Analytical and Bioanalytical Chemistry (2019) 411:4339–4347
https://doi.org/10.1007/s00216-019-01694-5

TRENDS

Fluorescence imaging-based methods for single-cell protein analysis

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Received: 15 November 2018 / Revised: 5 January 2019 / Accepted: 15 February 2019 / Published online: 11 March 2019

Abstract

The quantity and activity of proteins in many biological systems exhibit prominent heterogeneities. Single-cell analytical methods can resolve subpopulations and dissect their unique signatures from heterogeneous samples, enabling a clarifying view of the biological process. Over the last 5 years, technologies for single-cell protein analysis have significantly advanced. In this article, we highlight a branch of those technology developments involving fluorescence-based approaches, with a focus on the methods that increase the ability to multiplex and enable dynamic measurements. We also analyze the limitations of these techniques and discuss current challenges in the field, with the hope that more transformative platforms can soon emerge.

Keywords Single-cell · Fluorescence · Protein analysis · Multiplexed assays · Dynamic measurements

Introduction

Precise control of proteins is the foundation of maintaining normal biological processes [1–5]. Alterations to the expression level and activities of proteins are linked to the onset and development of various diseases, such as cancer [6, 7], diabetes [5, 8], and autoimmune diseases [9]. Protein analysis is therefore crucial to biomedical research, as it has directly contributed to the elucidation of disease mechanisms and the development of successful pharmaceuticals, such as kinase inhibitors in cancer treatments [10–12].

In many biological systems, the quantity and function of proteins are highly heterogeneous, and dissecting such heterogeneities has direct therapeutic implications. For instance, protein signaling patterns often differ significantly among cancer cells, which confers selection advantage and contributes to drug resistance [13, 14]. Another example is the prominent variance in cytokine secretion capabilities among phenotypically similar T cells, which has direct impact on the outcome of immunotherapies [15]. Single-cell methods are necessary for resolving these heterogeneities and accessing the underlying biological information. They have aided in unraveling the resistance mechanisms of targeted inhibitors and provided rationales for combinatorial therapies [16–19]. For instance, we have demonstrated that in BRAF-mutant melanoma models, treatment with vemurafenib (a BRAF inhibitor) caused the selection of a drug-resistant subpopulation, with a unique co-dependency on NFkB and ERK signaling. Therapies that co-target those pathways showed outstanding synergistic effects on inhibiting proliferation [20].

In this article, we review recent advances in the field of single-cell protein analytical methods, with a focus on fluorescence-based approaches. Specifically, we describe technology advancements in two directions: improving analysis multiplexing and implementing time-resolved readouts.

Methods for improving multiplexing

Compared to high-throughput proteomics methods using mass spectrometry [21, 22], multiplexing in fluorescence-based protein analysis is inherently restricted by available fluorophores, since non-overlapping detection channels are preferred. From an information theory perspective, such limitation roots from the lack of complexity in the coding system. Therefore, apparent solutions are spatially segregating the protein and assigning temporal codes to proteins. Here, we highlight two recently developed technologies that explored spatial and temporal coding and that have the potential of improving analysis multiplexing.

Published in the topical collection *Young Investigators in (Bio-)Analytical Chemistry* with guest editors Erin Baker, Kerstin Leopold, Francesco Ricci, and Wei Wang.

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Beads-on-barcode antibody microarray

Spatially segregated surface assays have been widely utilized in microfluidics-based single-cell analysis [23]. A common scheme is to implement an addressable capture antibody array and miniaturize it within a single-cell chamber. Here, multiplexing is limited by the size of the chamber, which determines the maximum number of assay strips or spots that can fit in. Carefully designed platforms have allowed simultaneous analysis of up to 42 proteins [24], but further boosting the detection throughput remains challenging.

The Shi and Wei groups realized an ingenious design that can potentially overcome the spatial restrictions. They introduced another dimension to the traditional antibody barcode array. The key innovation is transferring the antibody assay from the surface barcode onto microbeads [25]. These beads have functional groups that allow conjugation to antibodies, and the color and size of the beads provide the encoding of analytes (Fig. 1a). They implemented a microwell single-cell chip and used this beads-on-barcode method to identify and analyze the properties of rare circulating tumor cells from blood samples (Fig. 1b). For proof of concept, they demonstrated the coding method on six proteins, including organ-specific markers and therapeutic targets (Fig. 1c).

Although the study did not go beyond current state-of-the-art multiplexing, the transformative potential is intriguing. The beads can be readily modified with different single-strand DNA oligomers to couple with a DNA-encoded antibody library. Meanwhile, incorporation of additional hybridization segments can allow the beads to attach to designated locations on the surface barcode. Since the size and color of the beads provide variables that are orthogonal to the surface location, the accessible coding space can be significantly expanded. With the four distinct sizes and three colors demonstrated in the paper, and the 20 barcode strips available in the single-cell barcode chip technology, the theoretical capacity can readily reach 240 proteins per cell.

