How to Build an Integrated Microscopy System for Live Cell Mechanotransduction Studies

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A new integrated microscopy system allows scientists to simultaneously stimulate and image live cell response in real-time.

Understanding how cells react to mechano-chemical stimulation is essential to developing therapies for many vascular diseases such as hypertension, atherosclerosis, or tissue edema. Mechanical stresses are involved in both the cause and the progression of these diseases.

Studies of cell response to a variety of stimuli have traditionally relied on post-stimulation cell fixation and staining. Cell changes are usually analyzed with respect to a non-stimulated cell (i.e. control condition), which was also fixed and stained using the same procedure. Possible distortion of cellular components during specimen preparation is always a matter of concern when using this method. It also provides only static information about the status of the cell at a specific point in time.

Now, a new integrated microscopy system (Fig. 1) allows scientists to simultaneously stimulate and image live cell response in real-time, courtesy of Dr. Andreea Trache, an assistant professor at Texas A&M Health Science Center in College Station.[1, 2]

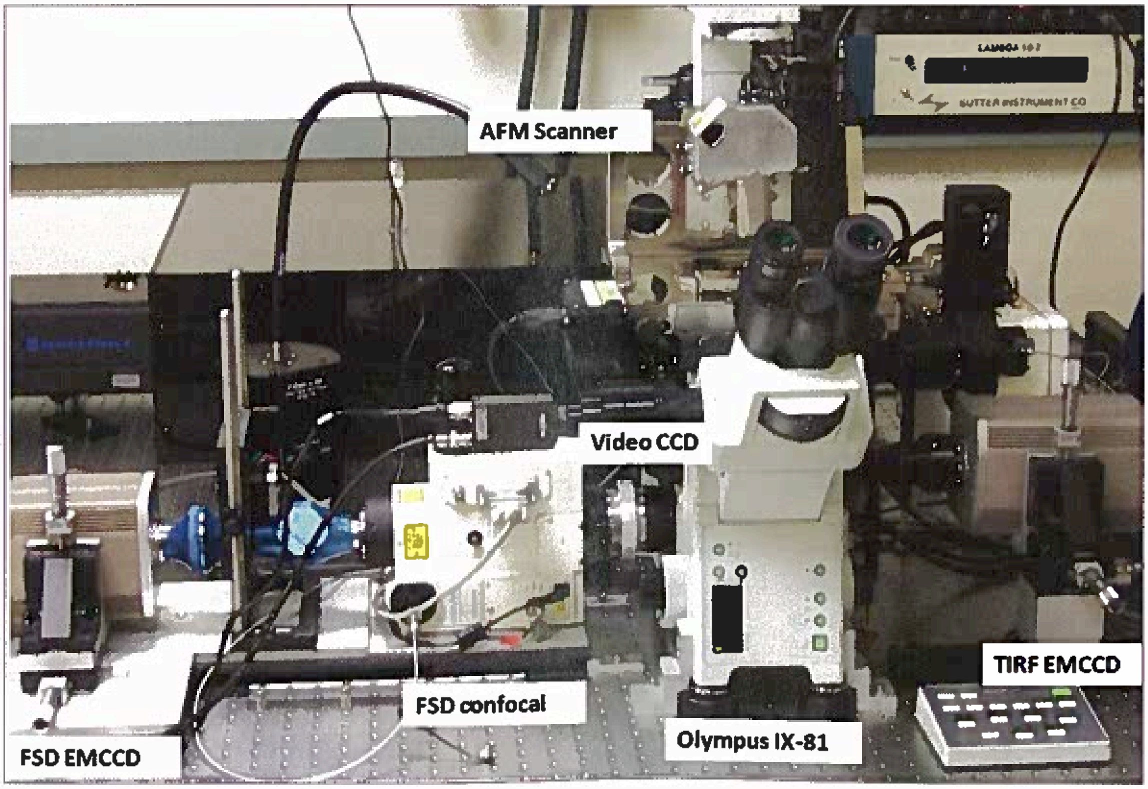

Figure 1: Layout of the integrated microscope. The AFM is placed on top of the optical inverted microscope. TIRF attachment is mounted on the right side of the optical microscope. The entire optical train of the FSD confocal scanning head in placed on the left side of the microscope on a silicon damper pad that isolates the unit from the optical table. Image reproduced from *J Biomed* Opt 14: 034024 (2009).

