Part I

Micro-nano techniques in cell mechanobiology

1 Nanotechnologies and FRET imaging in live cells

Eddie Y. Chung, Qin Qin, Agamoni Bhattacharyya, Shaoying Lu, and Yingxiao Wang

Live cells can sense the mechanical characteristics of the microenvironment and translate the mechanical cues to intracellular biochemical signals in physiology and disease. To investigate intracellular signaling transduction during mechanosensing, nanotechnologies, and FRET live-cell imaging technologies have been developed to visualize the output signals in real time, such as intracellular molecular activity. Meanwhile, micropatterned technologies have been applied to modulate the physical and mechanical environment surrounding the cell to fine-tune the input signals in cellular mechanosensing. These advanced technologies can join forces and shed new light into the molecular networks that control mechanotransduction in normal conditions and disease.

1.1 Introduction

Mechanical force plays crucial roles in regulating pathophysiological processes such as atherosclerosis and cancer metastasis (Makowski and Hotamisligil 2004; Bissell and Hines 2011). For example, shear stress without a clear direction can lead to endothelial cell (EC) dysfunction and atherogenesis (Bao, Lu, and Frangos 1999; Bao, Clark, and Frangos 2000; Gimbrone and Garcia-Cardena 2013). However, there is a lack of understanding on how the cells can perceive the spatiotemporal cues and transduce them into biochemical activities to regulate cellular functions. It has been hypothesized that the great spatiotemporal heterogeneity of force distribution under atheroprone-disturbed flows contributes to the pathophysiological modulation of EC responses in mechanotransduction (DePaola et al. 1992; Frangos, Huang, and Clark 1996; Davies 1997; Tardy et al. 1997; Nagel et al. 1999; Bao et al. 2000; Butler et al. 2000; Li et al. 2002). The hypotheses will need to be investigated through novel imaging approaches with high spatiotemporal resolution in single live cells, such as the fluorescence proteins, fluorescence resonance energy transfer (FRET) biosensors, and enabling nanotechnologies.

Fluorescence proteins have been widely applied in single live cell imaging, a practice that has revolutionized the research field of cell biology, including mechanotransduction and mechanobiology. FRET-based biosensors have been engineered and applied to visualize molecular activities in single live cells (Miyawaki et al. 1997; Mochizuki et al. 2001; Ting et al. 2001; Zhang et al. 2001; Violin et al. 2003; Kunkel et al. 2005; Wang et al. 2005; Zhang et al. 2005; Pertz et al. 2006; Buranachai and Clegg 2008). The FRET biosensors contain a pair of donor and acceptor fluorescence

proteins. The ratiometric measurement of the biosensor signals utilizes the donor-to-acceptor intensity ratio to represent the activity of target molecules. This measurement is self-normalizing and independent of the heterogeneous biosensor expression level among different cells (Liao et al. 2012). The genetically engineered FRET biosensors also allow subcellular localization to cytosol, plasma membrane, or organelles, which can provide measurement of local molecular activities with high spatial resolution. Therefore, FRET-based biosensors provide a versatile tool to monitor molecular signals in live cells in real time (Wang, Shyy, and Chien 2008; Song, Madahar, and Liao 2011).

Over the past 10 years, nanotechnologies have had a huge impact on scientific research. Novel nanomaterials, as well as nano- and microtechnologies and ensuing imaging methods, have enabled researchers to investigate molecular networks inside the cell with high functionality and high fidelity. Specifically, nanosized quantum dots (QDs) have been applied as FRET donors for QD FRET biosensors to monitor molecular activities in live cells. In this chapter, we will introduce the development of FRET biosensors based on fluorescence proteins and nanosized quantum dots, as well as their applications in live cell imaging under controlled microenvironment, for studies in mechanotransduction and cancer.