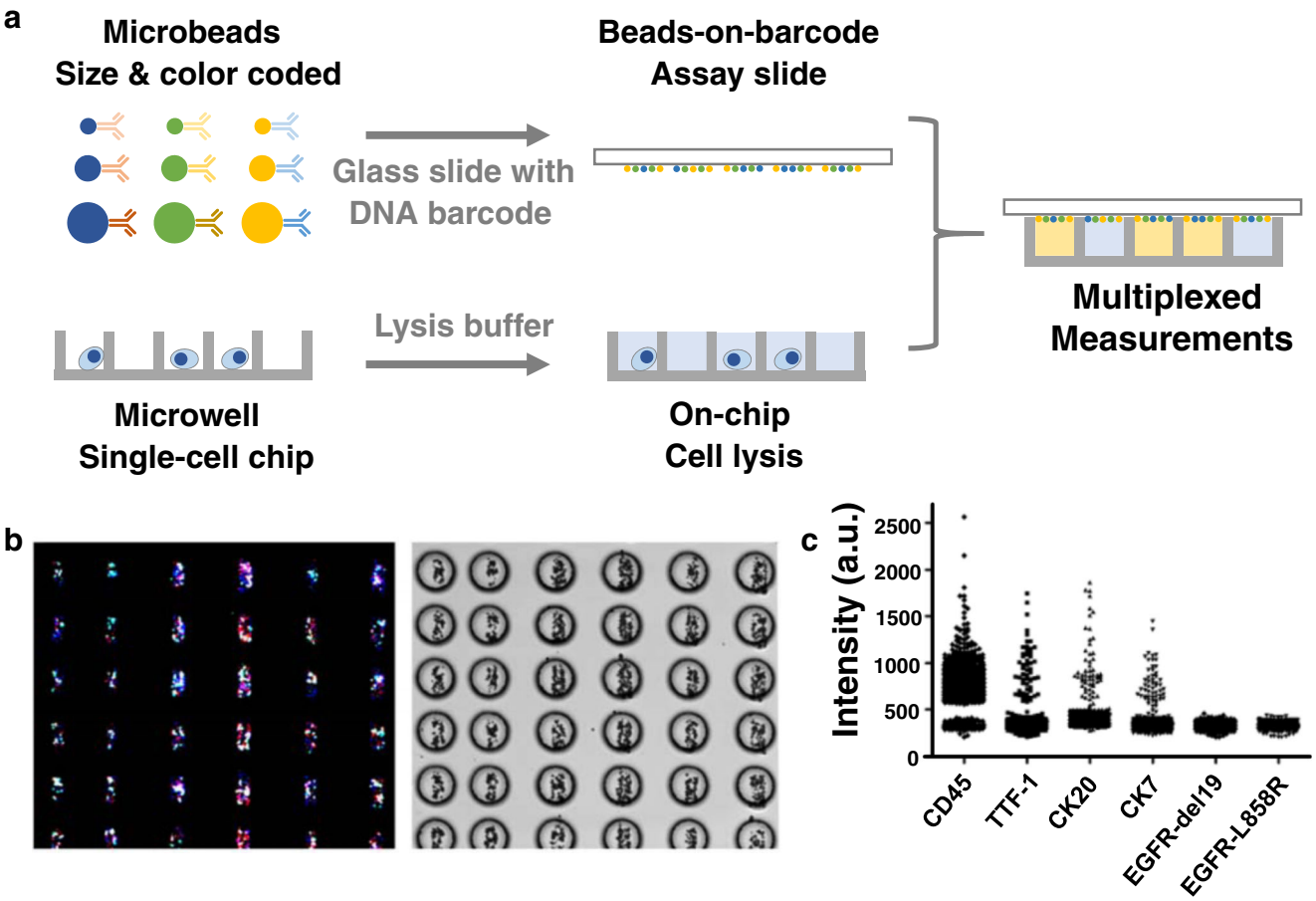

Fig. 1 The beads-on-barcode single-cell assay. The key innovation is the extra coding variables enabled by the size and color of microbeads. (**a**) Microbeads are attached to glass slides to form a beads-on-barcode assay slide. This slide is aligned to the microwell single-cell chip to enable addressable multiplexed measurements. (**b**) Representative fluorescence and bright-field images of the fully assembled device. (**c**) Single-cell data showing the heterogeneity of protein expression levels among 1109 individual cells. Adapted from reference [25] with permission from American Chemical Society

Cleavable antibodies enabled immunofluorescence

Immunofluorescence imaging techniques have the obvious advantage, over the microchamber-based methods, of preserving the native spatial information in the sample. However, such spatial resolution also prevents location-based analyte coding, therefore limiting detection throughput. Employing sequential stain/de-stain cycles to enable the temporal coding provides an immediate solution. Many successful examples have been demonstrated in the field of single-cell transcriptome analysis, where RNAs are analyzed with fluorophore-labeled complementary sequences through many rounds of hybridization/dissociation processes [26]. Adapting such scheme to antibody-based protein analysis is challenging, because removing antibodies from a fixed tissue sample is difficult. Available de-staining approaches involve harsh conditions and cause damage to the sample. Therefore, alternative methods are required.

