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Bioengineering Tools for Probing Intracellular Events in T Lymphocytes

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Abstract

T lymphocytes are the central coordinator and executor of many immune functions. The activation and function of T lymphocytes are mediated through the engagement of cell surface receptors and regulated by a myriad of intracellular signaling network. Bioengineering tools, including imaging modalities and fluorescent probes, have been developed and employed to elucidate the cellular events throughout the functional lifespan of T cells. A better understanding of these events can broaden our knowledge in the immune systems biology, as well as accelerate the development of effective diagnostics and immunotherapies. Here we review the commonly used and recently developed techniques and probes for monitoring T lymphocyte intracellular events, following the order of intracellular events in T cells from activation, signaling, metabolism to apoptosis. The techniques introduced here can be broadly applied to other immune cells and cell systems.

1. INTRODUCTION

The immune system has evolved to protect the body against pathogens and to detect and/or remove internal abnormalities such as damaged cells and cancer. The proper functions of the immune system depend on collaborative groups of innate and adaptive immune cells, as well as intricate signaling networks within these cells. As a major component of the adaptive immunity, T lymphocytes are the central coordinator and executor of many immune defense mechanisms. Dysregulation of T lymphocytes can lead to a wide range of diseases including immunodeficiency, allergy, inflammatory diseases, and autoimmunity (Dornmair, Goebels, Weltzien, Wekerle, & Hohlfeld, 2003; Gouin, 2011; Noval Rivas & Chatila, 2016; Theofilopoulos, Dummer, & Kono, 2001; Wong & Huissoon, 2016). Conversely, immunotherapies that promote T cell functions through checkpoint inhibition (Postow,

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Conflict of Interest

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Callahan, & Wolchok, 2015) or directly use adoptively transferred/engineered T cells (Wang, Yin, Wang, & Wang, 2014) have shown great promises in treating multiple cancer types (Berraondo et al., 2017). Moreover, a deeper understanding of the immune system biology will be key to better manipulations of the immune system and T cell immunity for medical applications, such as vaccine development, immune cell biomanufacturing, and immunomodulation and personalized immunotherapies for cancer and autoimmune diseases (Crooke, Ovsyannikova, Poland, & Kennedy, 2019; Hollingsworth & Jansen, 2019; Rosenberg & Restifo, 2015; Rosenblum, Gratz, Paw, & Abbas, 2012; Ryba-Stanisławowska, Sakowska, Zieliński, Ławrynowicz, & Trzonkowski, 2019).

The time frame of the events in T lymphocytes spans from within seconds to days (Valitutti, Coombs, & Dupré, 2010), and the spatial scale spans from single cell level (8~10 μm) to molecular level, which poses challenges to techniques for capturing those events. The bioengineering tools, including molecular probes and advanced microscopy, have allowed investigators to interrogate intracellular dynamics of immune cells with exquisite temporal and spatial resolutions. Many of the milestone discoveries about T lymphocytes would not have been possible without these techniques. For example, double indirect immunofluorescence microscopy helped Kupfer et al. discovered the movement of microtubule organizing center (MTOC) toward immunological synapse (IS) (Kupfer & Dennert, 1984); the development of cell-permeable calcium sensors helped Tsien et al. to define the correlation between calcium level and T cell stimulation (Tsien, Pozzan, & Rink, 1982). High resolution microscopy and cellular probes allow researchers to visualize events in single cells, instead of behaviors of bulk population indicated by conventional biochemical methods. They also provide information on the dynamics subcellular organelles, such as the trafficking of lytic granules (Jenkins & Griffiths, 2010) and the morphology of mitochondria (Buck et al., 2016). Imaging-based probes such as fluorescence resonance energy transfer (FRET) biosensors have extended the resolution of conventional fluorescence microscopy to nanometer scale to reveal the dynamic interaction, clustering and state changes of signaling molecules (Chigaev et al., 2015; Y. Ma et al., 2017). Single molecule tracking has brought new insights into the nanoclusters during lymphocyte signaling dynamics (Paeon et al., 2016). Moreover, the advances of the new tools toward quantitative, real-time measurements of cellular events are further enabling mechanistic models of immune cell behaviors to inform more effective immunotherapeutic strategies (Makaryan, Cess, & Finley, 2020).

In this review, we will highlight the bioengineering tools that enables the studies of cellular events in T lymphocytes, with a focus on the intracellular probes that detect cell signaling. As the functions of T cells are regulated by many complex and intertwined biological activities, to provide a clear context of these tools, our review will follow the order of different intracellular events of a T lymphocyte from initial activation, cytoskeletal reorganization, signal transduction, to viability status (including metabolism and apoptosis). We will introduce and discuss the bioengineering tools that have been developed or have the potential to help understand these processes.

2. CYTOSKELETAL AND MECHANOBIOLOGICAL CHANGES

2.1 Immunological synapse formation

A key role of the T lymphocytes is to perform immune surveillance against pathogens, which is achieved through the migration and adhesion of T cells onto antigen presenting cells (APCs). Upon the recognition of antigenic peptide-MHC (pMHC) complex by T cell receptor (TCR), T lymphocytes stop migrating and form a cell-cell interface termed the immunological synapse (IS) (Dustin, 2002). A mature IS is characterized by multiple spatial regions of supramolecular activation clusters (SMACs), often in a bullseye shape (Kaizuka, Douglass, Varma, Dustin, & Vale, 2007)) (Figure 1a). The TCR/pMHC complexes (along with costimulatory molecules) form the central SMAC region (cSMAC) to mediate the central T cell activation signals, while the interactions of integrin receptor LFA-1 with adhesion ligand ICAM-1 form the peripheral SMAC (pSMAC) to promote T cell adhesion with APCs (Delon & Germain, 2000; Smith-Garvin, Koretzky, & Jordan, 2009), as well as to prevent the cytotoxic secretion from leaking to bystander cells (Stinchcombe, Majorovits, Bossi, Fuller, & Griffiths, 2006). The outmost region of the IS, referred to as the distal SMAC (dSMAC), is enriched for CD45 and F-actin and a site for initial engagement and formation of TCR microclusters (Dustin, 2008). Importantly, IS plays a crucial role in T cell activation and many downstream functions, including the directional functionalities of the T cells (e.g. targeted cell killing and cytokine secretion) (Germain, Robey, & Cahalan, 2012; Stinchcombe et al., 2006); it also gives rise to the memory and effector T cell populations through cell polarization and asymmetric division (Chang et al., 2007). It is thus vital to develop tools to elucidate the early cellular events regulating this dynamic process.

2.1.1 Supported lipid bilayers and cell-pair imaging for IS formation—The cell membrane-bound engagements of receptor-ligand pairs in the IS lead the first wave of intracellular events in T cell activation. The molecular composition and the functional role of the SMACs and the subsequent cytoskeletal reorganization were first described in the 1980s (Kupfer, 1987, 1989) and have since been extensively investigated. Earlier studies have focused on examining the molecular compositions and their spatial arrangements in the IS, through the fluorescently labeled cell surface receptors such as TCR, CD3, ICAM-1, LFA-1 etc. (Dustin et al., 1998; Grakoui et al., 1999; Monks, Freiberg, Kupfer, Sciaky, & Kupfer, 1998). However, it was difficult to track IS formation in real-time due to the low frequency of T cell/APC conjugates under normal settings, and the rapid nature of the IS formation process (on the order of seconds to minutes). The small size of the naïve T cells (<10 µm) also adds an additional layer of technical challenges to image them *in vivo*. Thus, several *in vitro* bioengineering techniques have been employed to image/track the very first steps of the IS formation.

