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Review

Live cell imaging of mechanotransduction

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Mechanical forces play important roles in the regulation of cellular functions, including polarization, migration and stem cell differentiation. Tremendous advancement in our understanding of mechanotransduction has been achieved with the recent development of imaging technologies and molecular biosensors. In particular, genetically encoded biosensors based on fluorescence resonance energy transfer (FRET) technology have been widely developed and applied in the field of mechanobiology. In this article, we will provide an overview of the recent progress of FRET application in mechanobiology, specifically mechanotransduction. We first introduce fluorescent proteins and FRET technology. We then discuss the mechanotranduction processes in different cells including stem cells, with a special emphasis on the important signalling molecules involved in mechanotransduction. Finally, we discuss methods that can allow the integration of simultaneous FRET imaging and mechanical stimulation to trigger signalling transduction. In summary, FRET technology has provided a powerful tool for the study of mechanotransduction to advance our systematic understanding of the molecular mechanisms by which cells respond to mechanical stimulation.

Keywords: fluorescence resonance energy transfer; mechanotransduction; stretch; shear stress; substrate stiffness

1. INTRODUCTION

Cells in the body are exposed to a variety of mechanical forces such as hypotonic swelling, hydrostatic pressure, mechanical stretch and shear stress. These mechanical forces can be perceived by cells and thus cause different physiological consequences, including deformation and regulation of cellular polarization (Katsumi *et al.* 2004; Ingber 2006), migration (Wang *et al.* 2006; Hsu *et al.* 2007) and differentiation (Datta *et al.* 2006; Obi *et al.* 2009; Pek *et al.* 2010). It remains unclear, however, how cells perceive the mechanical stimuli and transmit them into cellular biochemical signals.

With the development of imaging technologies, particularly green fluorescent protein (GFP) and its derivative fluorescent proteins (FPs), it is now convenient to visualize molecular signals at subcellular levels in live cells. Traditional assays to detect/measure molecular signals usually require the killing of cells, which may cause the alteration of innate information. With FPs and biosensor-based FPs, molecular signals at different subcellular compartments can be monitored in live cells continuously without damaging the cells. These molecular biosensors have been increasingly applied to the field of mechanobiology.

In this review, we will introduce the fluorescence resonance energy transfer (FRET) technology and its use in studying mechanotranduction. There will be a specific emphasis on the integration of FRET and mechanoactivation systems for the live cell imaging of mechanotransduction.

2. FLUORESCENT PROTEINS AND FLUORESCENCE RESONANCE ENERGY TRANSFER

Since the discovery of GFP in *Aequorea* jellyfish in the 1960s (Shimomura *et al.* 1962), GFP has been widely applied in life science studies because it can be fused to a target molecule for its dynamic visualization independent of other cofactors (Chalfie *et al.* 1994; Inouye & Tsuji 1994). The wide-type GFP has two chemically different populations, which emit different fluorescent colours (Tsien 1998). Improvement by Tsien's

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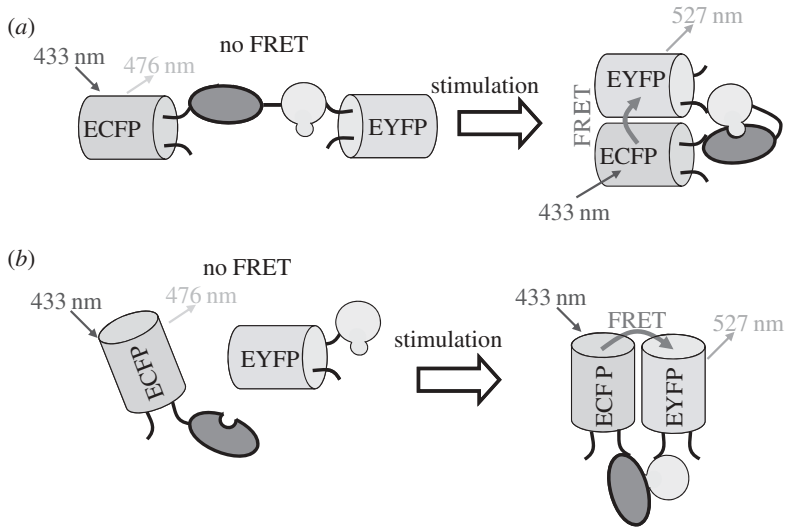

Figure 1. Functional mechanism of FRET biosensors. (*a*) Intramolecular FRET: two target molecules/domains covalently fused together between a pair of donor and acceptor FPs can interact with each other upon stimulation to cause FRET changes. (*b*) Intermolecular FRET: two target molecules/domains are separately fused to donor and acceptor FPs. The two target molecules/domains can interact with each other upon stimulation and bring the donor and acceptor FPs together to cause FRET changes.

laboratory has resulted in enhanced GFP (EGFP), which has better fluorescent brightness with decent properties at 37°C for live cell studies. A variety of FPs have also been developed based on EGFP to emit different colours (Wang *et al.* 2008), including blue (BFP), cyan (CFP) and yellow (YFP) (Ormo *et al.* 1996; Tsien 1998). Recently, FPs with a relatively long wavelength have been developed based on a red FP isolated from *Discosoma* (DsRed) (Matz *et al.* 1999). These new different-colour FPs, covering the emission colours ranging from orange to deep red (Wang *et al.* 2008), allow the visualization of multiple molecular signals in a single live cell by fusing target molecules with FPs with distinct colours.