To overcome the abovementioned obstacle, the Guo group developed a method to label antibodies with cleavable fluorophores [27]. Such an approach allows sequential staining and reading of a large number of proteins. The key innovation is a cleavable linker containing α-ethoxyazide moieties. This linker can be modified with NHS-esters to allow straightforward conjugation to antibodies and fluorophores. Phosphines such as tris(2-carboxyethyl) phosphine (TCEP) can reduce the azide groups to amines. The resulting α-ethoxyamine is hydrolyzed to allow cleavage of the linker (Fig. 2a), and subsequent washing removes the cleaved fluorophores. The cleavage reaction is performed under mild conditions and is compatible with biological samples. Cleaved antibodies stay in the sample but does not contribute to fluorescence signal, thereby allowing new rounds of staining and imaging on the same sample (Fig. 2b). The Guo group demonstrated both the feasibility and effectiveness of this method, using three fluorophores and four rounds of staining cycles in single HeLa cells. The implementation of cleavable fluorophores solves the irreversibility associated with immunostaining. In another experiment, they demonstrated 12 consecutive staining cycles. Given that high-performance microscopes could support more than 10 fluorescence channels, this technology has the potential to be expanded to over 100 protein targets.

Methods for obtaining dynamic readouts

The expression and function of proteins in living systems are dynamic, as exemplified by the intricate control and response system that governs cell-signaling events [28]. Intrinsic fluctuations are frequently observed in protein analysis, as a combined result of the stochastic nature of biological events and well-regulated signaling networks. The heterogeneity resolved by single-cell analysis contains such fluctuations, and dissecting the fluctuation using information theory approaches can reveal underlying biological mechanisms [29].

The majority of single-cell methods are based on destructive methods—cells must be fixed to enable protein analysis, yielding snapshot measurements. Even though these snapshots lack true temporal information, there is the assumption that biological systems are mostly ergodic, hence discrete temporal sampling could infer dynamic information at the population level [30, 31]. This assumption has driven the successful development of biomedical

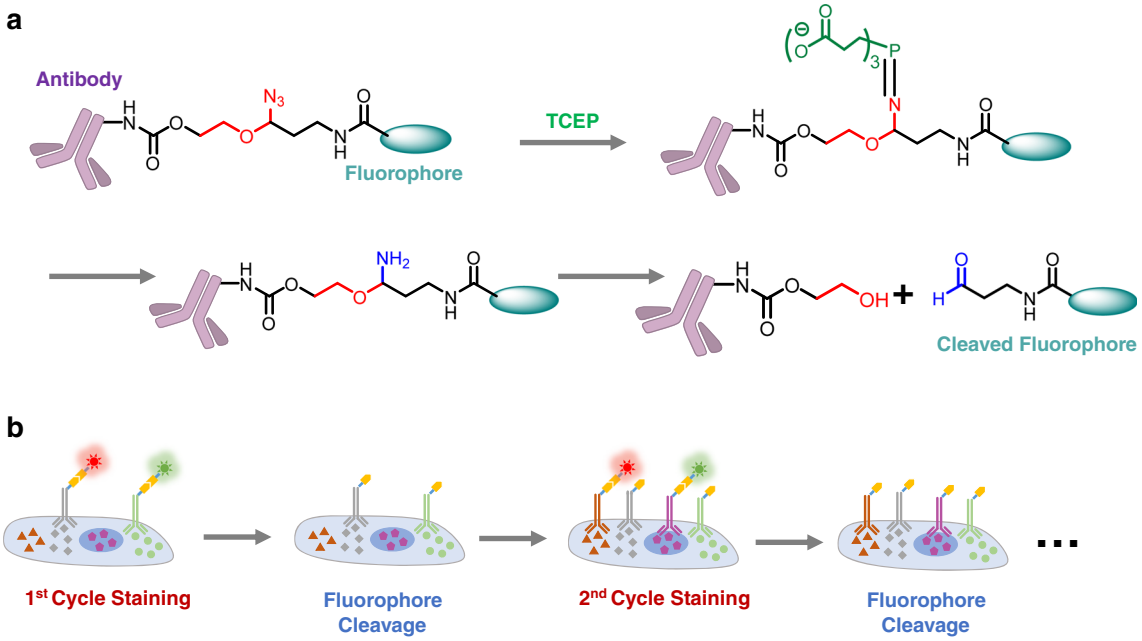

Fig. 2 Cleavable fluorophores allow multiplexed immunofluorescence assays. (**a**) The antibody-fluorophore conjugate can be cleaved by TCEP. (**b**) Cycles of staining and cleaving allow simple expansion of multiplexing capability

research, especially the field of systems biology. Nevertheless, this assumption is not widely proven and there is evidence that some biological processes are nonergodic [32, 33]. It is likely that technologies capable of continuously analyzing proteins from living single cells promise deeper understanding of biological systems. Here, we summarize recent technology developments of dynamically analyzing proteins and their single-cell implementations.

Microengraving with detachable slides

Secreted proteins are great targets for dynamic measurements, because they can be easily quantified without damaging the cell, and because their status provides functional views of the cell. Microengraving devices are common platforms that provide access to single-cell cytokine release profiles. The basic design involves a bottom chip with single-cell wells, and a top slide with addressable capture antibody arrays. When assembled, the single-cell wells are sealed by the top slide, segregating individual cells. On-chip incubation allows the cells to secrete proteins; the proteins of interest can be then captured by the antibodies immobilized on the top slide. At the end of the incubation, the top slide is removed and the captured proteins are further analyzed by sandwich immunofluorescence assays.