One such technique involves creating an artificial APC cell membrane, by using the supported lipid bilayers (SLBs) tethered with pMHC and other accessory molecules, often fluorescently labeled, to initiate IS formation by the seeded T cells (Figure 1b). The general form of lipid bilayers consists of a substrate (e.g. mica or silica) and a phospholipid bilayer supported by it, with a water layer in between. Coupled with live microscopic imaging, the artificial cell membrane has helped reveal the spatiotemporal sequence of events that take

place in T cells upon engagement with APCs (Choudhuri et al., 2014; Dustin et al., 1996; Grakoui et al., 1999; Smoligovets, Smith, Wu, Petit, & Groves, 2012). Notably, a variety of spatial patterns, architectural designs and materials has been applied to the SLB substrate, to modulate the spatial, topographical, mechanical and chemical properties of the lipid bilayer. Using micro- and nano-fabrication techniques such as photolithography and electron-beam lithography, researchers have investigated the spatial sorting of microclusters in the IS by creating interconnected or isolated spatial regions of lipid bilayers with uniform or distinct molecular contents (DeMond, Mossman, Starr, Dustin, & Groves, 2008; K. D. Mossman, Campi, Groves, & Dustin, 2005; Shen, Tsai, Shi, & Kam, 2009). More recently, patterned SLBs have been used to confine the lateral movement of TCRs to probe the mechanical coupling between TCR and T cell cytoskeleton (Y. Yu, Fay, Smoligovets, Wu, & Groves, 2012), to analyze the colocalization of TCR with microvesicles during their polarized release in the IS (Choudhuri et al., 2014), and to determine the spatial thresholds of the lateral and axial distances between ligands in the IS for TCR triggering (Cai et al., 2018). In addition to the conventional silica or mica substrate, a broad range of materials has also been explored as substrates for SLBs, including graphene (Ang et al., 2010), titanium oxide, aluminum oxide (Tabaei, Vafaei, & Cho, 2015), etc. Polymer cushions such as polyethylene glycol (PEG), poly-L lactic acid (PLLA) and agarose (Tanaka & Sackmann, 2005), have also been used especially for functional recapitulation of transmembrane proteins (Wagner & Tamm, 2000). Additionally, these cushioned SLB substrates also allow for more realistic recapitulation of the mechanical properties of cell membranes and membrane proteins (Glazier & Salaita, 2017), which has been increasingly recognized as a key player in T cell activation (covered in 2.1.2).

These SLB-based reductionist approaches are ideal for mechanistic studies through the highly controllable features, e.g. the densities and types of the involved molecules, spatial constraints (K. D. Mossman et al., 2005), and cluster sizes (DeMond et al., 2008; Hartman, Nye, & Groves, 2009; K. Mossman & Groves, 2007). Yet, they are limited in dealing with the heterogeneity of the real IS formation, where different APC subsets could lead to different kinetics of T cell activation (Oelke et al., 2003). Therefore, there has been a resurgence of the efforts on resolving IS formation in actual T cell/APC pairs. One of the enabling techniques is the laser tweezer, which has the advantage of adjusting cell pairs into almost any arbitrary plane of interest, thus allowing high-resolution imaging of IS in the horizontal XY-plane (A. C. N. Brown et al., 2011; Oddos et al., 2008) (Figure 1c). Other promising single-cell handling techniques alternative to the laser tweezer include magnetic and acoustic tweezers. Magnetic tweezers utilize externally controlled (electro)magnets to apply mechanical forces on ferromagnetic particles, which has a higher force exertion range than the laser tweezer (Swaminathan & Waterman, 2016). Acoustic tweezers, on the other hand, can manipulate single cells through direct acoustic radiation force or indirect induced fluid flows created by the ultrasound waves (Ozcelik et al., 2018). Both magnetic tweezers and acoustic tweezers are yet to be applied in IS study.

A limitation of using laser tweezers for IS formation is it has very low throughput, and the prolonged exposure of the live cells to the trapping laser beam could lead to phototoxicity. This has been well addressed by another recent technique, the imaging flow cytometry, which has benefitted from the vast improvement of imaging and data acquisition speed

in recent years (Barteneva, Fasler-Kan, & Vorobjev, 2012; Han, Gu, Zhang, & Lo, 2016). This technique can be used for high-throughput analysis of T cell-APC pairs and does not require all cell-cell conjugates to be positioned at certain angles. Instead, nuclear aspect ratios can be used as a criterion to select the “right” cell-cell conjugates from a vast dataset, where cell doublets are often recognized by the aspect ratio width : length ≤ 0.5 due to the distinct longer morphology of the cell pairs (Ahmed, Friend, George, Barteneva, & Lieberman, 2009). Another strategy is to measure the intensity of the interacting mask during imaging flow cytometry, which has been used to quantify dendritic cell-T cell immunological synapse formation (Markey, Gartlan, Kuns, MacDonald, & Hill, 2015).

2.1.2 Force spectroscopy for mechanical forces/signaling in IS—The IS formation requires extensive cytoskeletal remodeling that underlies the formation of membrane microclusters and their centripetal movement within the IS (Ilani, Vasiliver-Shamis, Vardhana, Bretscher, & Dustin, 2009; Kaizuka et al., 2007; Varma, Campi, Yokosuka, Saito, & Dustin, 2006), which implies an involvement of mechanical forces and/or mechano-signaling. A significant challenge in measuring forces in T cells is their small sizes (around 8~10 μm), where the conventional traction force microscopy (TFM) techniques with gel-embedded beads are difficult to apply (Munevar, Wang, & Dembo, 2001; Style et al., 2014). Instead, Bashour et al. used an elastomer micropillar array (Tan et al., 2003) with single micrometer size/spacing to measure the dynamic force generated by T cells, where they discovered that CD28 augments the traction force associated with CD3 (Bashour et al., 2014). Researchers have also utilized optical tweezers to manipulate antibody or pMHC-coated beads on T cell membrane, to apply mechanical forces during TCR/pMHC interactions to study the force required for T cell activation. It was found that microbeads coated with a non-agonist antibody to TCR/CD3 can trigger calcium flux with a tangential force of 50 pN, but not without force application, suggesting TCR as an anisotropic mechanosensor (S. T. Kim et al., 2009). The atomic force microscopy (AFM) is another technique that can measure the force exerted at the single molecular level, e.g. the binding force between TCR and pMHC (Hosseini et al., 2009; Lim, Mortellaro, Lim, Hammerling, & Ricciardi-Castagnoli, 2011; Puech et al., 2011). In AFM, the interaction force between the tip of an elastic, microfabricated cantilever and the sample is measured through the deflection of the cantilever, which can sense mechanical forces down to pN range (Figure 1d). Interestingly, researchers have taken advantage of the high controllability of AFM tips and the rapid feedback through optical measurement to apply different force loads to trigger T cell activation. For instance, Hu & Butte coated an AFM cantilever tip with anti-CD3 to stimulate T cells while simultaneously imaging T cell response through calcium flux (K. H. Hu & Butte, 2016). They found that inhibiting actin cytoskeleton abrogates TCR-triggered calcium flux, which can be rescued by cyclic force applied to the engaged TCR through the sinusoidal movement of the AFM tip, thus revealing a crucial role of mechanical force in TCR mediated T cell activation.

Another molecular force spectroscopy, biomembrane force probe (BFP) (Evans, Ritchie, & Merkel, 1995) has been applied to measure the mechanical regulation of receptor-ligand interactions in the IS formation (J. Huang et al., 2010). In this technique, a micromanipulator/micropipette holds an ultrasensitive transducer (e.g. a red blood cell),

which is attached to a microbead coated with pMHC. An axial movement of the micropipette allows for attachment or detachment of the microbead on/from a T cell held by another micromanipulator/micropipette. The pMHC/TCR engagement can be detected from the deformation of the transducer (when used without bead) or the displacement of the bead during the detachment (Figure 1e). Liu et al. followed up on this work by developing a newly modified fluorescence biomembrane force probe (fBFP) technology to quantitatively investigate the strength of the TCR/pMHC bond in the presence of external forces and determined the optimal force (including magnitude and duration) of the TCR-pMHC catch bonds for triggering effective T cell signaling (B. Liu, Chen, Brian, & Zhu, 2014). Using a human red blood cell as the sensitive force sensor, a high-speed camera and real-time imaging tracking techniques, the fBFP can achieve force, spatial and temporal resolutions of ~ 1 pN, ~ 3 nm and ~ 0.5 ms, respectively (Y. Chen et al., 2015). Ju et al. further developed a method termed dual biomembrane force probe (dBFP), to reveal a crosstalk between chemokine receptors and antigen receptors, which is crucial to the formation of the IS (Ju et al., 2017). Importantly, dBFP enables the investigation of the cooperative binding of multiple receptor-ligand species located in the proximal or separate regions.