Genetically encoded biosensors based on FRET between FPs have further extended the capability of FPs to measure active molecular signals (Wang *et al.* 2008). FRET is a quantum mechanics phenomenon where two FPs (donor and acceptor) with the donor emission spectrum overlapping that of the acceptor can have energy transfer when the donor and the acceptor are close to each other with proper relative angles (Tsien 1998). The early FRET biosensors use the BFP and GFP pair (Romoser *et al.* 1997; Mahajan *et al.* 1998). At the current stage, the most popular FRET pair is CFP and YFP because of the sufficient overlap between the donor emission and acceptor excitation spectra (Wang *et al.* 2008). Furthermore, variants of CFP have in general better photo-stability and brightness than those of BFP (Miyawaki *et al.* 1997). Different variants of CFP and YFP have been shown to provide different levels of dynamic ranges for the FRET changes of biosensors (Ouyang *et al.* 2008*b*). Recently, a high-efficiency FRET pair based on variants of CFP and YFP, CyPet and YPet, has been developed via a directed evolution strategy (Nguyen & Daugherty 2005). However, CyPet appears to have poor properties at 37°C and hence is not suitable for live cell studies (Shaner *et al.* 2005). An ECFP and YPet pair has since been demonstrated to allow a high dynamic range of FRET biosensors for various molecules, including Src, Rac, MT1-MMP and calcium (Ouyang *et al.* 2008*b*). The most recent development of different elegant FPs, including a CFP variant mTFP1 (Ai *et al.* 2006), an RFP variant mCherry (Shaner *et al.* 2004) and a far-red FP mKate (Shcherbo *et al.* 2007), has provided more opportunities for the development of new FRET pairs with novel properties. The usefulness of these new FPs for FRET studies, however, remains relatively underexplored.

Since genetically encoded biosensors based on FRET and FPs can allow the monitoring of dynamic molecular signals at subcellular compartments/levels in live cells without causing much detrimental impact of the host cells, numerous FRET biosensors have been developed (Wang *et al.* 2008). Typical FRET studies involve the development of biosensors where the donor and acceptor FPs are fused to interacting molecules/domains, with the chimera proteins containing donor and acceptor FPs either separated or integrated into a single molecule. The designing principle is that the stimulation and the ensuing molecular signals can cause the interaction of the fused molecules/domains, such that the relative distance/orientation between the connected FPs can be altered to result in a FRET change. As such, the molecular signals can be monitored by observing the FRET changes (figure 1). In general, it is preferred to have the donor and acceptor FPs integrated into a single molecule (intramolecular FRET) such that a simple ratiometric analysis of the emissions from donor/acceptor can represent the FRET signals. When the donor and acceptor FPs are separated into two different chimeric molecules (intermolecular FRET), the copy number ratio of the acceptor/donor at different subcellular locations can be different because of the different expression and localization of donors/acceptors. Furthermore, separated chimeric molecules will also have more interference from their

endogenous interacting counterparts. Therefore, more sophisticated numerical approaches will be needed to analyse and interpret the results for these intermolecular FRET biosensors (Zhang *et al.* 2002). Nevertheless, biosensors based on both intramolecular and intermolecular FRET have allowed the successful visualization of crucial molecular events, including the activation of membrane receptors such as integrins, growth factor receptors and G-protein-coupled receptors (GPCRs) and intracellular signalling molecules such as kinase, phosphatases, small GTPases, phospholipids and calcium (Zhang *et al.* 2002; Wang *et al.* 2008). In this article, we will specifically focus on those biosensors that have been used for the study of mechanotransduction.