1.2 FRET biosensors

It has become well known that signaling molecules function in a nonlinear network with pathways that are largely dependent on subcellular localizations. Therefore, it is essential to develop and utilize imaging tools to monitor spatiotemporal activation of different signaling elements in this network. FRET biosensors provide ideal tools for this purpose. For example, the FRET-based kinase biosensors have wide applications in systematic understanding of the functional roles played by Src kinase and focal adhesion kinase (FAK) in mechanosensing and mechanobiology (Wang et al. 2005; Ouyang et al. 2008; Seong et al. 2011; Seong et al. 2013). In addition, a FRET reporter for a mechanosensitive canonical transient receptor potential channel 6 (TRPC6) has been developed to visualize real-time local calcium influx mediated at TRPC6 in different lipid regions (Lei et al. 2014). Two FRET biosensors with distinct fluorescence spectrums have also been developed to simultaneously monitor both the activities of Src kinase and MT1-MMP protease within the same live cells (Ouyang et al. 2010). All these arrays of FRET biosensors will provide a colorful landscape of functional roles played by different molecular activities in live cells during mechanotransduction.

1.3 Src FRET biosensors and mechanotransduction

The Src FRET biosensor was developed by Wang et al. (2005) to visualize Src kinase activity in live cells. The biosensor contains a cyan fluorescence protein (CFP), a SH2

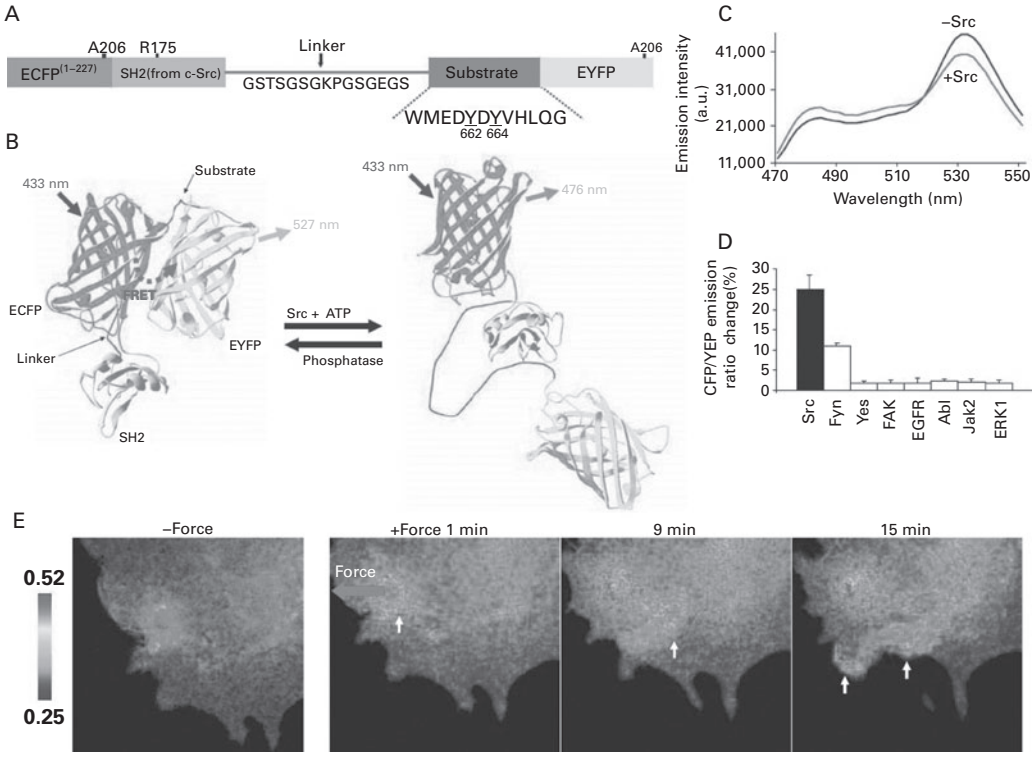

Figure 1.1 **The schematics and characterizations of the Src reporter, and observed wave-propagation in a live cell.**

(A) The Src reporter is composed of CFP, the SH2 domain, a flexible linker, the Src substrate peptide, and YFP. (B) The drawing illustrates the FRET effect of the Src reporter upon the actions of Src kinase or phosphatase. (C) Emission spectra of the Src reporter before (black) and after (red) phosphorylation by Src. (D) In vitro emission ratio changes (mean + s.d.) of the Src reporter in response to Src and other kinases. (E) Directional wave propagation (white arrow) of Src activation was observed after mechanical stimulation by bead traction (red arrow).