2.1.3 Optical microscopy-based methods—Apart from the force spectroscopy, optical microscopic techniques are central to the studies of the T cell/APC interactions. Fluorescence recovery after photobleaching (FRAP) has been widely used to measure lateral diffusion of membrane proteins on the cell surface, and thus an ideal approach to study the binding kinetics in the IS. During the photobleaching phase of the FRAP, a small spot of strong laser pulse is often exerted on the cell surface that contains the fluorescently labeled proteins. This creates a gradient of photobleached vs. unbleached molecules between the exposed spot and the nearby unexposed membrane regions. By examining the recovery of the fluorescence in the bleached spot through mathematical models, we can determine the two-dimensional diffusion coefficient or the equilibrium dissociation constant of the labeled ligands or receptors of interest (Tolentino et al., 2008). For instance, Dustin et al. used this method to measure the dissociation constant of CD2 and LFA-3 (Dustin, 1996; Dustin et al., 1997). One problem with FRAP is that its precision relies on the irreversible bleach of fluorophores, however, some of the bleached fluorophores could regain fluorescence, which results in an overestimation of the diffusion coefficient. Choosing appropriate fluorophores with minimal reversible photobleaching is thus crucial for FRAP experiments (Mueller, Morisaki, Mazza, & James, 2012; Snapp, Altan, & Lippincott-Schwartz, 2003). Another concern is that cells are inevitably heated by the laser pulses. Two-photon FRAP allows photobleaching confined to a refined depth, which enables the measurement of 3D resolved diffusion coefficients (E. B. Brown, Wu, Zipfel, & Webb, 1999; Waharte, Brown, Coscoy, Coudrier, & Amblard, 2005). FRAP has also been used to illustrate the dynamics of cytoskeletal turnover in IS formation (Fritzsche et al., 2017; Fritzsche, Lewalle, Duke, Kruse, & Charras, 2013; Malinova et al., 2016). On the other hand, photoactivation is a common way to achieve precise spatiotemporal control of molecule activation. Photoactivatable molecules are turned on only in the illuminated region, which can be used to trigger intracellular events at specific sites in specific cells. Upon UV irradiation, photoactivatable moth cytochrome c (MCC) can trigger TCR activation when presented by MHC. It has been used to study the spatiotemporal dynamics of cytoskeletal

polarization (Chauveau, Le Floc'H, Bantilan, Koretzky, & Huse, 2014; Huse et al., 2007; Quann, Merino, Furuta, & Huse, 2009) and TCR signaling events (Huse et al., 2007).

Total internal reflection (TIRF) microscopy (Axelrod, 1981) has been widely used to study the IS formation. Under TIRF, the bottom of a T cell in touch with a ligand-bound substrate is illuminated by an exponentially decaying evanescent field caused by the total reflection of an incident laser light, which excites fluorophores within a ~100 nm thickness near the membrane-substrate interface (Figure 2a). This greatly reduces the background fluorescence noise as well as the phototoxicity to live cells due to the minimal exposure of the cell outside of the region of fluorescent excitation. TIRF is compatible with high-speed super-resolution imaging, and has been used in the studies of TCR and BCR microcluster dynamics (Beemiller, Jacobelli, & Krummel, 2012; Sohn, Tolar, & Pierce, 2008; Varma et al., 2006; Yi, Balagopalan, Nguyen, McIntire, & Samelson, 2019), recruitment and phosphorylation of signaling kinases (Owen et al., 2010; Sohn et al., 2008; Varma et al., 2006; Williamson et al., 2011) and IS proximal cytoskeleton dynamics (Beemiller et al., 2012; Smoligovets et al., 2012). To accommodate the study of single molecule dynamics, TIRF has further been coupled with fluorescence (or Förster) resonance energy transfer (FRET) to image TCR binding in situ and derived the dissociation and association rates of TCR-pMHC complexes (Huppa et al., 2010). The working principle of FRET will be introduced in 3.2.

Fluorescence lifetime imaging microscopy (FLIM) measures the fluorescence lifetime (τ) of fluorescent analytes (including fluorophores, FRET sensors and some endogenous autofluorescent molecules in cells), i.e. the average time that the fluorophore stays in its excited state. Unlike fluorescence intensity-based imaging, FLIM is less disturbed by the concentration of fluorescent probes, which minimizes artifacts and provides reproducible quantitative measurements over time (Sarder, Maji, & Achilefu, 2015). The fluorescence lifetime is also sensitive to the molecular state and environment of the fluorescent molecule (e.g. conformation, enzyme binding status, temperature, pH, etc.). These features make FLIM based biosensors (which will be covered in 3.3) ideal for long-term monitoring of intracellular events and the effect of environmental parameters.

The rapid development in super-resolution microscopy in recent years has been driving the nanoscale revelation of the spatial organizations of receptors and signaling proteins, as well as the observation of granule secretion within the IS. Single-molecule localization microscopy, including the photo activated localization microscopy (PALM) (Betzig et al., 2006; James et al., 2007; Shroff, Galbraith, Galbraith, & Betzig, 2008) and the stochastic optical reconstruction microscopy (STORM) (Bálint et al., 2020; Persson, Potrzebowski, Potrzebowska, & Svensson, 2018; Rust, Bates, & Zhuang, 2006), could reach a resolution of 10~20 nm in the XY-plane (Ashdown et al., 2017) (Figure 2b). Using a high-speed version of the PALM and photoswitchable fluorophores, Lillemeier et al. found that TCR and LAT (linker for activation of T cells), a transmembrane protein phosphorylated by TCR activation to recruit downstream signaling molecules, cluster into separate protein islands on the membrane. They also found that the respective protein islands have minimal contact with each other when T cells are quiescent, but form concatemers after T cell activation (Lillemeier et al., 2010). Others have imaged IS with structured illumination (SIM) (Figure 2c) and stimulated emission depletion (STED) (Figure 2d) (A. C. N. Brown

et al., 2011; Rak, Mace, Banerjee, Svitkina, & Orange, 2011). Li et al. combined SIM with TIRF using an ultra-high numerical aperture system, which achieves sub-100-nm resolution while maintaining the fast capture speed of SIM, to examine dynamics of cytoskeletal and cytoskeleton-associated proteins such as myosin IIA, paxillin, and vinculin (D. Li et al., 2015). Colin-York et al. revealed an antigen-dependent regulation of actin flow velocity with TFM, FRAP, and TIRF-SIM (Colin-York et al., 2019). Notably, genetically encoded fluorescent proteins (Williamson et al., 2011), synthetic dyes (Ashdown et al., 2017; Essig et al., 2017; Wieneke, Raulf, Kollmannsperger, Heilemann, & Tampé, 2015) and quantum dots (Xu, Tehrani, & Kner, 2015) have been used as probes for super-resolution microscopy. There are several key factors to consider in designing or selecting probes for super-resolution microscopy, which include the labeling specificity and efficiency as well as the brightness and the size of the probes (Galbraith & Galbraith, 2011; Hell et al., 2015; Kozma & Kele, 2019). For single-molecule super-resolution microscopy, a high brightness is also crucial as the probe must emit enough photons to be detected before being photo-bleached (Patterson, Davidson, Manley, & Lippincott-Schwartz, 2010).

2.2 Probes for cytoskeletal changes and vesicle trafficking

The reorganization of actin cytoskeleton is required for the migration, activation and effector functions of lymphocytes. The cytoskeletal architecture of a T cell changes drastically upon its activation (Balagopalan, Sherman, Barr, & Samelson, 2011). Apart from its role in cell adhesion and IS maturation, cytoskeletal reorganization provides a dynamic framework for molecular movement or phosphorylation (Beemiller & Krummel, 2013; Varma et al., 2006) and mechano-signaling transduction (Hivroz & Saitakis, 2016). Cytoskeleton further facilitates the vesicle trafficking. It has been shown that cytotoxic T lymphocytes (CTLs) release cytokines and cytolytic lysosomes at the IS they form with the target cell to kill the latter (Jenkins & Griffiths, 2010). This polarized secretion is orchestrated through cytoskeletal rearrangement and repositioning of the microtubule-organizing center (MTOC) toward the IS, which causes the reorientation of vesicle compartments (Huse, 2012) (Figure 1a). There has thus been significant effort on probing these dynamic processes.

2.2.1 Cytoskeletal probes and imaging—Several actin and microtubule probes have been developed for cytoskeletal studies in T cells, which are visualized through TIRF and super-resolution microscopy. Fluorophore conjugated phalloidin, a highly selective bicyclic peptide to actin, is commonly used for F-actin staining in fixed or permeabilized cells, which cannot penetrate live cell membrane (Banerjee & Orange, 2010; Sustar, Vainio, & Mattila, 2018). Instead, the dynamics of actin and microtubule can be tracked through microinjection of phalloidin (Schmit & Lambert, 1990) or fluorescently labeled actin or tubulin (Waterman-Storer, Desai, Chloe Bulinski, & Salmon, 1998) into live cells (Chazotte, 2010). Lately, cell membrane-permeable actin and tubulin tracers, such as SiR-Actin and SiR-Tubulin (from Cytoskeleton Inc.), has been introduced to the market (Melak, Plessner, & Grosse, 2017; Yadav et al., 2019), which can potentially be used to probe T cell cytoskeleton.