3. SIGNALLING TRANSDUCTION IN MECHANOBIOLOGY

Mechanical signals can regulate cellular functions in two modes, transmission and transduction. The former is mainly based on the intracellular cytoskeleton system, with which external signals can be passed to the remote part of the cell (Wang *et al.* 1993; Na *et al.* 2008); the latter can convert the mechanical signals into biochemical signals. Mechanotransduction from extracellular stimuli to intracellular signals can be based on force-sensitive ion channels, such as stretch-activated ion channels (Martinac 2004), or on the extracellular matrix–integrin complex (Maniotis *et al.* 1997). In particular, integrins are a family of adhesion molecules on the cell surface that play key roles in mechanotransduction. Under mechanical stimulation, integrin can adjust its conformation by binding to extracellular matrix proteins to form a high-affinity and activation condition, which will promote the connection between its cytoplasmic tail and cytoskeleton as well as the formation of stress fibres and focal adhesions, thereby triggering the intracellular signalling transduction pathway and ultimately affecting physiological functions (Mitra *et al.* 2005). Indeed, a large number of signalling molecules have been reported to be activated and involved in mechanotransduction, including Src (Wang *et al.* 2005; Radel *et al.* 2007) and small Rho GTPases such as RhoA, Rac1 and Cdc42 (Tzima *et al.* 2002; Wojciak-Stothard & Ridley 2003; Goldyn *et al.* 2009; Poh *et al.* 2009). Recent evidence also indicates that these signalling molecules do not function in isolation (Wang *et al.* 2007). Instead, they form a complex network and transmit signals in a nonlinear fashion, varying based on different subcellular locations (Seong *et al.* 2009). FRET technology has been proved to be a powerful tool for the study of mechanotransduction at subcellular levels in different live cells, including stem cells.

Over recent years, there has been an increasing interest in understanding mechanobiology in stem cells owing to its potential promise for therapeutic applications in tissue engineering or organ repair. Stem cells are characterized by their ability for self-renewal and differentiating into a diverse range of specialized cell types. These stem cells possess precise sensing systems to detect a variety of mechanical forces and transduce them into biochemical signals that lead to various outcomes. Recent studies show that mechanical forces, including gravity, tension, stiffness, compression, pressure and shear stress, play a vital role in regulating gene and protein expression of stem cells. For example, in mesenchymal stem cells (MSCs) derived from bone marrow, cyclic stretching can have a profound influence on cell lineage commitment (Sumanasinghe *et al.* 2006; Juncosa-Melvin *et al.* 2006, 2007; Chen *et al.* 2008) and promote the differentiation of MSCs into smooth muscle cells (SMCs) (Kurpinski *et al.* 2006). Similarly, in adipose-derived stem cells, cyclic strain alone or in combination with transforming growth factor beta 1 (TGF-β1) induced the differentiation towards the SMC lineage (Lee *et al.* 2007). Static mechanical compression, on the other hand, seems to promote the chondrogenesis of MSCs. In fact, cyclic compression enhanced the gene and protein expressions, including chondrogenic markers type II collagen and aggrecan (Angele *et al.* 2004; Pelaez *et al.* 2008). Hydrostatic pressure also enhanced the chondrogenic differentiation of human MSCs (HMSCs) (Wagner *et al.* 2008). Fluid shear stress, on the other hand, enhanced the gene and protein expression of vascular endothelial cell markers such as FIK-1, Flt-1, vascular endothelial (VE)-cadherin and CD31 (PECAM-1) in mouse embryonic stem cells (Yamamoto *et al.* 2005). Stem cells were also shown to be sensitive to the stiffness of the extracellular matrix. In fact, the stiffness of extracellular elastic matrices can guide the differentiation of HMSCs, committing towards the neuronal cell lineage on soft matrices and towards the osteogenic cell lineage on hard matrices (Engler *et al.* 2006).

In this section, a variety of signalling molecules important for mechanotransduction in different cells, including stem cells, will be discussed. A special emphasis will be on the studies in which FRET imaging plays an important role.

3.1. Calcium

Calcium ion is one of the most important biological signals (Berridge *et al.* 2000; Kim *et al.* 2009). It has been proved to play an important role in mechanotransduction, e.g. shear stress-induced signal transduction (Yamamoto *et al.* 2000). In fact, shear stress can stimulate the Ca^{2+}-dependent synthesis of nitric oxide, prostacyclin and PGI_2 (Rubanyi *et al.* 1986; Falcone *et al.* 1993; Kuchan & Frangos 1994; Davies 1995). Most cells mobilize their Ca^{2+} signals via the Ca^{2+} entry across the plasma membrane and/or the Ca^{2+} traffic between cytoplasm and intracellular stores such as endoplasmic reticulum or sarcoplasmic reticulum (Wehrens *et al.* 2005; Oancea *et al.* 2006). Tsien's laboratory developed a useful FRET-based calcium biosensor (Palmer & Tsien 2006), which includes a mutant of calmodulin (CaM), a CaM-binding peptide and two FPs serving as a FRET pair. When Ca^{2+} binds to the docking sites in CaM, the bound CaM can wrap around the M13 domain to cause a decrease in the distance between the two FPs and hence an increase in the FRET efficiency (Palmer & Tsien

2006). We have also developed a calcium biosensor pairing ECFP with YPet, which provided a high dynamic range in monitoring intracellular calcium oscillations (Ouyang *et al.* 2008*b*; Kim *et al.* 2009). Using this improved FRET calcium biosensor, we found that lowering the substrate stiffness significantly inhibited both the magnitude and frequency of the cytoplasmic Ca^{2+} oscillation in human SMCs. This substrate stiffness effect is dependent on the Rho-associated kinase (ROCK) but not on cytoskeleton, indicating that RhoA and its downstream molecule ROCK may mediate the impact of substrate rigidity on Ca^{2+} oscillation (Kim *et al.* 2009).