Noise problem

Using an atomic force microscope (AFM) combined with total internal reflection fluorescence (TIRF) and fast-spinning disk (FSD) confocal microscopy, Dr. Trache is able to mechanically stimulate and image cytoskeletal reorganization in real time with high spatial and temporal resolution. An AFM uses a nanosensor to directly apply and measure mechanical force with high precision, being able to mechanically stimulate and record the cell response. TIRF microscopy provides high-contrast images of fluorescently labeled molecules in the vicinity of the cell-coverslip interface with high resolution along the z-axis. FSD confocal microscopy allows rapid 3-D imaging throughout the cell.

Ultimately, the mechanical signaling initiated by the AFM is transmitted to the cell via a matrix-integrin-actin linkage. To stimulate the actin, Dr. Trache uses a fibronectin-functionalized AFM tip. Fibronectin, an extracellular matrix protein, binds to transmembrane receptors called integrins. Inside the cell, integrins connect to actin via focal adhesion proteins. By labeling the actin filaments with green fluorescent proteins, Dr. Trache can record actin remodeling in real-time with QuantEM 512SC EMCCD cameras from **Photometrics.**

"This setup is challenging because it's hyper-sensitive to noise and vibrations. The AFM can measure nanometer displacements at nano- to pico-Newton force. At that level, the rotation of the spinning disk or the camera fan would disturb the experiment," explained Dr. Trache, who needed to isolate any vibration sources from the AFM and specimen.

"Also, a typical experiment lasts about 80 minutes, in which time 250 confocal images are acquired. We can't have photobleaching because it would misrepresent the biological process we're recording," continued Dr. Trache. "We needed a highly sensitive camera that would allow us to minimize the intensity of the laser excitation. Critical, too, was a high signal-to-noise ratio to record dim fluorescence images with a dark background, in order to minimize post-processing of hard data."

Dr. Trache also needed to synchronize the spinning disk of the confocal scanning head, which rotates at 5,000 rpm, with the low-light camera to obtain uniformly illuminated images. In turn, the field of view of the confocal camera needed precise alignment with the TIRF camera, the AFM tip, and the AFM video camera and eyepiece, providing the ability to switch between different imaging modalities during the experiment without disturbing the specimen (i.e. moving the specimen in the field of view).