In principle, removing the top slide does not affect the cells, and examples of such manipulation have been demonstrated in integrated single-cell proteomic/transcriptomic studies. Therefore, using detachable top detection slides becomes a natural extension of the available technology. The Love group demonstrated this implementation of detachable assay slides on their microengraving platform (Fig. 3a). They obtained three sets of cytokine profiles from single human T cells, using sequential incubation-detection cycles (Fig. 3b) [34]. By resolving the kinetic secretion profile, they showed that different cytokines dominated a distinct time period after T cell activation.

Dynamic quantification of enzymatic activities in living cells

For most intracellular proteins, the more biologically relevant measurements are those capturing protein signaling and function events. Fluorescent substrates have been extensively explored to probe enzymatic activities in living cells, and their implementations on microscopy platforms provide access to single-cell functional assays. However, it is challenging to perform quantitative enzymatic assays in living cells, especially those aiming to obtain catalytic constants. First, most enzymes have homologous relatives existing simultaneously inside the same cell; therefore, it is difficult to restrict the interrogation to a single type of enzyme. Second, the concentrations of the enzyme, substrate, and product must be quantifiable throughout the dynamic experiment. Last but not least, the intracellular space is

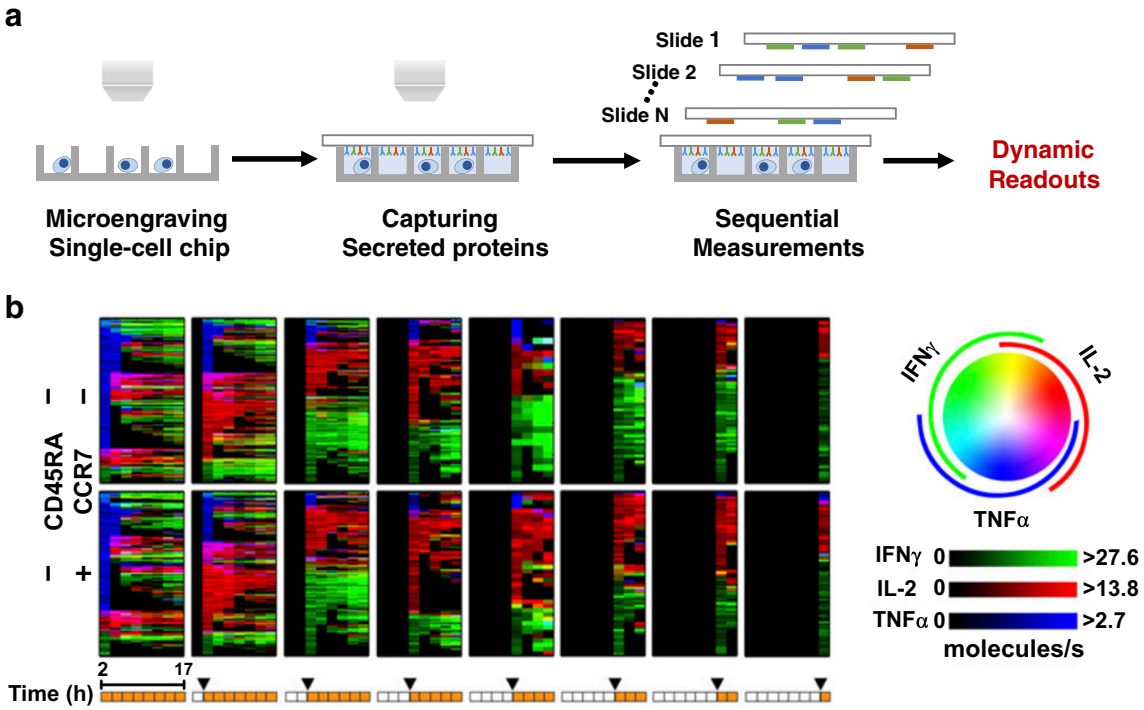

Fig. 3 The detachable slides method enables dynamic measurements of secreted proteins with single-cell resolution. (**a**) Single cells are separated in a microengraving chip. The secreted proteins are captured and subsequently measured by using a glass slide with immobilized antibodies. Multiple slides are sequentially introduced to obtain dynamic readouts. (**b**) Cytokine secretion kinetics of 2349 viable T cells. Each row within each block reflects the dynamic activity of an individual T cell over time. The color wheel illustrates the type and relative magnitude of secreted cytokines. Adapted from reference [34]

not a homogenous environment; therefore, common enzyme kinetics models, such as those described by the Michaelis-Menten equation, cannot be readily adapted.

The Schreiber group developed a method based on cleavable fluorogenic substrates to quantify enzyme activity in living cells [35]. In this case, the enzyme TEM1β-lactamase (β-lac) was trasfected in HeLa cells. This enzyme is restricted to prokaryotes; therefore, any observed activities in the HeLa cells must originate from the β-lac. The enzyme was also fused to a fluorescent protein, mCherry, to allow quantification of enzyme concentrations. CCF2, a fluorogenic donor-acceptor pair based on Föster resonance energy transfer (FRET), was chosen because its emission peak shifts from 520 to 447 nm after cleavage by β-lac, allowing the concentration of the substrate to be quantified (Fig. 4). With this method, the Schreiber group determined the apparent catalytic efficiency (k_{cat}/K_m) in living cells. They obtained values that were much lower than those generated from in vitro measurements. In addition, they found significant cell-to-cell variabilities that are independent of enzyme concentrations. Further analysis revealed that this heterogeneity originated from the differential substrate flux in crowded cell culture systems. These findings suggest that catalytic properties determined in a homogeneous solution cannot be simply extended to the intracellular system.