This research was originally published in the journal *Nature*. Wang et al. "Visualizing the mechanical activation of Src." *Nature* 2005, 434(7036): 1040–1045.

domain, a flexible linker, a Src substrate, and a yellow fluorescence protein (YFP, Fig. 1.1A, B). After the Src reporter is phosphorylated by Src kinase at the substrate tyrosine sites, it shows higher emissions of CFP and lower emissions of FRET (emission of YFP upon CFP excitation), which can be represented by a loss of FRET and an increase of CFP/FRET ratio caused by Src activation (Fig. 1.1C). The increase of CFP/FRET ratio was fully activated by c-Src, while only weakly activated by c-Fyn, and not activated by c-Yes or other kinases in mammalian cells (Fig. 1.1D). These results prove that the Src reporter is specifically activated by Src kinase in mammalian cells.

Fibronectin-coated beads can bind to integrins and thus couple with cytoskeleton (Miyamoto et al. 1995). These functionalized beads were then applied to human umbilical vein endothelial cells (HUVECs) and resulted in a local FRET response of

the Src reporter around the beads (Arias-Salgado et al. 2003). However, if the beads were coated with noninteractive polylysine, then there was no significant FRET response. This result infers that specific integrin-cytoskeleton coupling is necessary for mechanotransduction. The authors then targeted the monomeric Src reporter to the plasma membrane (Thomas and Brugge 1997; Zacharias et al. 2002; Arias-Salgado et al. 2003). The FRET response of this membrane-targeted reporter was reversed and prevented by PP1, a general inhibitor of Src family kinase, which proves that the Src reporter has specificity toward Src kinase activity.

Upon applying mechanical force by laser tweezers on fibronectin-coated beads adhering to the cell surface, a robust FRET-signal change was observed, which indicated a rapid distal Src activation and a slower directional wave propagation of Src activation in live cells (Fig. 1.1E). The Src activation serves as a mechanism for cells to adapt to the new mechanical environment. Therefore, monitoring dynamic signal transduction processes by FRET biosensors enables the visualization and quantification of mechanotransduction process in live cells. This result marked the first time when the mechanotransduction process was visualized in live cells.

1.4 Focal adhesion kinase (FAK) FRET biosensors

Focal adhesions are molecular complexes at the contact sites at the interface between the cell and its extracellular matrix (ECM). Mature focal adhesions contain transmembrane receptor integrins, associated intracellular signaling proteins, and adapter proteins (Luo, Carman, and Springer 2007). Signaling proteins at focal adhesions include Src and FAK tyrosine kinases and receptor-like tyrosine phosphatase α (RPTP-α). In newly assembled focal adhesions, integrin ligates with ECM proteins, which subsequently recruit many intracellular structural and signaling proteins to the focal adhesion sites.

Since integrin lacks enzymatic activity, the signaling proteins in focal adhesions are crucial to transfer extracellular mechanical information toward the inside of the cells (Mitra, Hanson, and Schlaepfer 2005; Mitra and Schlaepfer 2006). For example, Src and FAK coordinate and regulate downstream signals in focal adhesions (Seong 2011; Seong et al. 2013). With a FAK FRET biosensor designed with a similar strategy as that of the Src biosensor, Seong et al. (2011) reported that growth-factor-induced FAK activation is mediated and maintained by Src activity, while FAK activation on cell adhesion is in fact independent of Src activation (Seong et al. 2011). FAK also interacts with different integrin receptors and extracellular matrix proteins to sense the mechanoenvironment (Fig. 1.2) (Seong et al. 2013). In this study, Seong et al. (2013) found that matrix protein fibronectin (FN)-mediated FAK activation is dependent on mechanical tension, while FAK can be sufficiently activated on type I collagen independent of tension. Therefore, different ECM proteins can differentially transmit or shield mechanical forces from the environment to the functional molecules in the cell and regulate cellular functions (Fig. 1.2) (Seong et al. 2013). In this case, FRET biosensors provided a precise picture of in situ molecular signals at real time in single