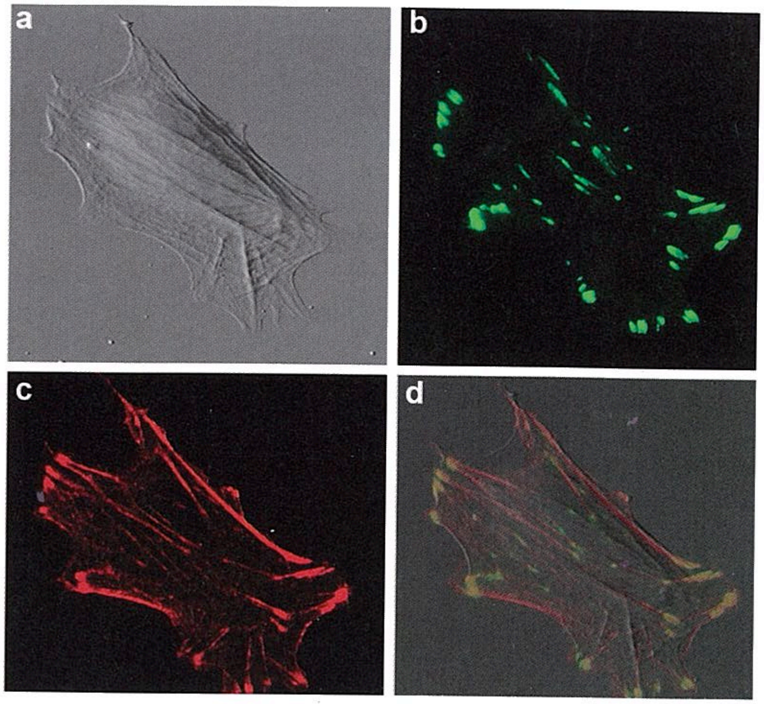

Figure 2: A live vascular smooth muscle cell transfected with vinculin-GFP and actin-mRFP was imaged by AFM (a) to provide a general topographic view of the cell with actin cytoskeletal fibers immediately beneath the apical membrane. The same cell was then imaged by TIRF microscopy (b) to show the focal adhesions at the basal cell membrane marked by vinculin-GFP (green channel). A confocal image (c) was acquired as a 3D stack of 20 planes and presented as maximum projection to show the cytoskeletal actin structure (red channel) throughout the cell. The overlay (d) depicts the excellent registration between the AFM image and the fluorescence imaging techniques.

Engineering the perfect setup

Dr. Trache methodically isolated all sources of vibration by mounting the confocal scanner on a silicone damper pad, and isolating it from the microscope body. Vibrating equipment, such as external Photometrics camera fans, were mounted to adjacent structures.

Out of the cameras that Dr. Trache tested, Photometrics' QuantEM EMCCD cameras provided "the best synchronization between the spinning disk and the camera." The camera's pixel-clock timing resolution allows for synchronization and superior signal-to-noise sampling.

"We tested several cameras," she adds, "and these seem to do the best job for all the features that we're looking for."

Cooled to -30°C, the QuantEM cameras have greater than 92% quantum efficiency, sub-electron read noise, and 16 μm × 16 μm pixels to provide fast image capture at a low laser excitation intensity. The QuantEM's 16-bit digitization also delivered the wide dynamic range that Dr. Trache needed for both FSD confocal and TIRF microscopy.

Seeing (in real-time) is believing

"Live cell real-time imaging is the only way to directly monitor cellular responses to mechano-chemical stimulation. Currently, we obtain consistent data from studying real-time live cell remodeling," said Dr. Trache. "We can image focal adhesion proteins recruitment at the basal cell surface as the cell remodels in response to the AFM's applied force. It's action and reaction."

As for minimizing laser intensity, there is "no photobleaching with time," Dr. Trache said. "The QuantEM camera gives us high contrast images with a short exposure time."

She added, "We are able to capture images at 20 fps. If we need to, we can push it to 50 fps or more."

"As I said, especially with TIRF microscopy that provides images within 100 nm from the basal cell surface, we want the signal-to-noise ratio as high as possible. We do post-processing, but our hard data gives us an excellent signal-to-noise ratio," she continued.

The TIRF microscopy images of the cytoskeleton and focal adhesions proximal to the basal cell surface can be analyzed in conjunction with the FSD confocal microscopy images obtained by scanning the entire cell.
The result is a 3-D capture of whole-cell adaptive responses to mechanical force at high spatial and temporal resolution (Fig. 2).

This integrated technology provides an important new tool, broadly applicable in cellular biology to any adherent cell type, to study protein dynamics, as well as how cells interact with their neighbors or the extracellular matrix. Using this technology, important mechanobiology questions regarding the cells' ability to sense, respond and adapt to mechanical stimuli can now be addressed.

References

1. Andreea Trache and SM Lim, Live cell response to mechanical stimulation studied by integrated optical and atomic force microscopy, *Journal of Visualized Experiments*, 44 (2010) 2072. http://www.jove.com/index/details.stp?id=2072.
2. Andreea Trache and SM Lim. Integrated microscopy for real-time imaging of mechanotransduction studies in live cells, *Journal of Biomedical Optics* vol. 14 (3), 034024 (2009).