Genetically encoded reporters for profiling protein activities

Genetically encoded reporters are indispensable tools in studying proteins [36]. Many well-established protocols have enabled real-time analysis of protein functions and signaling activities. Nevertheless, their single-cell implementations were only realized in recent years, following the development of advanced microscopy platforms.

The Zhang group has developed a suite of probes for interrogating various kinase activities [37]. The key approach is to construct a genetically encoded FRET system that responds to substrate phosphorylations. A common architecture consists of a FRET-enabling fluorescent protein pair that is connected by a substrate peptide segment and a phospho-site binding domain. In certain cases, a long and flexible linker is added between the substrate and the binding domain to enhance the FRET signal. When the substrate sequence is phosphorylated, a conformational change will take place, leading to a significant increase in FRET signals (Fig. 5a). This bioprobe allows dynamic monitoring of signaling processes with impressive spatiotemporal resolution in living cells. Recently, the Zhang group extended the application of these probes from mammalian cells to yeasts, where a much higher signal-noise ratio is required. They developed an optimized MAPK (mitogen-activated protein kinases) activity probe in a yeast system and interrogated the yeast differentiation pathway at single-cell resolution (Fig. 5b, c) [38]. The result showed that the MAPK signaling dynamics is governed by complex feedback interactions and that distinct activity patterns promote different phases of yeast cell mating (Fig. 5d, e).

Due to the limited spectral space, it is difficult to implement multiple FRET reporting systems in the same cell; therefore, the abovementioned FRET method cannot provide multiplexing. In addition, the dynamic range of the FRET reporting system is rather limited. In a recent effort to overcome such limitations, the Zhang group developed a suite of excitation ratiometric kinase activity sensors (ExRai), achieving improved sensitivity and dynamic range [39]. The key innovation is a construct where a circularly permuted green fluorescent protein (cpGFP) is sandwiched between a kinase binding domain and a substrate segment. The interaction between the phosphorylated substrate and the binding domain induces conformational changes of the cpGFP, which alters its excitation profile and