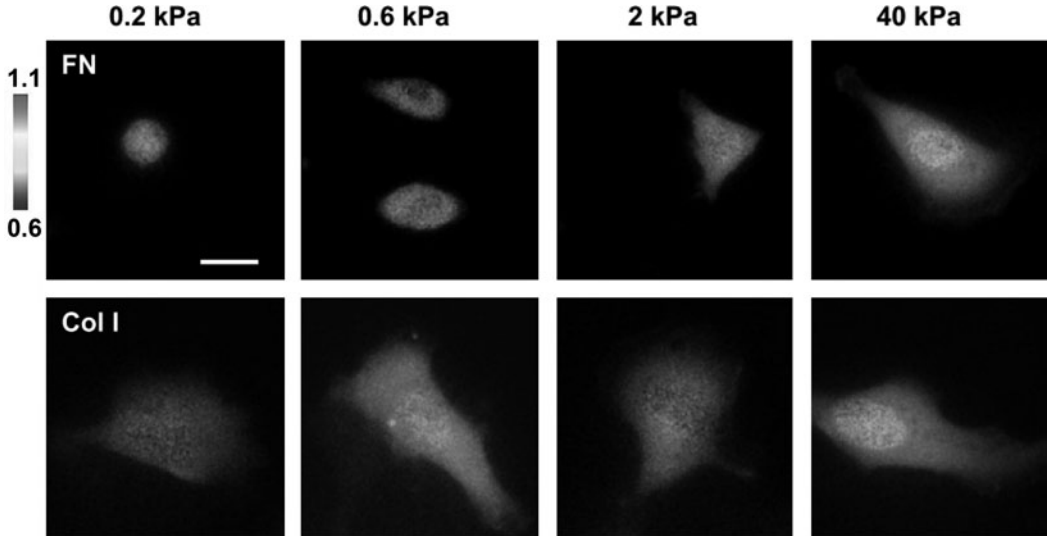

Figure 1.2 **FAK activation is dependent on substrate rigidity coupled with FN, but not Col I.** The representative ECFP/FRET ratio images of FAK biosensor in HT1080 cells cultured on the FN- (Top Panels) or Col I-coated (Bottom Panels) PA gels with different stiffness, as indicated. The color bar on the left shows the ECFP/YPet ratio values, with cold and hot colors representing low and high ratio values, as indicated.

This research was originally published in the journal *PNAS*. Seong et al. "Distinct biophysical mechanisms of FAK mechanoactivation by different extracellular matrix proteins." *PNAS* 2013, 110(48):19372–19377.

cells, which is especially important for the investigation of mechanosensing and mechanotransduction during cell-ECM interaction at focal adhesions (Bershadsky, Balaban, and Geiger 2003; Geiger, Spatz, and Bershadsky 2009).

1.5 Micropatterns, FRET, and fluorescence proteins (FPs)

To investigate how cells perceive and interpret the microenvironment to coordinate intracellular molecular signals and ultimately physiological functions, many technologies have been developed to manipulate the microenvironment around the cells and to study the effect of mechanical cues on cell behaviors. Among these, Kim et al. (2009) has combined micropatterning technologies with genetically encoded FRET biosensors to study spatiotemporal RhoA and Src activities in different microenvironments. A micropattern via micromolding in capillaries has been applied on glass coverslips on which cells attach (Fig. 1.3). After applying epidermal growth factor (EGF) on cells expressing Src or RhoA FRET biosensors, it has been shown that the microenvironment may regulate the spatiotemporal signaling network of RhoA and Src in live cells (Kim et al. 2009). Briefly, EGF induced a decrease of RhoA and an increase of Src activities with biphasic time courses in cells seeded on micropatterned glass, while the induced decrease of RhoA and increase of Src activities are relatively monophasic in

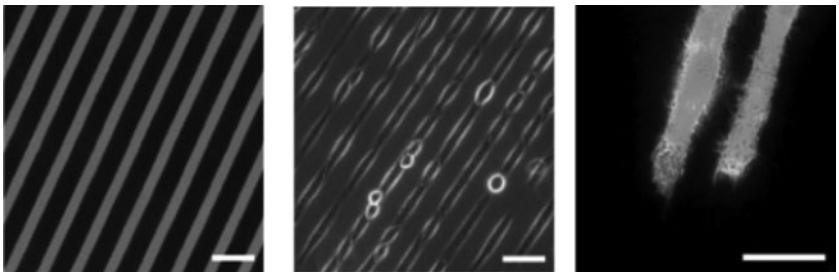

Figure 1.3 **Micropatterning on the silanized glass.**
Left: Fluorescence image of a pattern of NHS-rhodamine-conjugated Fn on silanized glass; Middle: Phase-contrast image (40x) of transfected HeLa cells cultured on the patterned surface; Right: Emission ratio images (100x) of the RhoA biosensor in transfected HeLa cells cultured on the patterned surface. Scale bars: 40 μm.

