Spectroscopic techniques for monitoring protein interactions in living cells

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Quantitative protein interaction analysis in living cells remains highly challenging as concentrations of interactions partners are difficult to quantify and to temporally modulate. In this review, the fundamental concepts for monitoring protein interactions in cells are discussed. Next to already well-established resonance energy transfer-based techniques, recent developments of approaches based on single molecule fluctuation and localization are presented. Moreover, the application of surface micropatterning and functionalized nanoparticles for solid phase type of protein interaction analysis in living cells are introduced. The complementary capabilities and limitations of these techniques and future directions based technological developments are discussed.

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Introduction
During the past decade, the scientific community has witnessed an enormous progress in our knowledge on protein–protein interactions. Proteomic methods for identifying interaction partners based on mass spectrometry, protein arrays and genome-wide screening by two-hybrid techniques have established detailed protein–protein interactions networks [1]. Moreover, elegant techniques for validating interactions are available including a broad spectrum of protein complementation techniques [2,3]. These techniques have proven powerful for confirming and visualizing interactions in the context of living cells and even in entire organisms [4]. Quantitative characterization of interactions, that is, the determination of equilibrium dissociation and kinetic rate constants, however, cannot be achieved by protein complementation assays because complexes are irreversibly trapped by these techniques. Truly mechanistic description of cellular processes requires precise parameterization of the formation and the stability of protein complexes within the cellular context.

For this reason, techniques for monitoring reversible protein–protein interactions are required.

A variety of powerful methods are available for quantitative protein interaction analysis in vitro, which are based on monitoring complex formation upon mixing interaction partners in defined concentrations. Thus, rate constants and/or equilibrium constants (association rate constant $k_{a}$, dissociation rate constant $k_{d}$ and equilibrium dissociation constant $K_{D}$) can be readily obtained. However, protein interactions are intricately regulated by the local environment within the cell, which controls post-translational modifications and may provide additional proteins, lipids, carbohydrates or nucleic acids modulating complex formation. Monitoring protein interactions within their physiological context of an intact, viable cell is substantially more challenging for several reasons. First, formation of protein complexes needs to be detected highly specifically in the presence of the whole cellular proteome. Second, protein concentrations within a living cell are difficult to quantify and cannot be varied readily, as required for probing the kinetics of protein interactions. Thus, most conventional approaches for quantitative protein interaction analysis cannot be applied for studying protein–protein interactions within cell. During the past decade, novel techniques to overcome these challenges have emerged, which exploit recent developments in ultrasensitive fluorescence detection, life cell fluorescence probe development as well as soft nanomaterial and micromaterial sciences. I will briefly introduce these concepts and then focus on exemplary applications and discuss current capabilities, limitations and future developments.

General considerations
Generic approaches for specifically detecting protein complex formation among the thousands of proteins and other biomolecules of the living cell are based on co-localization of the interaction partners on the molecular scale, that is, within dimension of 5–50 nm. Specific, non-invasive detection of proteins in cells is readily achieved by means of fluorescence microscopy with extremely high sensitivity down to the single molecule level. However, direct co-localization of proteins within protein complexes is obstructed by (i) the limitation of the spatial resolution by diffraction of light and (ii) rapid diffusion of proteins in the various compartments of the cell. These problems were very elegantly overcome by using Förster resonance energy transfer (FRET) as a direct spectroscopic reporter for proximity between a
donor and an acceptor chromophor on the nanometer scale (Figure 1). Co-localization of proteins can also be achieved by correlating fluctuations of molecules within diffraction-limited confocal volumes (Figure 2). Temporal as well as spatial cross-correlation of interaction partners labeled with spectrally different fluorophores allows quantifying interaction and dynamics of protein complexes. For proteins interacting at low concentrations, localization with a precision down to several nanometer is possible, thus providing the possibility to co-localize interaction partners within molecular dimensions (Figure 3). Alternatively, rather than trying to analyse homogeneously distributed proteins constantly diffusing in membranes or cellular compartments, microtechnological or nanotechnological approaches can be employed for locally enriching and immobilizing bait proteins within the cells as platforms for probing the interactions with a prey protein (Figure 4).

