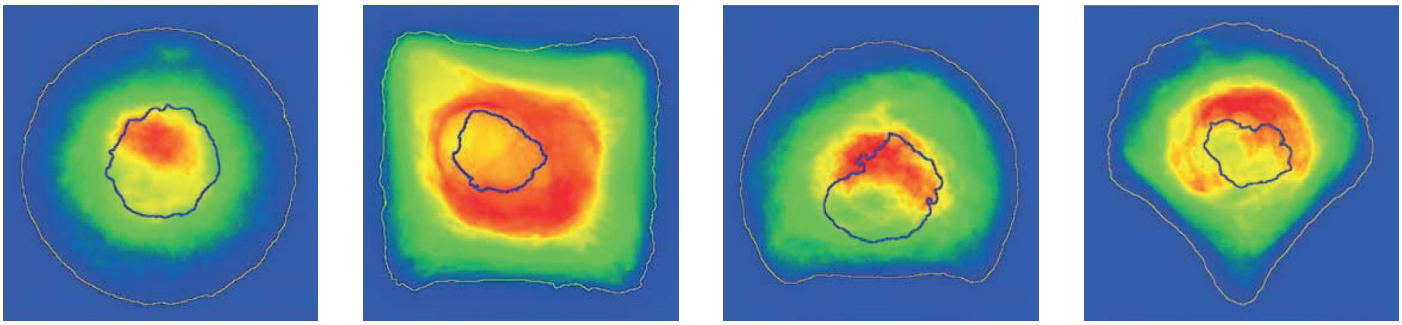

Figure 2: **A Mosaic of nine Example of averaged cells patterned on different shapes. A: circle | B: H | C: Pacman | D: Crossbow Images represent the average of 20 cells. The heat map corresponds to the vimentin distribution, the blue line to the average localization of the nucleus and the yellow line to the cell contour determined by actin. (scale bar represents 15 µm)**

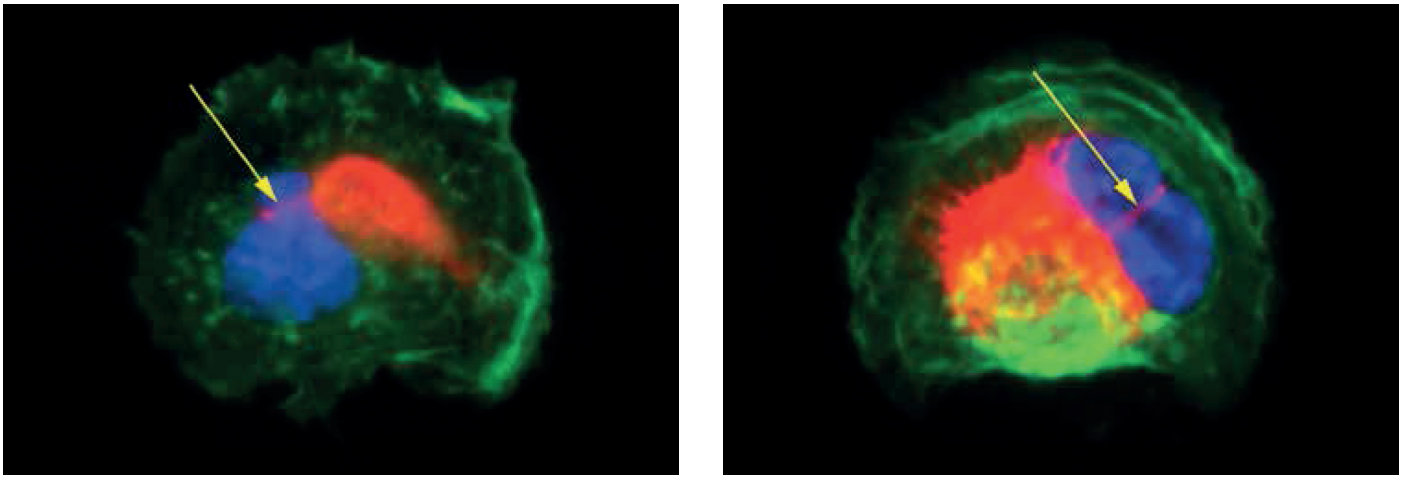

Figure 3: **Examples of two deformed nuclei. Green is actin, blue the nucleus and red vimentin. Yellow arrow emphasizes the vimentin around the nuclear deformation. (scale bar represents 15 µm)**

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3. Results

The large sensor used by the sCMOS camera allowed for the imaging of many cells in one frame. By constructing a mosaic of 3 x 3 images (Fig 1A) a number of micro-patterns could be observed. From this overview image single cells could be extracted (Figure 1B) automatically in a second step [6].

After normalizing selected cells for each colour independently, signals were averaged in order to get a mean image of the different fluorophores.

Figure 2 shows a difference in the orientation of the vimentin network in cells plated on different shapes depending on the polarity of the respective shape. In the case of the nonpolar shapes (circles and H patterns, Figure 2A and 2B) the network is quite homogeneously distributed around the nucleus.

In contrast to this result, vimentin was concentrated in the direction of the rounded edge, when the shape of the pattern is polar (pacman and crossbow, see Fig 2C and 2D). This is specifically of interest since this rounded edge can be interpreted as a lamellipodium at the front of a migrating cell.

It is also worth noting that in ~10 % of the cells the nuclei are deformed. Looking more closely it is noticed that this deformation seems to be associated with vimentin filaments surrounding the nuclei and might be squeezing them (Figure 3). Such a phenomenon has already been observed in cancerous cell lines without further investigation [7].

However, we still have to investigate if vimentin is really physically constricting the nucleus or if this phenomenon is a consequence of the deformation due to other cytoskeletal elements.

4. Conclusions

In this study we have shown how vimentin filaments' organization might be influenced by the cell shape in epithelial cells immobilized on micro-patterned cover slips. The filament network not only adapts to the polarity of its surroundings, making it an excellent marker of cell polarity, it may lead to deformations of the nuclei itself.

Imaging of fluorescently labelled cells allows the observation of the polarity and of fine internal structures. In this study, we sought to image a large number of cells at high spatial resolution, whilst at the same time measuring low light fluorescent emissions, necessary to study filamentous structures inside cells. Utilizing the latest sCMOS technology was key to fulfilling these requirements, allowing precise details of the fluorescently labelled cell structures to be captured.

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