This research was originally published in the journal *Small*. Kim et al. "Visualizing the effect of microenvironment on the spatiotemporal RhoA and Src activities in live cells by FRET." *Small* 2009, 5(12): 1453–1459.

cells on glass without pattern. These results indicate that the cells may be capable of probing and adapting to the mechanical environment in response to stimuli. Further experiments show that the inhibition of Src activity abolishes this biphasic RhoA response toward EGF in cells on patterns. These results suggest that the observed micropattern effect on the biphasic RhoA activation in the cells is mediated by Src (Kim et al. 2009).

The spatial distribution of molecular signals within cells is crucial for cellular functions. Polarized molecular activities can guide the cell toward persistent and directional migration. Micropatterned strips of cell-adhesive extracellular matrix protein fibronectin separated by the nonadhesive copolymer, pluronic 127, have been developed to study the polarized molecular activities in live cells (Ouyang et al. 2008; Lu et al. 2011). In this system, cells are constrained to the fibronectin strips, which are about 10 μm in width. When two cells form a junction, they are stably polarized with one end connecting to a neighboring cell (the junction end) and the other end free of cell-cell contact (the free end) (Lu et al. 2011; Ouyang et al. 2013). Therefore, this system provides an ideal microenvironment in which to study polarized molecular activities and interactions in live cells.

Integrating the polarized micropatterns with the newly discovered high-efficiency FRET pair of enhanced CFP (ECFP) and YPet, the authors were able to visualize highly polarized Rac activity concentrating at the free end, which was activated by the growth factor stimulation and mediated by the Src-family kinase expression. In contrast to the polarized Rac activation, a global activation of Src was detected in these cells, which was modulated by the well-balanced endogenous Rac activity. These results demonstrate that the activations of Rac and Src have different spatial patterns, which mutually regulate each other (Ouyang et al. 2008).

Furthermore, the one-dimensional feature of the striped micropattern provides an ideal system for quantitative analysis of the observed fluorescence signals. For this

purpose, an image analysis software package, Fluocell, was developed to quantify and analyze the dynamic regulation of signaling molecules in cells on pattern (Lu et al. 2011; Ouyang et al. 2013). In these studies, cells on micropattern were transfected with PH-Akt-GFP (the PH domain of Akt was fused to a green fluorescence protein, GFP), or a PAK-PBD-YFP (the PAK domain of PBD fused to a YFP), to monitor the intracellular PI3 K and Rac activities, respectively. Briefly, the cells with a junction end and a free end were stimulated with the growth factor and observed with polarized distribution of PH-Akt-GFP and PAK-PBD-YFP. The recorded fluorescence intensity images were aligned along the stripe direction, mapped to 1D, quantified, and normalized, so that different cells have a normalized uniform length and total fluorescence intensity at the longitudinal direction. This analytical approach allows the statistical analysis of image data from multiple cells, as well as the quantitative comparison of the sequential activation of PI3 K and Rac in the cells on pattern. These results revealed different polarization patterns of PI3 K and Rac1 activities induced by growth factor, suggesting that the initiation of edge extension occurred before PI3 K activation, which led to a stable extension of the free end followed by Rac activation (Lu et al. 2011). Further substantial experiments and analysis showed that PI3 K and Rac polarization depend specifically on the N-cadherin-p120-catenin complex at the cell-cell junction, whereas myosin II light chain and actin filament polarization depend on the junctional N-cadherin-β-catenin complex (Ouyang et al. 2013). Therefore, the integration of micropattern, fluorescence live cell imaging, and computational analysis provides a powerful tool to quantitatively investigate the spatiotemporal regulation of molecular signals in live cells under different microenvironment conditions.