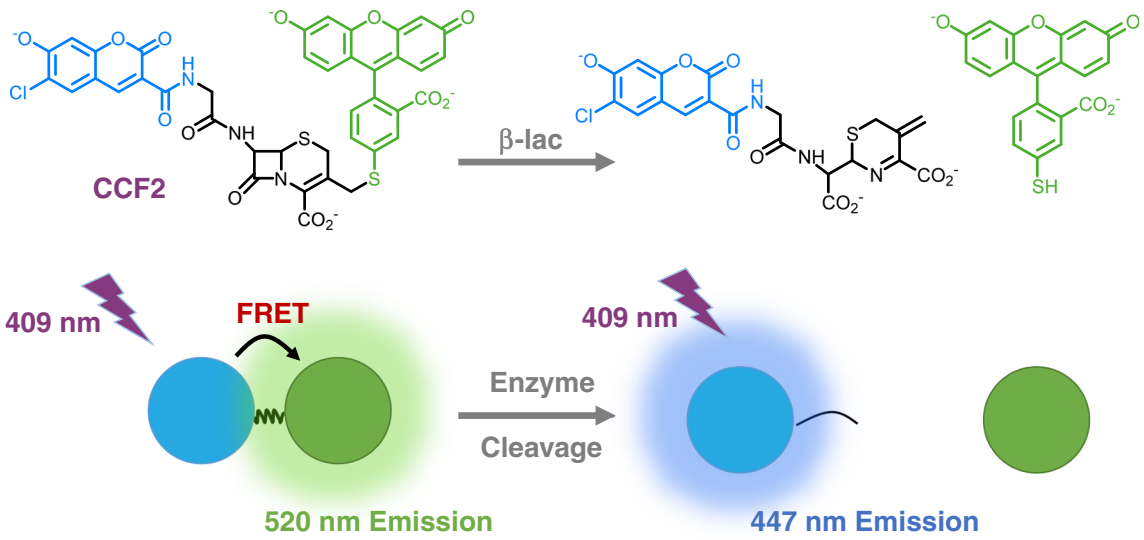

Fig. 4 The color of CCF2 fluorescence is dependent on the β-lac enzyme activity

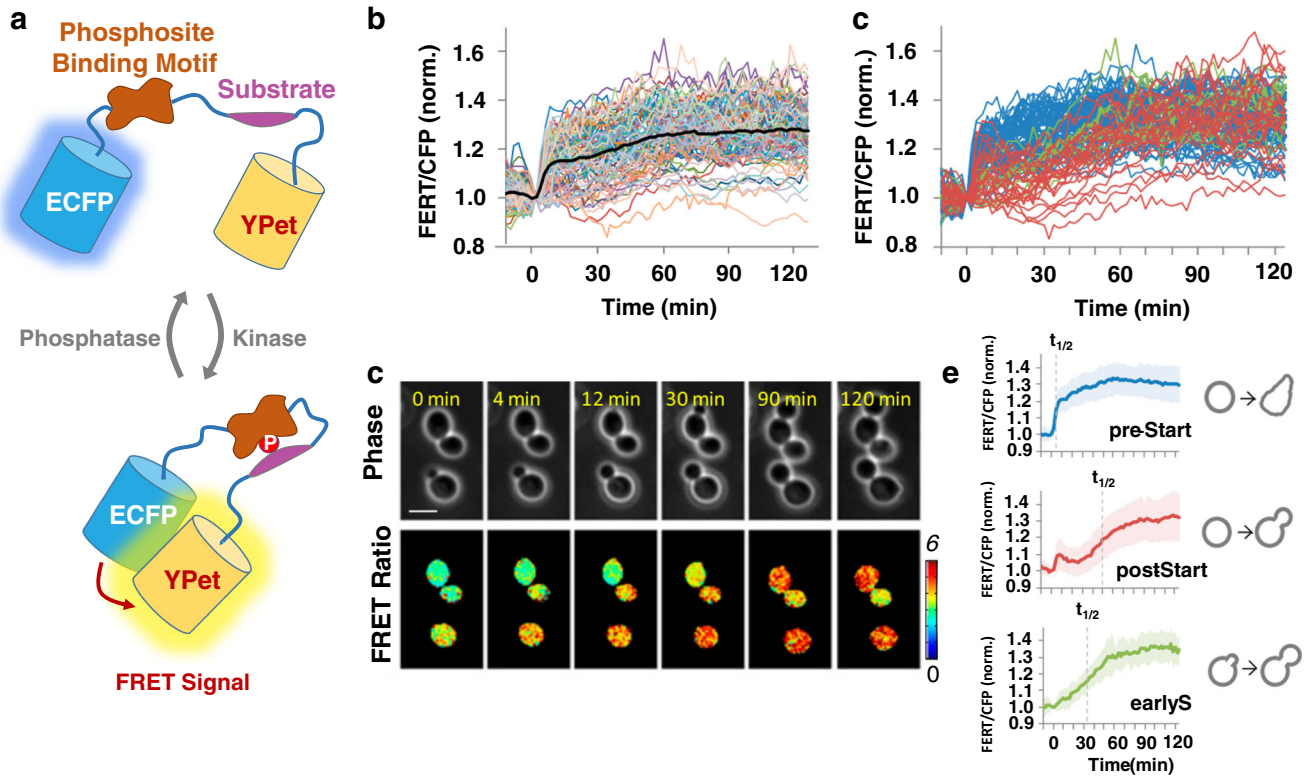

Fig. 5 Genetically encoded fluorescence reporter systems for profiling kinase activities. (**a**) FRET-based detection mechanism. The red circle marked "P" indicates phosphorylation. ECFP, enhanced cyan fluorescent protein; YPet, yellow fluorescent protein for energy transfer. (**b**) Asynchronous yeast cells expressing the biosensor were treated with 10 μM pheromone and imaged over a 2-h period. Single-cell FRET ratio responses are plotted with average response as a black line. (**c**) Representative phase-contrast and FRET ratio images of cells from the pheromone-stimulation experiments (scale bar, 5 μm). (**d**) Single-cell MAPK activity responses of yeast cells after 10 μM α-factor treatment. (**e**) Average response for pre-start, post-start, and early-S cells. The cartoons illustrate the morphology signatures of each group. Adapted from reference [38]

enables a phosphorylation-sensitive ratiometric readout (Fig. 6a, b). Compared to previous generations of biosensors, ExRai exhibited up to sevenfold enhancement in sensitivity and up to twofold expansion in dynamic range (Fig. 6c, d). Because only one fluorophore is used for probing each protein, this method enables simultaneous monitoring of up to six signaling proteins from single living cells.

Many transcription factors will translocate from cytosol to nucleus upon activation. This property can be exploited as a readout for the upstream kinase activities. The Heiser group demonstrated such an example, where they mapped the subcellular localization of forkhead box protein O1 (FOXO1) to evaluate AKT (also known as protein kinase B) signaling dynamics [40]. They fused a fluorescent protein to FOXO1 and determined nuclear fluorescence intensities to profile AKT signaling from single cells (Fig. 7).

Chemical probes for profiling signaling dynamics

Genetically encoded approaches require complex sample manipulations. Therefore, they cannot be readily adopted for analyzing sensitive cells, such as clinical samples, which limits their translational value. To circumvent this restriction, our group developed a chemical approach to perform dynamic profiling of intracellular signaling activities at the single-cell level [41]. The key element is a pair of cyclic peptide probes that can specifically recognize distinct epitopes on the target protein (Fig. 8a). We demonstrated the detection of AKT signaling as a proof of concept. A pair of cyclic peptides was developed to recognize the phosphorylated Ser473/474 site and a distal epitope on AKT. Simultaneous binding of the two peptide probes to the same protein enabled a FRET signal, which was contingent upon phosphorylation. These probes were packaged into cationic liposomes and delivered into the cytosol of U87 cells, where they provided the dynamic readout of AKT phosphorylation levels. A microwell single-cell chip was employed to achieve single-cell resolution (Fig. 8b, c). Using this platform, we showed that the heterogeneity of signaling activities exhibited time-dependent features, which would be completely masked by snapshot-type measurements (Fig. 8d, e). Our strategy eliminates the need for genetic modifications and can be easily utilized to analyze sensitive samples.