The green fluorescence protein (GFP)-actin fusion proteins have become widely adopted in live cell cytoskeletal imaging (Melak et al., 2017). Kaizuka et al. transfected GFP-actin into Jurkat T cells to first visualized that the TCR and adhesion microclusters formed at the

periphery of IS are carried by a retrograde flow of actin toward the center of the IS (Kaizuka et al., 2007). To avoid the interference of the full-size GFP-actin fusion protein on the actin dynamics, researchers have alternatively developed fusions of GFP to actin-binding domains (ABDs), such as GFP-moesin (Edwards, Demsky, Montague, Weymouth, & Kiehart, 1997), GFP-LimE (Bretschneider et al., 2004), and GFP-ABD (also known as ABP-120) (Pang, Lee, & Knecht, 1998). Notably, Riedl et al. created LifeAct, a shorter F-actin-binding peptide fused to GFP (Riedl et al., 2008), to track T cell activation and morphology both *in vitro* and *in vivo* (Galeano Niño et al., 2020). Myosin is a large family of actin-associated, ATP-powered motor proteins, of which non-muscle myosin IIA is a predominant isoform in T cells. Myosin IIA generates tension in the actin cytoskeleton by crosslinking filaments and sliding upon each another. Thus, the strain between myosin and actin has been a topic of interest. Strain sensors based on proximity imaging (PRIM) (Iwai & Uyeda, 2008) and FRET (Hart et al., 2019; Muretta, Rohde, Johnsrud, Cornea, & Thomas, 2015) techniques have been developed to interrogate this question, which is yet to be adopted in T cell studies.

2.2.2 Vesicle trafficking—Upon the formation of IS, secretory vesicles move toward MTOC. In the later stages of T cell activation, MTOC is reoriented toward IS, guiding the polarized secretion (Figure 1a). TIRF and super-resolution microscopy techniques have been used to study the granule trafficking at IS (Ritter et al., 2017; Sleiman, Stevens, & Rettig, 2017). Liu et al. used TIRF to image the lytic granule degranulation events in natural killer cells on lipid bilayers containing ligand to LFA-1, 2B4 and NKG2D, and found that the granule secretion of natural killer cells is confined to a central region (D. Liu et al., 2009). There are multiple strategies to probe the movement of lytic granules. Some researchers have labeled granzyme A, granzyme B and serglycin with fluorescent tags to trace the lytic granules (Martina et al., 2011). Others have fluorescently tagged LAMP-1 and VAMP-1 to follow the fate of the lytic granule membrane (Chitirala et al., 2019; Emily M. Mace et al., 2012). In addition, the pH-sensitive LysoTrackers have also been used to directly stain lytic granules in immune cells (Emily M. Mace et al., 2012; Steen, Kirchberger, & Guse, 2007).

3. SIGNALING EVENTS

Within seconds of IS formation, TCR initiates a cascade of tyrosine phosphorylation events, which activate multiple downstream signaling pathways that regulate the specific functional outcomes of T cells (Gaud, Lesourne, & Love, 2018; Thaventhiran, 2013). It is thus crucial to interrogate these early signaling events.

3.1 Fluorescence intensity-based probes

The fluorescence intensity-based probes are among the earliest developed biosensors for intracellular signaling (Figure 3a). Both synthetic dyes and fluorescent proteins have been used along with the modern high-resolution optical microscopy technologies. For example, using GFP-tagged signaling molecules and TIRF, Douglass et al. tracked the trajectory of single signaling molecules at a resolution of 30 frames/second to study their heterogeneous diffusion behavior and observed mobility changes upon TCR signaling. With that, they deduced that the protein-protein interactions on T cell membrane creates microdomains that exclude or trap signaling molecules to facilitate signal transduction (Douglass & Vale,

2005). However, it has not been very reliable to determine the inter-molecular interactions or kinase activities at the single-molecule level using colocalization analysis of the intensity-based probes.

3.2 Fluorescence (or Förster) resonance energy transfer (FRET) and FRET sensors

Fluorescence (or Förster) resonance energy transfer (FRET) is a technique for studying molecular interactions, protein structural dynamics, and protein binding kinetics by measuring the distance between two fluorophores. A FRET sensor typically consists of a pair of donor and acceptor fluorophores. When the donor and the acceptor are close to each other (<10 nm), the energy absorbed by the donor from a photon at the excitation wavelength is transferred non-radiatively to the acceptor, the latter of which then emits a photon at a longer emission wavelength than that of the donor. The efficiency of this energy transfer (FRET efficiency) is dependent on and highly sensitive to the distance between the donor and the acceptor, typically in the range of tens to hundreds of nanometers. The donor-acceptor pairs in FRET can be small organic dyes, quantum dots, and fluorescent proteins. In live cell imaging, the fluorescent protein pairs are the most commonly used. They offer high spatiotemporal details of the spatial proximity of signaling molecules in living cells with minimal invasiveness and are more reliable than the conventional fluorescent intensity-based probes.

A FRET sensor usually consists of a donor-acceptor pair connected by a sensing domain which can be specifically catalyzed by the analyte (Figure 3b). There are also intermolecular FRET pairs where the donor and acceptor are separated in two molecules, each conjugated with one part of the sensing domain. The first FRET biosensor was a calcium indicator named Cameleon constructed by Roger Tsien group (Miyawaki, Griesbeck, Heim, & Tsien, 1999; Miyawaki et al., 1997). In Cameleon, a calmodulin (CaM) and a CaM-binding M13 domain are placed between two GFP-variant proteins (e.g. a cyan variant ECFP and a yellow variant EYFP). Under increasing levels of intracellular calcium, the M13 domain binds to CaM and causes a conformational change to Cameleon that brings ECFP and EYFP close to each other. This enhances the FRET efficiency between the two fluorescent proteins, which can be measured by the intensity ratio (I_{EYFP}/I_{ECFP}) to calculate calcium concentration. Cameleon has been utilized to measure the calcium flux in naïve T cells in different phases of activation (Le Borgne et al., 2016). Besides calcium imaging, FRET biosensors using other molecular pairs have also been broadly applied to detecting signaling events and molecular interaction upon T cell activation, for instance, TCR-pMHC binding kinetics (Huppa et al., 2010), protein-protein interactions (Zal & Gascoigne, 2004), and post-transcriptional modification such as tyrosine phosphorylation (Randriamampita et al., 2008a).

Notably, there are continuing efforts on improving the design of FRET sensors for intracellular sensing. Researchers have focused on enhancing the specificity of the sensing domains to the analyte over other factors or similar molecules (Miyawaki et al., 1999). Others have optimized the distance between FRET pairs by adjusting the linker of the sensor constructs (Komatsu et al., 2011). Another strategy is to increase the ratio changes in the emission and improve signal-to-noise ratio of the FRET sensors by optimizing the choice

of fluorophores on both donor and acceptor sides of the construct. For example, different pairs of fluorescent proteins have been developed, including CFP/YFP and mOrange/mCherry, mTFP1/mCitrine and mAmetrine/tdTomato, and mTagBFP/sfGFP and mVenus/mKok (Hochreiter, Pardo-Garcia, & Schmid, 2015; Mochizuki et al., 2001). Researchers have further engineered fluorophore pairs specifically for FRET. For instance, CyPet-YPet is a FRET pair with larger FRET span, which was obtained through evolutionary optimization based on the parent pair of CFP-YFP (Nguyen & Daugherty, 2005).

Interestingly, FRET sensors have been designed to detect intracellular events at specific sites. Wu et al. designed a FRET-based biosensor targeting the focal adhesion complexes during cancer cell adhesion. The sensor contains a targeting domain for the focal adhesion and a Src-specific substrate sequence that can be phosphorylated by active Src kinase, which subsequently increase the emission ratio of the FRET pair. The researchers seeded cancer cells expressing this biosensor on fibronectin-coated cover glass to allow cell adhesion and spreading, and found a spatiotemporal correlation between the kinase activations and focal adhesion assembly (Wu et al., 2016). Optogenetics has also been utilized to control the localization of FRET biosensors in cells. Huang et al. developed a light-induced FRET-based biosensor for selective localization/activation of the sensor in the nucleus or the cytosol, to study the different activities of Fyn kinase in these regions (Z. Huang, Ouyang, Lu, Wang, & Peng, 2020). Bohineust et al. co-expressed photoactivatable actuators and FRET-based calcium reporter to achieve simultaneous manipulation and visualization of calcium signaling in single T cells in vivo (Bohineust, Garcia, Corre, Lemaître, & Bousso, 2020).

3.3 Fluorescence lifetime-based sensors

The limitations of the fluorescence intensity-based probes and FRET biosensors include their dependence on the concentration of the fluorophores, the interference from photobleaching, and the lack of available probes for some specific biological events. Those limitations can be overcome with FLIM sensors (Figure 3c).