3.2. Cytoskeleton

Cytoskeleton provides mechanical support and maintains the shape of the cell. For example, the actin filaments link cortical cytoskeleton and cell–cell junctions at the apical surface and matrix adhesions at the basal side of the cell via its actin network extending through the whole cell (Asparuhova *et al.* 2009). It has been well established that cytoskeleton also participates in mechanotransduction. When exposed to mechanical forces such as laminar fluid shear stress, cells will reorganize their actin cytoskeleton and focal adhesions, and subsequently align in the direction of flow (Katsumi *et al.* 2004; Tzima 2006). A series of signal transduction events have been shown to be involved in this mechanoadaptation. For example, PECAM-1 and VE-cadherin in conjunction with VEGF receptor-2 can stimulate the phosphatidylinositol-3-OH kinase upon shear stress application (Tzima *et al.* 2005; Chen & Tzima 2009), which can lead to the activation and ligand binding of integrins β3 and β1 on the basal surface of the cell (Tzima *et al.* 2001). Rac1 was also activated to promote the lamellipodia formation at the downstream end of the cell under flow (Tzima *et al.* 2002). A transient decrease in RhoA activity accompanied by actin depolymerization was observed in cells under flow, which was followed by a sustained activation of RhoA and a reappearance of stress fibres in the direction of flow (Tzima *et al.* 2001). With FRET technology, Na *et al.* (2008) further showed that the shear stress induced rapid activation of Src at remote cytoplasmic sites, which is dependent on the cytoskeletal prestress, with microtubules being the essential structure for transmitting stresses to activate Src. These results indicate that rapid signal transduction via the prestressed cytoskeleton is a unique feature of mechanotransduction (Na *et al.* 2008). Therefore, cytoskeleton can be considered as a kind of 'memory device' for the history of cellular mechanical stress, with its acute responses largely dependent on how the cell–matrix adhesions and cytoskeleton are organized as a consequence of previous mechanical stimulations (Asparuhova *et al.* 2009).

3.3. Src

Src is a critical signalling molecule that regulates cell migration and mechanotransduction. Src is also closely related to adhesion processes, including integrin signalling. When integrin is activated, it can associate with Src via the SH3 domain, thus causing the conformational change and activation of Src (Arias-Salgado *et al.* 2003; Huveneers *et al.* 2007). We previously developed a genetically encoded Src biosensor that enables the imaging and quantification of the spatio-temporal activation of Src in live cells. A rapid distal and a slower directional wave propagation of Src activation along the plasma membrane was observed when a laser-tweezer traction was applied on fibronectin (FN)-coated beads adhering on the top of human umbilical vein endothelial cells (HUVECs) (Wang *et al.* 2005) (figure 2). Using an improved cytosolic Src biosensor based on ECFP and YPet, Na *et al.* (2008) also demonstrated that Src can be directly activated via microtubule deformation upon mechanical loading. Activated integrins upon mechanical stimulation can also induce the autophosphorylation of focal adhesion kinase (FAK) at tyrosine 397 (Li *et al.* 1997; Wang *et al.* 2002), thus creating a binding site for the SH2 domain of Src, which in turn activates FAK by phosphorylating other tyrosine residues in FAK and creates additional protein-binding sites (Mitra & Schlaepfer 2006). The activated FAK and Src together can subsequently form a complex and stimulate Rac1 activity through the recruitment and phosphorylation of the scaffolding protein p130Cas (Chodniewicz & Klemke 2004). Finally, the activated Src can also affect the integrin–cytoskeleton interface through a selective and functional interaction between Src and the vitronectin receptor, e.g. integrin αvβ3, which ultimately regulates cell spreading and migration (Felsenfeld *et al.* 1999).

3.4. Rho, Rac and Cdc42

Rho, Rac and Cdc42 are closely related small GTP-binding proteins that play important roles in mechanotransduction. These small GTPases can regulate cell shape changes through the effects on cytoskeleton and cell adhesion. Wojciak-Stothard & Ridley (2003) showed that all these three GTPases can be activated rapidly under 3 dyn cm^{-2} of shear stress. RhoA was activated within 5 min of stimulation and returned to baseline afterwards. Rac1 and Cdc42 reached peak activation in 5 min, which would then sustain their activities for a long duration of time (Wojciak-Stothard & Ridley 2003). The shear-induced cell elongation was shown to require Rac1 and Cdc42, while Rho and Rac1 appeared important for the shear-guided migration (Wojciak-Stothard & Ridley 2003). To observe the spatial and temporal regulation of these signal molecules with high resolutions, Matsuda's group developed FRET-based Rac and Cdc42 biosensors, namely Raichu-Rac and Raichu-Cdc42, respectively (Itoh *et al.* 2002). Yoshizaki in Matsuda's group further generated a Raichu-RhoA FRET biosensor that can report RhoA activities (Yoshizaki *et al.* 2003). Independently, Kraynov and Pertz from Hahn's group also successfully developed Rac and RhoA biosensors (Kraynov *et al.* 2000; Pertz *et al.* 2006). With these FRET biosensors, Rac1 activities were found to be polarized upon flow application, activated at the leading edge of cells along the flow direction in