1.6 Quantum dots as FRET donors

FRET technology has allowed single-cell imaging with high spatiotemporal resolution for applications in biology and medicine. The brightness of current available FRET pairs has limited broad usage, however, and a relatively high concentration of biosensors is needed to obtain meaningful FRET signals. The relatively broad spectrums of contemporary dyes also limit their scientific applications as FRET donors and acceptors. Quantum dots are inorganic semiconductor nanocrystals that have sharp, size-tunable symmetric emission spectra. By changing the size of quantum dots, it is possible to obtain specific emission wavelengths (Fig. 1.4), which therefore can be combined with almost every dye to form a FRET pair (Alivisatos, Gu, and Larabell 2005; Michalet et al. 2005; Smith et al. 2008). Most importantly, quantum dots have an order of magnitude higher extinction coefficient and quantum yield than conventional dyes, which make it suitable for molecular imaging (Alivisatos et al. 2005; Resch-Genger et al. 2008). Quantum dots, with their special core-shell inorganic composition, are also resistant to photobleaching in comparison to organic dyes (Reiss, Bleuse, and Pron 2002; Sukhanova et al. 2004). With these attractive features, they have been widely used in live cells, and even for in vivo imaging (Akerman et al. 2002; Dubertret et al. 2002; Ballou et al. 2004).

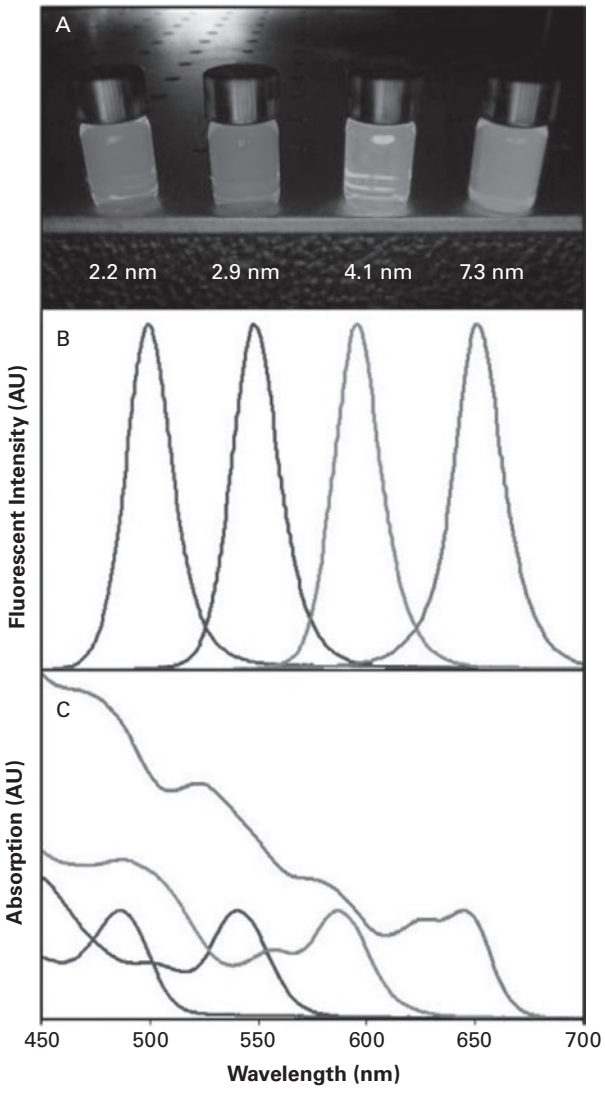

Figure 1.4 **Size-dependent optical properties of cadmium selenide QDs dispersed in chloroform, illustrating quantum confinement and size-tunable fluorescence emission.**
(A) Fluorescence image of four vials of monodisperse QDs with sizes ranging from 2.2 nm to 7.3 nm in diameter. This image was obtained with ultraviolet illumination. (B) Fluorescence spectra of the same four QD samples. Narrow emission bands (23–26 nm FWHM or full-width at half-maximum) indicate narrow particle size distributions. (C) Absorption spectra of the same four QD samples. Notice that the absorption spectra are very broad, allowing a broad wavelength range for excitation. Both the absorption and emission intensities are plotted in arbitrary units (AU).

Reprinted from Smith et al. "Bioconjugated quantum dots for in vivo molecular and cellular imaging." *Journal of Advanced Drug Delivery Reviews* 2008, 60: 1226–1240. Copyright (2008), with permission from Elsevier.