FLIM-based probes include both exogenous probes and endogenous autofluorescent molecules. For instance, Damayanti et al. developed a FLIM biosensor to visualize the activity of Abl kinase in live cells. The biosensor consists of an Abl substrate peptide tagged with a Cy5 fluorophore and a cell-penetrating peptide. The substrate is relatively selective for the c-Abl kinase (Abl1) over other tyrosine kinases and the fluorescence life time shifts of the sensor is phosphorylation-dependent (Damayanti, Parker, & Irudayaraj, 2013). The same group also developed an Akt-targeting FLIM biosensor, the fluorescence decay of which is prolonged upon Akt phosphorylation (Damayanti et al., 2017). On the other hand, FLIM studies on the endogenous autofluorescent molecules, such as nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) and flavin adenine dinucleotide (FAD), have been used to generate unique insights into cellular metabolism in a label-free, non-invasive, and real-time manner (L.-C. Chen et al., 2014; Zhou et al., 2020).

Importantly, FLIM can also be used to measure FRET in a quantitative and precise way, as the fluorescence lifetime of a FRET donor becomes shorter when/where FRET occurs. Notably, FLIM-FRET probes require a different optimization strategy in design from regular FRET probes. It was found that FLIM detection benefits more from the higher quantum

yield of the donor than that of the acceptor (J. B. Klarenbeek, Goedhart, Hink, Gadella, & Jalink, 2011), and that the red-shifted FRET variants are better suited for FLIM detection (J. B. Klarenbeek et al., 2011; Van Der Krogt, Ogink, Ponsioen, & Jalink, 2008).

3.4 Fluorescent biosensors in T cell signaling

3.4.1 Protein-protein interactions and clustering—The formation of protein complexes and clusters plays a crucial role in T cell signaling. One of the key interactions during the recruitment of T lymphocytes to the site of inflammation is the binding of an integrin, VLA-4 (very late antigen-4, an $\alpha 4\beta 1$ integrin) to its endothelial ligand VCAM-1 (vascular cell adhesion molecule 1). Hyun and colleagues developed an elegant FRET sensor in which they labeled the cytoplasmic regions of the $\alpha 4$ and $\beta 1$ subunits of VLA-4 with monomeric cyan and yellow fluorescent protein (mCFP and mYFP), respectively. With this tool, they were able to visualize activated VLA-4 at the lamellipodia of T cells migrating on VCAM-1, and showed a correlation between the activation of the integrin with a dynamic separation of the FRET donor and acceptor (Hyun, Chung, McGrath, Waugh, & Kim, 2009). The density of TCR-CD3 complexes in the IS reflect the signaling state of the T cells upon activation. Ma et al. designed a Venus-mCherry FRET biosensor (called CliF) that can form not only intramolecular FRET in monomers but also intermolecular FRET in protein clusters, which demonstrate distinct fluorescence lifetime values measured by FLIM. They attached the sensor to CD3 ζ , and detected the formation, movement, and dynamics of TCR-CD3 clusters in IS upon T cell activation (Figure 4a) (Y. Ma et al., 2017). On the other hand, TCR heterodimers associate constitutively with multiple CD3 proteins in the T cell membrane for signal transduction. The CD3 subunits in the complex contain immunoreceptor tyrosine-based activation motifs (ITAMs), the phosphorylation of which leads to TCR downstream signaling. To monitor the phosphorylation of CD3 signal-transducing subunit ζ , Yudushkin et al. linked EGFP and mCherry as a FRET pair and added a tandem SH2 domain of human ZAP-70 (zeta-chain-associated protein kinase 70) to bind to tyrosine-phosphorylated ITAMs of CD3 ζ . This causes conformational rearrangement of the adjacent fluorescent proteins and a change in the FRET efficiency, which can be measured through the fluorescence lifetime of EGFP in FLIM (Figure 4b). With this ZIP (CD3 ζ ITAM phosphorylation) biosensor, they observed an activation-dependent colocalization of internalized CD3 ζ within lysosomes (Yudushkin & Vale, 2010).

3.4.2 GTPases—GTPases are molecular switches that are active when bound to GTP and become inactivated when bound to GDP. The activation and inactivation of GTPases are regulated by guanine nucleotide exchange factors (GEFs) and GTP activating proteins (GAPs), respectively. Small GTPases, such as Ras (Genot & Cantrell, 2000; Malumbres & Barbacid, 2003), Rap1 (Kinashi & Katagiri, 2004), RhoA (Bros, Haas, Moll, & Grabbe, 2019) and Rab5 (Prashar, Schnettger, Bernard, & Gutierrez, 2017), act as switches in TCR signaling. Therefore, there has been significant efforts on developing biosensors to monitor their activation states. Among those, Raichu (which stands for Ras and interacting protein chimeric unit) FRET sensors have been developed for Ras and Rap1 (Figure 4c) (Mochizuki et al., 2001), RhoA (Pertz, Hodgson, Klemke, & Hahn, 2006) and Rab5 (Kitano, Nakaya, Nakamura, Nagata, & Matsuda, 2008). The Raichu FRET sensors consist of four key functional components: a donor, an acceptor, a GTPase and a GTPase-binding domain from

the binding partner of the GTPase (Ishido et al., 2011). Like other fluorescent reporters (Bivona et al., 2004) of GTPase activity, Raichu-Rap reflects the GTP-binding state of Rap1, with Ras-binding domain of Raf binding to Ras-GTP, drawing the donor and acceptor close to each other. Hyun et al. used TIRF to selectively illuminate the biosensor at the interface between the migrating T lymphocytes and the cover slip functionalized with VCAM-1 and CXCL-12 (Hyun et al., 2009). The study revealed that, while the Rap1 sensor itself was localized evenly throughout the cell, the activated form of the biosensor was restricted to the leading edge of the migrating cell where the chemokine receptor CXCR4 and the active form of VLA-4 are located. This supports the idea that localized chemokine engagement activates Rap1, which in turn leads to integrin activation. Carlin et al. optimized Raichu-Cdc42 sensor (Itoh et al., 2002) for FLIM purpose to image the real-time activity of Cdc42, which is member of Rho family, in the synapses formed by natural killer (NK) cells using multiphoton FLIM (Carlin et al., 2011).

3.4.3 cAMP/PKA signaling—The ubiquitous second messenger cyclic adenosine monophosphate (cAMP) regulates many cellular functions through the effector molecules, which mainly include PKA (a cAMP-dependent protein kinase) and Epac (a family of exchange proteins directly activated by cAMP). cAMP is mostly known to be immunosuppressive (Wehbi & Taskén, 2016). The first cAMP sensor, FICRhR, was constructed by labeling PKA catalytic subunits with fluorescein and the regulatory subunits of the tetrameric kinase with rhodamine (Adams, Harootunian, Buechler, Taylor, & Tsien, 1991). DiPilato et al. developed the first cAMP FRET sensor by flanking Epac1 with ECFP (cyan) and citrine (yellow). They observed the ratio of cyan-to-yellow emissions was elevated by 10–30% in living mammalian cells upon cAMP increase (DiPilato, Cheng, & Zhang, 2004). By fusing the sensor to various specific targeting motifs, they were able to measure the cAMP dynamics in different subcellular compartments e.g. plasma membrane, mitochondria, and nucleus. There have been other Epac-based cAMP FRET biosensors developed based on similar design principles, using Epac flanked by CFP and YFP (Nikolaev, Bünemann, Hein, Hannawacker, & Lohse, 2004; Ponsioen et al., 2004). Notably, the Epac probes have become a popular option for sensing intracellular cAMP. A number of novel Epac-based cAMP FRET sensors have been developed since then (Agarwal et al., 2014; J. Klarenbeek, Goedhart, Van Batenburg, Groenewald, & Jalink, 2015; L. Li, Gervasi, & Girault, 2015; Patel & Gold, 2015), which aim at increasing the sensing precision, or enabling monitoring cAMP at different subcellular locations.