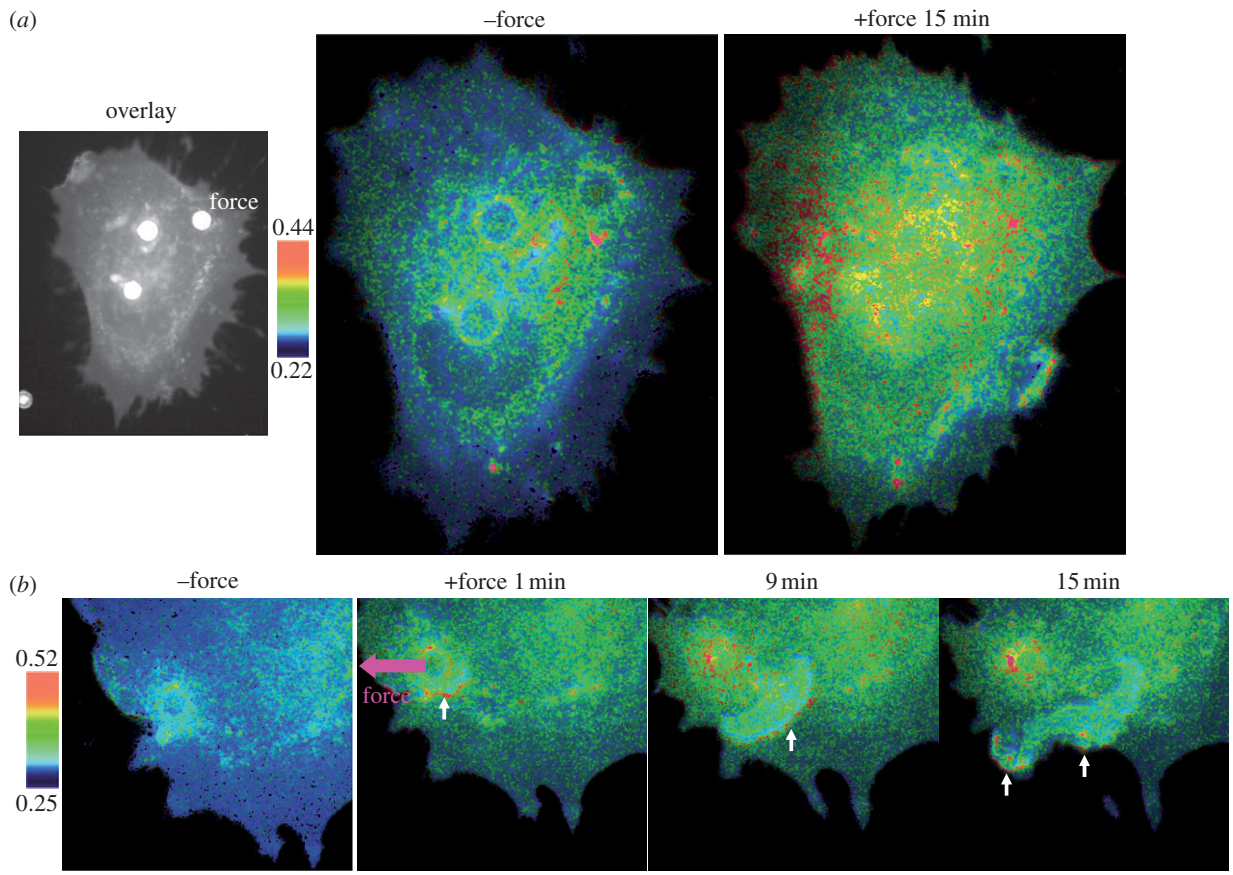

Figure 2. Directional and long-range propagation of Src activation induced by mechanical force. (*a*) Laser-tweezer traction on the bead at the upper right corner of the cell (shown on the left) caused FRET responses.The colour images on the right represent the FRET ratio of Src biosensor before and after the mechanical stimulation. (*b*) FRET responses of a cell with clear directional wave propagation away from the site of mechanical stimulation. Adapted from Wang *et al.* (2005).

BAECs (Kraynov *et al.* 2000; Tzima *et al.* 2002) or suppressed at the side facing the flow in pulmonary artery endothelial cells (PAECs) (Zaidel-Bar *et al.* 2005). Upon stretching, Rac1 and Cdc42 activities were shown to remain unchanged, whereas overall RhoA activity increased (Goldyn *et al.* 2009). Using a FRET Rac biosensor, Poh *et al.* (2009) showed that Rac can be activated rapidly and directly under mechanical twisting, independent of Src activation. These results suggest that Rho small GTPases can be visualized by FRET biosensors to reveal their coordinate responses in space and time upon mechanical stimuli.