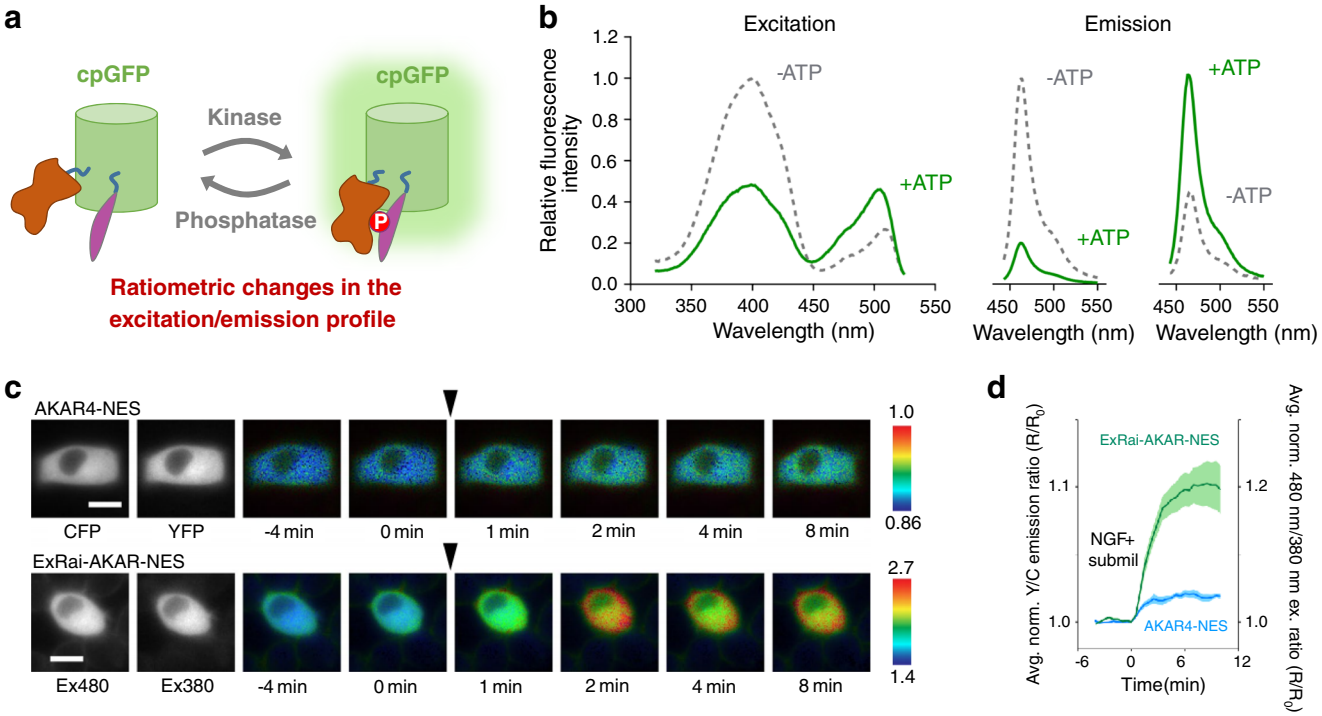

Fig. 6 Ratiometric reporting system based on a single fluorophore. (**a**) Modulation of circularly permuted green fluorescent protein (cpGFP) by a phosphorylation-dependent molecular switch. (**b**) ExRai fluorescence excitation spectra collected at 530-nm emission (left) and emission spectra collected at 380-nm (middle) and 488-nm (right) excitation without (gray) or with (green) ATP. (**c**) Representative pseudocolor images of previously reported FRET sensor, AKAR4-NES (top), and newly designed ratiometric reporter, ExRai-AKAR-NES (bottom). PC12 cells were treated with NGF (nerve growth factor) and imaged. CFP, cyan fluorescent protein channel; YFP, yellow fluorescent protein channel. (**d**) Average response profiles of the two biosensors. Adapted from reference [39] with permission from Springer Nature Publishing Group

Outlook

Fluorescence-based single-cell protein analysis has resolved the heterogeneity in many model systems and clinical samples. They have provided clarifying and mechanistic view of various biological processes, especially in cancer, where disease progression and resistance development are directly affected by the inter- and intra-tumoral heterogeneity.

Despite this success, the limitations of current single-cell technologies are also obvious. For instance, detection schemes relying on antibodies are always troubled by the inconsistent quality of commercial antibodies. Cross-reactivity among many types of antibodies also directly threatens the quality of multiplexed single-cell datasets. In terms of dynamic single-cell measurements, aside from the limitations to multiplexing and the omnipresent problem of photobleaching, they are also hampered by many other factors: detachable slides on the microengraving platform can only be employed on secreted proteins; genetically encoded protein reporters have the risk of causing severe perturbations to the target protein; and the liposomal delivery of our epitope-targeting peptides cannot be generalized to a wide range of cells. Ameliorating these drawbacks requires tremendous effort and remains an active research direction.