3.4.4 Phosphorylation states of proteins—SH2 (Src homology 2) domain docks to the phosphorylated tyrosine residues of intracellular proteins. It has thus often been incorporated in the sensing domains of FRET biosensors designed to detect the phosphorylation of interested proteins. Lck (lymphocyte-specific tyrosine-protein kinase) and Fyn (proto-oncogene tyrosine-protein kinase) are the primary tyrosine kinases that phosphorylate ITAMs of the TCR-CD3 complex in T cells. Lck is a membrane-tethered kinase that phosphorylates tyrosine residues in the ITAMs in the TCR-CD3 complex, whereas double-phosphorylated ITAMs are the docking sites for ZAP70 and other TCR signaling associated proteins. Lck is often associated with CD4 or CD8 co-receptors, which may potentiate Lck activity by bringing it into the proximity of CD3 (Methi, Berge,

Torgersen, & Taskén, 2008; Straus, 1992). On the other hand, dephosphorylation of the inhibitory tyrosine residue, Tyr505, upon TCR engagement leads to the unfolding of Lck structure and enhancement of the kinase activity. Therefore, a FRET biosensor has been designed to monitor the conformational change of Lck during T cell activation, which consists of the full length of Lck and a FRET pair of ECFP and EYFP (Paster et al., 2009). To monitor the Lck kinase activity in T cell signaling, others have developed a substrate-based Lck FRET sensor. It contains a sensing domain with an Lck-sensitive tyrosine peptide and an SH2 domain that can bind to the Lck-phosphorylated peptide, thus changing the FRET efficiency (Wan et al., 2019). Using a similar design, Ouyang et al. developed a FRET sensor to measure Fyn activity, which consists of the FRET pair of ECFP and YPet, an SH2 domain with a single mutation to improve its sensitivity, and a Fyn substrate (Ouyang et al., 2019). The same group later added a light-inducible nuclear translocation feature to the Fyn FRET sensor (Z. Huang et al., 2020).

ZAP70 is a tyrosine-protein kinase that docks at phosphorylated ITAMs of the TCR-CD3 complex. Upon docking, ZAP70 is subsequently phosphorylated by Lck and auto-phosphorylated, which increase the kinase activity of ZAP70. ZAP70 in turn phosphorylates several signaling proteins, including LAT and SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa) in TCR signaling. ZAP-70 and Lck are expressed exclusively in T lymphocytes and natural killer cells, and are strictly required for T lymphocyte development in mice and functional mature T cells in humans. Several groups have developed ZAP70 FRET biosensors to image spatiotemporal activities of ZAP-70 in live T (Cadra et al., 2015; K. Li et al., 2016). A FRET sensor ROZA was designed with the sensing domain containing a peptide sequence from LAT known to be the substrate of ZAP-70, to monitor ZAP-70 activity in TCR mediated signaling (Randriamampita et al., 2008b). SLP-76 is an adaptor protein that mediates interactions between several signaling proteins, including LAT and the actin-regulatory proteins Nck and Vav1. SLP-76 is activated through phosphorylation by Lck and ZAP70, as well as through TCR independent pathways. A complex consisting of SLP-76, Nck, and Vav1 is recruited to IS during the early stages of T cell activation. Pauker et al. designed a triple-color FRET (3FRET) system to study the dynamic formation of this tri-molecular complex (Pauker, Hassan, Noy, Reicher, & Barda-Saad, 2012). They chose mCFP, mYFP, and mCherry to form three FRET pairs, enabling simultaneous observation of the regulatory behaviors of the three proteins (SLP-76, Nck, Vav1) that revise the existing model of the molecular complex (Figure 4d). A similar design of tri-color FRET system is seen in the study done by Kuo et al. on I κ B α /ERK/WWOX signaling in T cell maturation (Kuo, Ho, Huang, & Chang, 2018).

3.4.5 Calcium flux—In T cells, calcium flux is triggered by TCR-pMHC interactions, which has now been routinely used as a clear and robust readout for T-cell activation. Calcium flux regulates multiple aspects of T cell functions, including their motility, metabolism, proliferation, differentiation, and effector functions (Trebak & Kinet, 2019). There are two widely used types of calcium indicators, i.e. chemical calcium indicators and genetically encoded calcium indicators (GECIs). Some intensity based calcium indicators, such as quin-2, fluo-3, and fluo-4, have been utilized for monitoring calcium activity in T cells through microscopic imaging or flow cytometry (Ding, 2012; K.-D. Kim et al., 2017;

Salles et al., 2013; Shapiro, Adams, & Niederhuber, 1985), which however are prone to interference from photobleaching and loading conditions. Ratiometric indicators, including Indo-1 (dual emission) and Fura-2 (dual excitation) allow for more sensitive and dynamic measurements of calcium flux (Houtman et al., 2006; Wülfing, Sjaastad, & Davis, 1998). The major advantages of these indicators are their ease of use and the fact that, since they have been used in many cell types, the experimental protocols and/or limitations have been well established. However, the chemical biosensors are best suited for relatively short-term experiments (less than 5 hours) as signals usually deteriorate due to photobleaching and leakage over long period of time, especially under 37°C.

An alternative is genetically encoded calcium indicators (GECIs). As introduced earlier, the first unimolecular FRET-based sensor Cameleon consists of a Ca^{2+} -binding CaM and a CaM-binding M13 domain. Binding of Ca^{2+} to CaM makes CaM wrap around the M13 domain, increasing the fluorescence resonance energy transfer between the flanking GFPs (Miyawaki et al., 1997). CaM mutations can tune the Ca^{2+} affinities to measure free Ca^{2+} concentrations in the range 10^{-8} to 10^{-2} M (Miyawaki et al., 1997). Nakai et al. developed a GECI named GCaMP, which was based on CaM, M13 and a single GFP, for intracellular calcium monitoring (Figure 4e) (Nakai, Ohkura, & Imoto, 2001). A number of variations of GCaMP have been developed. GCaMP2 (Tallini et al., 2006) and GCaMP3 (Tian et al., 2009) have shown improved brightness, and were used in lymphocytes (Shulman et al., 2014). GCaMP6, an ultra-sensitive GCaMP variation was developed via mutagenesis and neuron-based screening (T.-W. Chen et al., 2013) and has become a popular choice of GECI for immune cell calcium imaging (Halle et al., 2016). Apart from increasing the brightness and sensitivity, researchers are also interested in expanding the excitation and emission spectra of the sensors for multi-color imaging or imaging with optogenetic actuators. Akerboom et al. created blue, cyan, and yellow variants of the GCaMP (Akerboom et al., 2013). Another example is NIR-GECO1, a recently developed near-infrared GECI (Qian et al., 2019). There are other useful GCaMP variations, including Salsa6f (Dong et al., 2017), a newly engineered ratiometric calcium indicator based on GCaMP6f designed for T cell study. Others have substituted CaM and M13 in GCaMP with Troponin C (TnC) to avoid the sensors from interfering the calmodulin-dependent pathways, as the Troponin family proteins have minimal interactions with endogenous proteins in cells (Mank et al., 2006). An example of the TnC-based sensors is TN-XXL (Mank et al., 2008), which originally cannot be expressed efficiently in immune cells in a transgenic model (Direnberger et al., 2012). Mues et al. modified the FRET pairs of TnC based sensors to make them suited for real-time calcium imaging in immune cells (Mues et al., 2013). Thestrup et al. developed Twitch sensors, a ratiometric GECI based on a different variant of TnC, which allowed for high-resolution tracking of T cells in vivo (Thestrup et al., 2014).

3.4.6 Cytokines and chemokines—Cytokines and chemokines are directly associated with T cell signaling and effector functions. The measurement of these soluble factors secreted by single T cells have been conventionally achieved through ELISpot (enzyme-linked immune absorbent spot) (Figure 5a) or FLUOROSpot assays, where cytokines/chemokines secreted by single cells are captured nearby on a substrate coated with a capture antibody and visualized by a detection antibody (Ahlborg & Axelsson, 2012). Intracellular

staining provides a more quantitative assessment of single cell secretion, where produced soluble factors are retained in Golgi apparatus by chemical inhibitors for subsequent immunostaining and detection by flow cytometry (Schmidt & Sester, 2013) (Figure 5b). To avoid disruption of normal T cell functions by the chemical inhibitors, some commercial kits combine the two approaches by pre-loading T cell surfaces with chimeric antibodies to capture cytokines secreted by the single cells over time, which are then detected through a fluorophore-labeled antibody and flow cytometry or fluorescence microscopy (Shen, Thomas, Dustin, & Kam, 2008; Sims et al., 2007) (Figure 5c). However, such approaches typically can only measure a handful of cytokines at a time. To increase the multiplicity of the analysis, Ma et al. developed a microfluidic platform that traps single T cells in microchambers, whereas the cytokines secreted by these cells are captured on pre-printed stripes (barcodes) of capture antibodies for immunosandwich assay (C. Ma et al., 2011). In a subsequent study, Lu et al. simplified the microchamber design and further pushed the multiplicity from 12 to 42 cytokines (Lu et al., 2015). The platform has later been commercialized and increasingly used to analyze secretome of single T cells and other cell types (Parisi et al., 2020; Zhu et al., 2020).