4. THE INTEGRATION OF MECHANICAL LOADING SYSTEMS WITH LIVE CELL FRET IMAGING

Besides the FRET biosensors discussed in the previous sections, there are a variety of FRET biosensors available to visualize the activation of important signalling molecules, including FAK (Cai *et al.* 2008), cAMP (Iancu *et al.* 2008; Bagorda *et al.* 2009), phosphodiesterases (Herget *et al.* 2008), PKA (Allen & Zhang 2006), PKC (Brumbaugh *et al.* 2006) and MT1-MMP (Ouyang *et al.* 2008*a*). These biosensors can allow the convenient investigation of the spatio-temporal regulation of various signalling molecules in mechanotransduction. At the same time, there are a variety of mechanical loading systems available to exert different mechanical forces on cells. In this section, we will introduce these different mechanical loading systems, with the emphasis on the integration of these systems with the live cell imaging technology using the FRET biosensors.

4.1. Stretch

It is in general difficult to apply cyclic stretch on a live cell directly and monitor the fluorescence signals simultaneously because of the focus alteration during the stretching process. However, the still image can be obtained at the interval of stretch cycles. Goldyn *et al.* (2009) cultured NIH3T3 cells on FN-coated polydimethylsiloxane elastomeric membranes that were subjected to cyclic stretching and successfully monitored the FRET changes of the pRaichu-Rac, pRaichu-RhoA and pRaichu-Cdc42 biosensors. The results revealed that only the activity of RhoA, but not Rac or Cdc42, was altered by cyclic stretch (Goldyn *et al.* 2009).

Relatively local stretch can be conveniently applied on cells with laser/optical tweezers or magnetic tweezers. Optical tweezers use lights to manipulate microscopic objects. The radiation pressure from a focused laser beam can trap and exert mechanical forces on small particles in the piconewton (pN) range. We have applied beads coated with FN, which

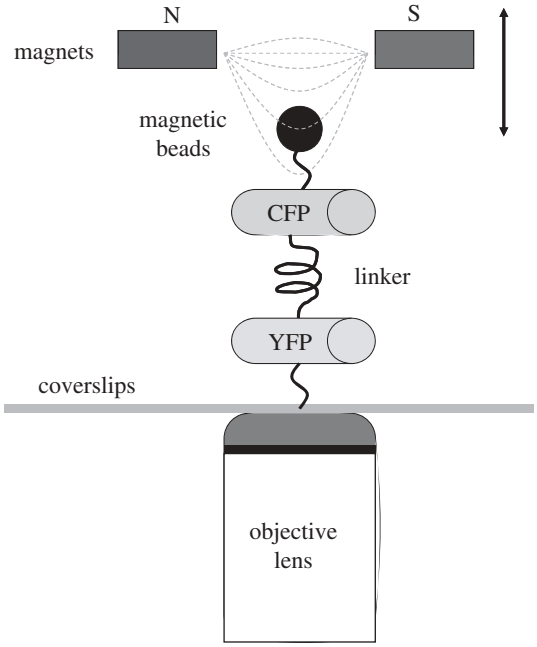

Figure 3. Magnetic tweezers and FRET imaging. Magnetic tweezers consist of a pair of permanent magnets placed above the sample holder of an inverted microscope. The FRET biosensor linked with a magnetized particle was coated onto a piece of glass coverslip. The magnets produce a strong field gradient that is used to exert a force on the magnetic beads. When subjected to the magnetic field, the internal magnetic moment of beads will tend to align with the external field, resulting in a rotational or twisting force on the FRET biosensor. The force can be varied by modulating the position of the magnets relative to the bead.

binds to integrins and hence causes coupling with cytoskeleton, to adhere on HUVECs. A single-beam optical laser-tweezer system was then used to pull the adhered beads. This pulling force on the HUVECs resulted in a directional FRET response of Src activity at the plasma membrane, with the majority of activations transmitted towards distal areas of the cell opposite to the force direction (Wang *et al.* 2005). Magnetic tweezers can also use a strong magnetic field gradient that is applied to exert a force on magnetic beads. The force can be changed by modulating the position of the magnets relative to the bead (figure 3). It can also allow the rotation of the magnetic bead so that the molecules/cells that tether on the bead can be conveniently twisted. Ning Wang's group has extensively applied magnetic twisting cytometry (MTC) to exert mechanical stress on cells. With the FRET technique integrated with MTC, mechanical force exerted at the membrane can be observed to transmit through the cytoskeleton to remote cytoplasmic sites (Na & Wang 2008). Shroff *et al.* (2005) have developed a nanoscopic force sensor consisting of a single-stranded DNA oligomer flanked by two dyes. The distance between the two dyes can increase when the DNA oligomer is stretched by magnetic tweezers, resulting in a reduced FRET. This sensor can detect twisting forces between 0 and 20 pN, allowing the possibility to measure internal forces of various materials, including DNA self-assemblies, polymer meshes and DNA-based machinery.