Finally, there is a pressing need to analyze proteins in the context of genomic, transcriptomic, and metabolic information, all at the single-cell level. Increasing evidence has demonstrated that integrating the detection of different categories of analytes allows a more holistic understanding of biological systems [42–44]. To date, it is still challenging to robustly combine all these types of measurements in the same single-cell platform. However, with the current pace of development

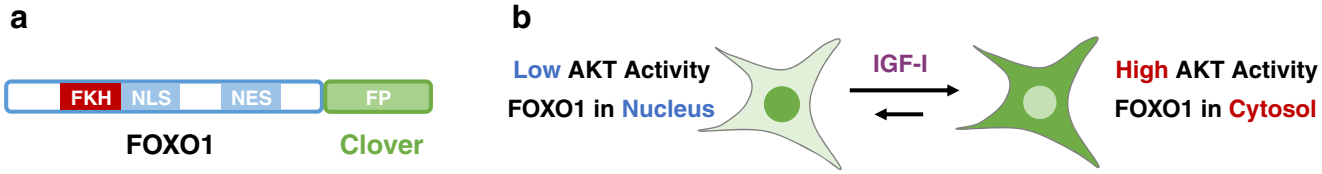

Fig. 7 The location of FOXO1 reflects the AKT (also known as protein kinase B) activity level and provides dynamic readouts at a single-cell level. (**a**) Construct of the FOXO1-clover fusion. (**b**) Mechanism of detecting AKT activities in living cells

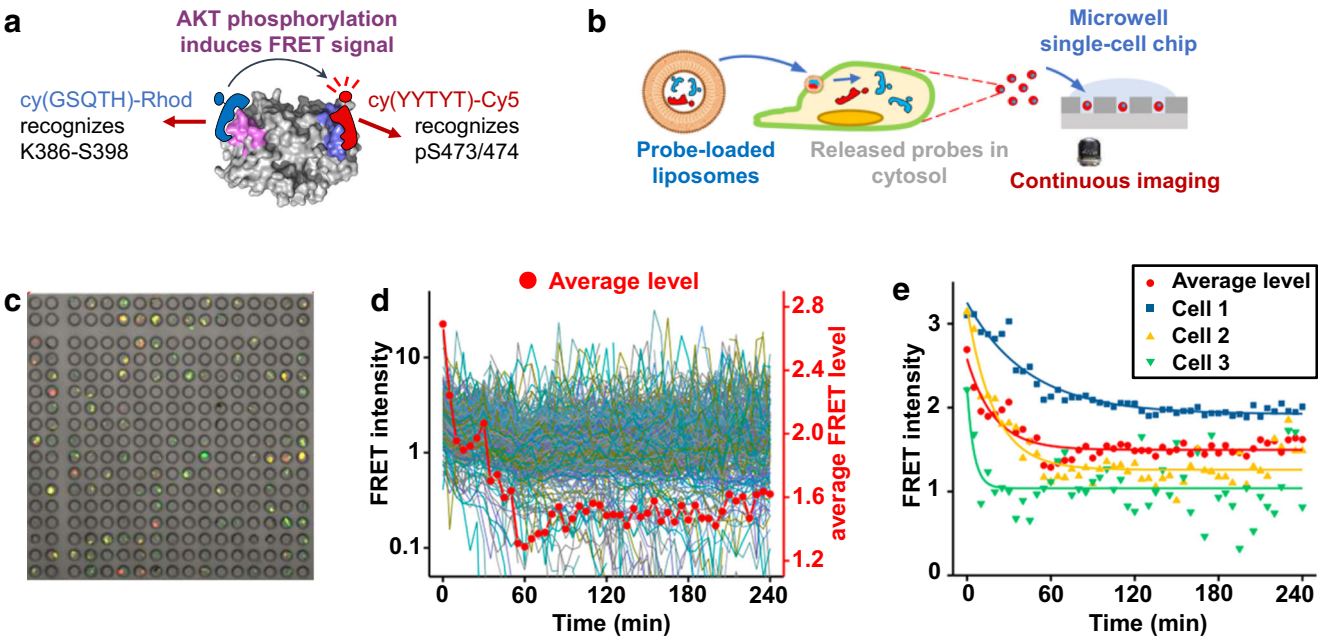

Fig. 8 Chemical methods for profiling AKT signaling dynamics from single cells. (**a**) Two epitope-targeting peptide probes enable dynamic probing of AKT phosphorylation status. (**b**) Intracellularly delivered peptide probes allow continuous profiling of AKT signaling dynamics from single cells. (**c**) Representative confocal image with overlaid fluorescence signals. (**d**) Single-cell AKT signaling trajectories from 280 serum-starved U87 cells. (**e**) Selected single-cell trajectories demonstrating the differential response rates among cells. Adapted from reference [41] with permission from American Chemical Society

in the field, there is good hope of addressing and resolving these outstanding issues.

Acknowledgements We thank Prof. Jin Zhang for valuable discussions.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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