Another key aspect of cytokine/chemokine detection is the sensitivity of the assays. To improve the sensitivity of fluorescence detection, nanomaterial sensor and metal enhanced fluorescence has been utilized. The concentrated electromagnetic field built around a metal will increase the excitation of the fluorophore. For instance, the detection sensitivity improved with a plasmonic Au chips (Valentina et al., 2015). Other sensitivity-enhancing techniques include photonic crystals and optical resonance, where shifts in the resonant wavelength can be correlated with antigen (cytokine) binding events at the sensor surface (Maier, Morgan, Lindner, & Pittner, 2008; Mathias, Ganesh, & Cunningham, 2008). Surface plasmon resonance has also been used for cytokine detection. Unlike fluorophore-dependent techniques, this technique is label-free. Signals measured with SPR is based on small changes in the refractive index on the sensor surface upon analyte binding. Localized surface plasmon resonance (LSPR) nanoplasmonic effects also allow for label-free, real-time detection of biomolecular binding events on a nanostructured metallic surface with simple optic and sensing tunability. For instance, LSPR was integrated with optofluidic biosensing technique to detect cell-secreted TNF- α cytokines in clinical blood samples (Oh et al., 2014). While these new sensors have largely been used to measure bulk cytokine/chemokine secretions, their applications in measuring single T cell secretions can be envisioned through combination with microchambers or ELISpot/FLUOROSpot techniques.

4. CELL VIABILITY

4.1 Metabolism

The reprogramming of T cell metabolism is triggered upon antigen recognition and accompanied with other signaling activities throughout T cell differentiation and emergence of memory T cells (Chapman, Boothby, & Chi, 2020). Recent studies have also shown cell metabolism as a crucial regulator of the phenotypes and functions of T lymphocytes (Bantug, Galluzzi, Kroemer, & Hess, 2018). It is thus important to examine this process,

for harnessing T cell immunity against infectious diseases and cancer (Sugiura & Rathmell, 2018).

Cell metabolism has been investigated with bulk, invasive, and/or endpoint readouts. Some plate-based proliferation assays, such as Alamar Blue assay and MTT/XTT assays, utilize the fluorescent/colorimetric changes of substrate materials in response to metabolic reduction, and can thus be used to monitor the proliferation and metabolic state of immune cells (including T cells) (Koyanagi, Kawakabe, & Arimura, 2015; Rampersad, 2012). The Seahorse assays, on the other hand, have offered real-time bioenergetic analysis upon T cell activation, with clear distinction between different metabolic programs (e.g. oxidative phosphorylation vs. glycolysis) determined by the oxygen consumption and the extracellular acidification rates (Akkaya et al., 2018; Jones et al., 2017; Jones et al., 2019; Menk et al., 2018). Mass spectrometry (MS) approaches allow identification and quantification of large panels of metabolites or metabolism-associated proteins, although in a bulk population of cells. For example, Palaskas et al. used mass spectrometry to profile metabolic changes in human T cells influenced by PD-1 signaling with a panel of 155 metabolites (Palaskas et al., 2019). Marchingo et al. took a proteomic approach and used mass spectrometry to analyze over 7000 proteins to reveal both Myc-dependent and independent metabolic processes in activated T cells (Marchingo, Sinclair, Howden, & Cantrell, 2020).

At the single-cell level, the metabolic state of T cells can be taken in snapshots by immunostaining on metabolic markers. Glut-1, the primary glucose transporter in hematopoietic cells, has been used as an indicator of glycolysis to reveal the regulatory role of CD28 and/or Akt signaling in glucose metabolism (Frauwirth et al., 2002; Jacobs et al., 2008; Siska et al., 2016). Other surface markers that may potentially be used as metabolic markers include GPR43 (a fatty acid transporter) and LAT1-CD98 or ASCT2 (amino acid transporters), which have been shown crucial in intestinal/commensal homeostasis (Maslowski et al., 2009; Sun et al., 2018) and activation (Nakaya et al., 2014; Sinclair et al., 2013) of T cells. T cell metabolism can also be monitored with intensity-based fluorescent markers. A fluorescent glucose analog 2-[N[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) has been used to measure glucose uptake rate using intensity differentials in flow cytometry (Siska et al., 2016). Isotope tracing can also be used to study T cell metabolism pattern in vivo. Ma et al. infused ^{13}C -glucose in mice to track the metabolism of CD8⁺ T cells in situ over the course of immune response, and observed a lower glucose oxidation level in vivo comparing to in vitro, as well as a dramatic glucose consumption switch in CD8⁺ T cells upon immune response (E. H. Ma et al., 2019). Sukumar et al. used tetramethylrhodamine methyl ester (TMRM), a lipophilic cation dye that stains for active mitochondria, to isolate therapeutic CD8⁺ T cells based on their mitochondrial membrane potential (Ψ_m), and showed that the low- Ψ_m CD8⁺ T cells were superior in metabolic fitness and tumor eradication (Sukumar et al., 2016). Akkaya et al. combined TMRM staining with Seahorse assays and staining of Glut1 and ROS (reactive oxygen species) to reveal that prolonged activation leads to an increase in mitochondrial activities, which favor CD4⁺ T cell proliferation (Akkaya et al., 2018).

Notably, FLIM provides a non-invasive option for monitoring T cell metabolism at the single cell level (L.-C. Chen et al., 2014; Zhou et al., 2020). The autofluorescent metabolic

co-enzymes, such as NAD(P)H and FAD, are involved in almost all steps of cell metabolism. The fluorescence lifetime of NAD(P)H and FAD are dependent on their enzyme-binding status as well as several other environmental factors (e.g. pH, oxygen, etc.) (Lakowicz, Szmajda, Nowaczyk, & Johnson, 1992). On the other hand, the redox ratio (ORR), defined as the FAD fluorescent intensity divided by the sum of NAD(P)H and FAD fluorescence intensities, also provides a means for probing the state of mitochondrial oxidative phosphorylation (Hou et al., 2016). Walsh et al. combined both the fluorescence lifetime parameters of NAD(P)H and FAD and the ORR to monitor the metabolic changes of T cells upon activation, and classified subpopulations of T cells based on the FLIM profiles (A. J. Walsh et al., 2020). Another promising label-free method for in vitro viability measurement is cyclic voltammetry (CV) (Kissinger & Heineman, 1983), which could detect cell redox properties with electrochemical sensors (Kafi, Kim, An, & Choi, 2011) and has been applied in lymphocytes (Nikbakht, Pakbin, & Nikbakht Brujeni, 2019).

4.2 Apoptosis

Apoptosis is a type of controlled cell death resulted from a metabolic cascade, and is essential for proper development and function of the immune system (Piliponsky & Levi-Schaffer, 2000). Indicators of apoptosis include morphological changes such as blebbing, cell shrinkage, chromatin condensation, nuclei fragmentation (Häcker, 2000), as well as changes in protein expression such as cleavage of caspases, release of cytochrome c, annexin V (early apoptosis) (Aubry et al., 1999; Zhang, Gurtu, Kain, & Yan, 1997), FasL (Nagata & Golstein, 1995), and TRAIL (Jeremias, Herr, Boehler, & Debatin, 1998). Studying immune cell apoptosis can provide insights into immunological regulation of diseases and guidance to the design of immunotherapy.

By far, the most common approach to detect apoptosis is through flow cytometry after fluorescent labeling of apoptotic markers. Annexin V has been extensively used to visualize apoptotic cells at selected time points (Babu et al., 2008; van Engeland, Nieland, Ramaekers, Schutte, & Reutelingsperger, 1998; Yasuhara et al., 2003). FasL (Nagata & Golstein, 1995) and TRAIL (Jeremias et al., 1998) are also common molecular markers used to detect apoptosis in T cells. Notably, while most apoptotic markers in flow cytometry are detected by fluorescence, the apoptosis-associated cell shrinkage can be captured in the forward scatter and side scatter channels, the two label-free channels in flow cytometry (Telford, Tamul, & Bradford, 2016).

To gain more insights into the biochemical and biophysical features as well as the tissue context of the apoptosis, various imaging strategies have been applied to the detection of apoptosis at the single-cell and population levels in organisms with or without genetically engineered reporters or exogenous contrast agents (Coppola, Ross, & Rehemtulla, 2008; Witney et al., 2015; Yang, Cui, Cai, Yang, & Forrest, 2011; Ye et al., 2014). One of the most well-known approaches to detect apoptosis is the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, which reveals early apoptosis through single- and double-strand DNA breaks in cells (Nagarkatti, Rieder, Vakharia, & Nagarkatti, 2010). It allows for microscopic observation of immune cells undergoing

apoptosis, particularly within embedded tissue sections (D. S. Walsh, Lane, Abalos, & Myint, 2004).