When larger forces in the micronewton range are needed to deform molecules such as extracellular matrix protein FN, magnetic and laser tweezers are no longer sufficient (Kellermayer *et al.* 1997; Tanase *et al.* 2007; Klotzsch *et al.* 2009). Little *et al.* (2008) developed a convenient method to stretch the FN fibres by at least five- to sixfold by depositing the FN fibres onto a silicone sheet, which can be stretched with a one-dimensional strain device. The FRET images of the fibres can be collected by an objective positioned beneath the silicone sheet to monitor the deformation of the FN fibres (Little *et al.* 2008). Klotzsch *et al.* (2009) from the same group have further developed an improved method to stretch the FN fibres. In this system, FN was labelled with Alexa Fluor 488 (donor) at random sites and with Alexa Fluor 546 (acceptor) on the specific cysteine sites located on FnIII7 and FnIII15. A force-sensing lever arm based on the silicon micro-electro-mechanical system technology was developed to exert and measure forces with micronewton resolutions. When the tip of the force sensor is inserted into the droplet of FN solution to suck the liquid and result in stretching the FN fibres, the FRET signals of the FN fibres can be concurrently imaged with confocal laser scanning microscopy. The results indicate that FN fibres can be extended more than eightfold before 50 per cent of the fibres break (Klotzsch *et al.* 2009). Meng *et al.* (2008) also designed a FRET biosensor, stFRET, with the Cerulean and Venus FRET pair connected by a stable alpha-helix structure. This biosensor can be inserted into structural protein hosts such as collagen-19, non-erythrocyte spectrin, α-actinin and filamin A within living cells. The FRET signals from this cassette can change in response to stress *in situ*, allowing the measurement of intracellular mechanical tension in real time (Meng *et al.* 2008).

4.2. Shear stress

Haemodynamic shear stress is a fundamental determinant of vascular remodelling and atherogenesis. Shear stress can be controlled precisely using a classic parallel flow chamber (Frangos *et al.* 1985). To allow the integration of a fluorescence microscope to observe the change of FRET biosensors on live cells, cells can be cultured on glass coverslips, which provide an optically accessible window for the chamber (Chachisvilis *et al.* 2006) (figure 4). With this kind of shear stress system integrated with fluorescence microscopy, Tzima *et al.* showed that Rac activation, visualized by a FRET biosensor, is concentrated at the subcellular locations in the direction of flow in BAECs. The Rac activation was further shown to regulate gene expression and the shear stress-induced cell alignment (Tzima *et al.* 2002). Another Rac FRET biosensor allowed the revelation that shear stress can decrease Rac activity at the side facing the flow in PAECs (Zaidel-Bar *et al.* 2005). Although the cell-type difference may have caused the discrepancy in these two reports, shear stress was clearly demonstrated to induce a polarized distribution of active Rac. Traoré *et al.* (2005) further studied the effect of shear stress on the internalization kinetics of the native LDL and ox-LDL in the endothelial cell

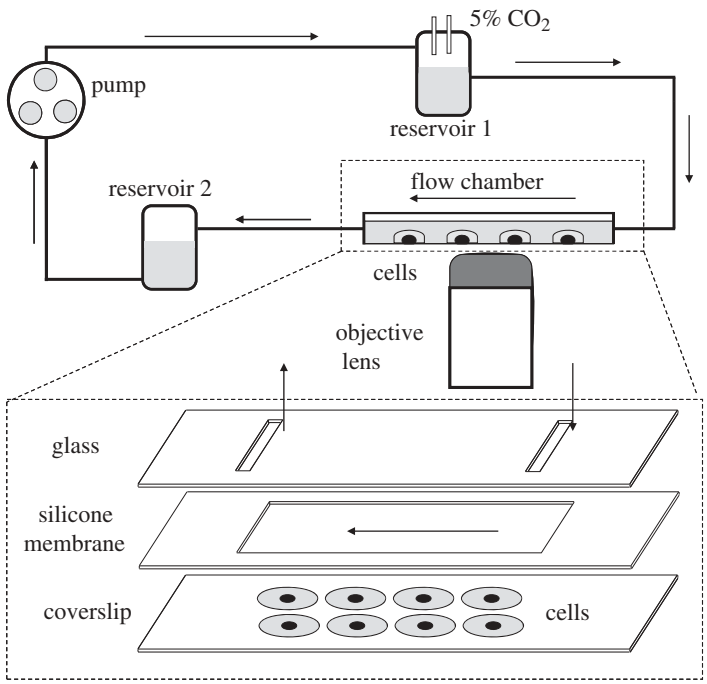

Figure 4. The flow system and FRET imaging. The system comprises a flow chamber to seed the cell, two reservoirs to form the hydrostatic pressure difference, a pump to circulate the flow medium and the tube system to connect all the compositions. A glass coverslip seeded with cells expressing FRET biosensors will form the floor of a flow channel, created by sandwiching a silicone gasket between the cover glass slide and an acrylic plate. Cells will be exposed to various shear stresses created by flows caused by a hydrostatic pressure difference between two reservoirs in the circulation system. The force of shear stress can be calculated as: $\tau_w = 6\mu Q/h^2 w$, where μ is the fluid viscosity of the solution, Q is the flow rate, h is the channel height and w is the channel width. Hence, by adjusting the flow rate or the channel height, different levels of wall shear stress can be generated to impose on the cell surface with a high precision. The FRET signals of biosensors can then be monitored by the objective to detect the cell response upon flow stimulation.

line ECV304 using confocal microscopy and FRET between two carbocyanine dyes, with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate as the donor and 3,3′-dioctadecyloxacarbocyanine perchlorate as the acceptor. Shear stress was shown to enhance LDL uptake and ox-LDL pre-incubation was found to affect the cell responses to shear stress stimulation (Traoré *et al.* 2005). By using time-resolved fluorescence microscopy and a conformation-sensitive FRET biosensor, Chachisvilis *et al.* (2006) showed that mechanical perturbation of the plasma membrane can lead to ligand-independent conformational transitions in a GPCR. Stimulation of endothelial cells with fluid shear stress, hypotonic stress or membrane fluidizing agents can all lead to a significant increase in the activity of bradykinin B2 GPCR, suggesting that GPCRs may be involved in mechanotransduction in endothelial cells (Chachisvilis *et al.* 2006). Zhang *et al.* (2009) developed a parathyroid hormone type 1 receptor (PTH1R) sensor based on FRET that enabled the detection of conformational change of PTH1R in single cells. The stimulation of murine pre-osteoblastic cells (MC3T3-E1) with fluid shear stress was observed to result in significant changes in conformational equilibrium of the PTH1R, suggesting that mechanical perturbation of the plasma membrane can cause ligand-independent responses of the PTH1R to mediate mechanotransduction in MC3T3-E1 cells (Zhang *et al.* 2009).

4.3. Substrate rigidity

Since the rigidity of the substrate where cells are seeded can affect the cellular force balance, substrate rigidity plays a crucial role in regulating cellular functions, such as cell spreading, traction forces and stem cell differentiation. Kong *et al.* demonstrated the power of FRET in analysing cell–material interactions. Hydrogels were applied to serve as the substrate of murine pre-osteoblasts (MC3T3-E1). The mechanical stiffness of the gels was adjusted by varying the molar ratio between the components of the hydrogels and calibrated by measuring the compressive elastic modulus (E) of the gels. The mechanics of cell–material interactions was evaluated by the FRET technique at the molecular level. The proliferation and differentiation of MC3T3-E1 was shown to correlate with the magnitude of force that cells generated. Therefore, the regulation of cellular functions by substrate stiffness is related to the alteration in cellular traction forces (Kong *et al.* 2005*b*). This rigidity of the cell adhesion substrate was also shown to affect gene transfer and expression (Kong *et al.* 2005*a*).

Recently, substrate stiffness and cellular tension have been substantially studied in terms of their effect on stem cell functions. Indeed, stem cells can differentiate differently on substrates with different stiffness (Engler *et al.* 2006). Mechanical loading was also shown to directly affect the differentiation commitment

of stem cells (Chowdhury *et al.* 2010). These results are also consistent with stem cells cultured on micropatterns where differential local mechanical tension can be generated at different patterned areas to guide the differential fate of the cells *in situ* (Ruiz & Chen 2008). With the method to manipulate the substrate rigidity, we also found that both the magnitudes and frequencies of spontaneous Ca^{2+} oscillations in HMSCs, observed by a highly sensitive calcium FRET biosensor, can be significantly inhibited if the substrate stiffness was decreased to 1 kPa. RhoA and its downstream molecule ROCK were further shown to mediate this substrate-rigidity-regulated Ca^{2+} oscillation, which may determine the physiological consequence of HMSCs (Kim *et al.* 2009).

5. CONCLUSIONS

FRET technology and genetically encoded molecular biosensors have provided powerful tools for the studies of mechanotransduction. Significant progress on the understanding of molecular mechanisms of mechanotransduction has been achieved with the application of these imaging tools. Because of the convenience in introducing the biosensors into cells and targeting them at subcellular compartments, these tools have particularly advanced our understanding of mechanotransduction with high spatio-temporal resolutions in live cells. At the current stage, the dynamic range of FRET biosensors is generally small. As such, it is difficult to apply these biosensors for the detection of subtle, but physiologically important, signals. Furthermore, the colours of biosensor fluorescence are limited, which will hinder the attempt to visualize multiple molecular signals in the same cell. With novel FPs increasingly being discovered and developed, it is expected that more improved FRET pairs will be available. As a result, FRET will play a more important role in promoting the advancement of the whole mechanobiology field.

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