At the single cell level, fluorescence microscopy can be used to visualize apoptosis through indicators like annexin V. Cytochrome c release from mitochondria is the initiating event in apoptosis, which has been conventionally detected with Western blotting. Interestingly, researchers have developed GFP-tagged cytochrome c for expression in cells to visualize the subcellular localization and redistribution of cytochrome c during apoptosis, including in Jurkat T cells (Goldstein et al., 2005). The activation of caspase 3 is a crucial early event in apoptosis (Thornberry & Lazebnik, 1998). A commercial, membrane-permeable biosensor named NucView® has been developed to detect apoptotic activity mediated by caspase 3 in live single cells in real time, e.g. in Jurkat T cells (Cen, Mao, Aronchik, Fuentes, & Firestone, 2008). The intact NucView biosensor is non-fluorescent and consists of a fluorogenic DNA dye and a DEVD (aspartate, glutamate, valine, and aspartate) substrate moiety that can be cleaved by caspase 3. Upon cleavage, the DNA dye stains the nucleus to report the apoptosis as well as visualizing the nuclear morphology. Similarly, Tyas et al. designed a FRET biosensor that consists of a FRET pair (CFP/YFP) connected by a DEVD segment, the cleavage of which by caspase 3 leads to a decrease in FRET. The authors reported a rapid change in FRET almost simultaneously with a depolarization of Ψ_m in the affected cells (Tyas, Brophy, Pope, Rivett, & Tavaré, 2000). DNA fragmentation, another indicator of cellular apoptosis, can be detected by the comet assay in single cells (Wood, Weingeist, Bhatia, & Engelward, 2010). Other imaging modalities such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have also been used to examine apoptotic Jurkat cells through visualization of cell morphological changes such as blebbing, chromatin condensation, and nuclei fragmentation (Dobrucki & Darzynkiewicz, 2001; Yasuhara et al., 2003).

Additionally, some engineering approaches like microfluidics have also been explored in studying apoptosis. Microfluidic channels have been designed to trap cells, at which point fluorescence imaging can be used to assess the apoptotic progress (Qin et al., 2005; Valero et al., 2005; Wlodkowic, Faley, Zagnoni, Wikswo, & Cooper, 2009). Most of these approaches have been carried out with tumor cells, but similar approaches also apply to the studies of immune cells. For example, caspase-3 expression and annexin V expression in single Jurkat cells were analyzed in a microfluidics chip device that integrated cell capture, protein extraction, and electrophoretic separation (Reif, Aguas, Martinez, & Pappas, 2010; Tabuchi & Baba, 2004).

Importantly, apoptosis has not only been monitored in T lymphocytes, but also deliberately triggered with inducible suicide genes to safeguard bioengineered T cell immunotherapies, such as chimeric antigen receptor (CAR) T cells (S. Yu, Yi, Qin, & Wu, 2019). This is achieved through herpes simplex virus thymidine kinase (HSV-tk) in combination with ganciclovir (GCV) (Marin et al., 2012), or an inducible caspase 9 (iCasp9) construct fused to the human FK506 binding protein (FKBP) that can be dimerized with a small chemical inducer of dimerization (CID) AP1903 (Tey, Dotti, Rooney, Heslop, & Brenner, 2007). While this is not the focus of this review, it highlights the importance of monitoring and

studying apoptotic events in T lymphocytes, for the development of future generations of safety triggers for T cell-based therapies and other cellular therapies.

Conclusion

Technological progresses in imaging and molecular probes have greatly facilitated immunobiology research. Meanwhile, the advances at the frontier of the biological research have also demanded and fueled the development of new generations of bioengineering tools to answer the new questions. The current trend in the T lymphocyte studies has been moving toward visualizing and quantitatively measuring the cellular events at the single-cell, subcellular, and molecular levels, with a spatial resolution at the nanometer scale and a time resolution at seconds or shorter. At the same time, the probes and imaging techniques are designed to be less invasive or disruptive to the natural signaling events or cell-cell interactions, which allows for faithful recapitulation and modeling of the inner works of the immune system. Additionally, some new emerging studies have also started simultaneously examining multiple cellular events, or biological activities in different categories, e.g. the relationship between mechano-transduction and biochemical signaling. It thus calls for multimodal integration of biosensors or development of multifunctional single sensors to enable the monitoring of multiple signaling events in a relatively simple way. We thus envision that those needs will continue to drive interests in new tools in the years to come.

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Figure 1. Bioengineering approaches to the immunological synapse formation.

(a) A schematic plot of an immunological synapse formed between a T cell and an antigen presenting cell (APC). SMAC: supramolecular activation clusters. MTOC: microtubule organization center. (b) T cell interacting with supported lipid bilayer that mimics the APC membrane. (c) Laser tweezer allows for reorienting T cell/APC pairs for direct imaging of the cell-cell interface. Measuring the bond strength between TCR and pMHC with (d) atomic force microscopy (AFM) and (e) fluorescence biomembrane force probe (fBFP). RBC: red blood cell.

Figure 2. Principles of imaging techniques and example images

(a) In TIRF, the intensity of the evanescent wave decays exponentially with distance from the interface. Therefore, background noise from none-surface fluorophores is greatly reduced. (b) In PALM and STORM, a sparse subset of photo-switchable fluorophores is excited each time and then reconstructed into high-resolution images with the repeatedly-taken pictures. (c) In SIM, samples are excited with different patterns of illumination each time and high-resolution images are reconstructed by overlaying these images. (d) In STED, fluorophores at the center of focal spot are left active while those at the periphery get selectively deactivated to minimize the background. (e) LifeAct stained F-actin under TIRF (Treanor et al., 2010). (f) TCR microclusters under light sheet dSTORM (Y. S. Hu, Cang, & Lillemeier, 2016). (g) Primary NK cell stimulated on a surface coated with MICA-Fc and ICAM-1 under 3D SIM. Green: actin; Red: MTOC (A. C. N. Brown et al., 2011). (h) Phalloidin stained F-actin under STED (E. M. Mace & Orange, 2014).

Figure 3. Intensity-based, FRET-based and FLIM-based sensors.

(a) Intensity based sensors are usually fluorescently tagged/labeled proteins or molecule that can specifically bind to target molecules, or a recombinant version of the target molecule with a fluorescent protein tag. The readouts are typically the percentage of cells showing signal above a threshold, or the spatial distribution of the fluorescence. (b) FRET-based sensors display a change in the emission spectrum when the distance between donor and acceptor is changed. The changes in emission are usually detected at a specific emission wavelength or ratiometrically at two wavelengths. (c) FLIM-based sensors display drastic changes in fluorescence lifetime upon binding to the target molecule. Some FLIM sensors consist of two parts: a substrate portion which binds to the analyte, and a delivery portion which helps the sensor get into cells. The readout is the fluorescence lifetime of the fluorophore.

Figure 4. Examples of FRET sensors for detecting T cell signaling.

(a) CD3 ζ -CliF reporters have intermolecular FRET when CD3 ζ molecules cluster, resulting in a decrease in Venus fluorescence lifetime. (b) A ZIP reporter contains an SH2 domain, which binds to the tyrosine-phosphorylated ITAMs to cause FRET between eGFP and mCherry, resulting in a decrease in eGFP fluorescence lifetime. (c) In Raichu-Ras, Ras-GTP binds to Raf, which results in FRET from CFP to YFP. (d) In a 3FRET system, there are three FRET pairs: mCFP-mYFP, mCFP-mCherry, and mYFP-mCherry. Each cell is imaged with six channel settings, including three FRET channels and three individual fluorophore channels. The corresponding FRET channel, the donor channel and the receptor channel are used to calculate each FRET pair. (e) In GCaMP, when Ca^{2+} binds to CaM, CaM- Ca^{2+} interact with M13, inducing a conformational change in the circularly permuted EGFP (cpEGFP), which leads to fluorescence intensity increase.

Figure 5. Detection of cytokine/chemokine from single T cells.

(a) In ELISpot, cells are immobilized (1), cytokines are captured by pre-coated antibody on nearby substrate (2) for subsequent staining and microscopic detection (3). (b) In intracellular staining, T cells are activated, fixed, permeabilized, stained for intracellular cytokines, and analyzed with flow cytometry. Protein secretion is inhibited by the addition of inhibitors of Golgi transport. (c) In cell surface capture, a chimeric antibody with specificity to both cell surface receptor and cytokine is pre-loaded on cell surface (1), to capture the cytokine released by individual cells (2). A detection antibody is used to stain the captured cytokines for measurement in flow cytometry or microscopy (3).