**Resonance energy transfer techniques**

FRET is based on the spontaneous transfer of energy from an excited state of one chromophor (the donor) via dipolar coupling to another chromophor (the acceptor), which emits the photon at a red-shifted wavelength (Figure 1a). The propensity for FRET strongly depends on the distance \( r \) (\( \sim r^{-6} \)), thus requiring very close (molecular) proximity between donor and acceptor chromophor (Figure 1b). Accompanied by the development of genetically encoded fluorescence labeling, FRET has been established as a key approach for protein interaction analysis in living cells with numerous applications and technical advancements during the past decade [5]. In particular the ability to probe FRET by fluorescence life time imaging (FLIM), which is becoming a standard feature in confocal microscopy, has contributed to much more reliable quantification of FRET signals down to a few percent. But even with FLIM, quantitative assessment of equilibria remains challenging as the FRET efficiency is not readily converted into concentration of protein complexes, which is required for the determination of binding constants. However, by combination with microinjection, the formation of protein complexes could be imaged by FRET and association rate constants in the cytosol could be quantified [6]. Recently, multi-parameter fluorescence imaging has been applied for studying protein–protein interactions in living cells [7]. By integrating information about fluorescence anisotropy, lifetime and intensity for donor and acceptor dye, much more reliable quantification of FRET efficiencies is possible. This method exploits the possibility to obtain information from individual molecules and complexes. Another fundamental limitation of FRET application for studying protein complexes arise from the high proximity required for efficient energy transfer. Despite tremendous progress in fluorescent probe development [8], reliable detection of FRET is not possible for donor–acceptor distances beyond \( \sim 9 \) nm (Figure 1c), thus limiting this approach for relatively small proteins and protein complexes. Larger distances are accessible by bioluminescent resonance energy transfer (BRET), which moreover eliminates background excitation of the acceptor fluorophore. For protein interactions in the plasma membrane, time-resolved FRET (trFRET) has emerged as a powerful alternative. This method is based on lanthanide ions as
FRET donors, which exhibit much longer fluorescence lifetimes than traditional fluorophores (millisecond vs. nanosecond regime) and very narrow emission bands. These features provide larger Förster radii (up to 10 nm) and very sensitive detection of sensitized fluorescence by time-gated detection. Thus, G-protein coupled receptor (GPCR) dimerization could be reliably detected in cells and in tissues [9,10]. Though BRET and trFRET are highly sensitive for detecting interactions, they are not useful for quantifying and for imaging interactions on the single cell level as the overall yield of light is relatively low. However, nanoparticles with engineered photophysical properties are emerging as highly sensitive probes for FRET applications [11]. Thus, gold particles are very efficient fluorescence quenchers over relatively long distances, while Lanthanide ion-based upconversion nanoparticles can be employed as background-free luminescence donors.

Correlation techniques
Protein complex formation results into a correlated movement of the interaction partners. This readout is exploited by various cross-correlation techniques, which map the motion of proteins by analyzing fluctuations due to variation in the number of proteins diffusing in and out of each volume element of the cell (Figure 2a). Fluorescence correlation spectroscopy is based on the autocorrelation of such fluctuations within a diffraction-limited (confocal) detection volume (approximately 0.2 fL), thus obtaining information about the diffusion dynamics of fluorescent molecules, as well as their concentration [12] (Figure 2b,c). As fluctuations result from individual molecules, the detection volume must not be overcrowded. For protein–protein interaction analysis, cross-correlation of the fluctuations obtained for two interacting partners labeled with spectrally distinct dyes (fluorescence cross-correlation spectroscopy, FCCS)
allows to quantify complex formation [13,14]. In combination with the ability to determine the absolute concentrations of the interaction partners from the autocorrelation curves, equilibrium constants are readily determined. In the past 5 years, several successful applications of FCCS to quantify interactions have been reported including transcription factors [15,16] and ligand–receptor interactions [17,18]. Even in living zebra fish embryos, the $K_d$ of a protein complex could be determined [19]. Despite improved detection schemes, for example, by including fluorescence lifetimes (lifetime cross-correlation) [20], absolute quantification by FCCS still remains technically challenging, as very precisely overlapping confocal volumes are required [21]. Relatively high brightness and photostabilities as well as fast maturation of fluorophores are required for reliable quantification. Combination of EGFP with mCherry is typically applied for live cell FCCS measurements.

While FCS and FCCS are based on a temporal correlation of fluorescence fluctuations within a given detection volume, spatial correlation within images is also possible. Image correlation spectroscopy (ICS) [22] was originally developed in order to analyse oligomerization (spatial ICS) or relatively slow concerted movements (temporal ICS), while image cross-correlation spectroscopy was applied for quantifying protein co-localization at diffraction-limited resolution (Figure 2d). With fast, sensitive image acquisitions becoming possible with confocal scanning microscopes, raster ICS (RICS) has been established as a method to analyse spatial and temporal single molecule fluctuations in living cells [23]. The principles of these methods have been recently summarized [24,25]. As for FCCS, cross-correlation of images from two protein species detected in different channels (cross-correlation raster image correlation spectroscopy, ccRICS, and spatio-temporal ICCS, STICCS) allows for quantitatively analysing the spatio-temporal dynamics of protein complexes [26**,27*,28,29] (Figure 2e,f). This technique can resolve diffusion and interaction dynamics in the sub-second regime and has been successfully applied for unraveling complex formation at focal adhesions [26**]. In combination with number and brightness analysis, the stoichiometry of protein complexes can be determined [27*,30,31]. While these single molecule fluctuation techniques have so far been mostly applied based on confocal detection, fast and ultrasensitive camera technology as well as novel detection schemes such as single plane

Figure 3

Protein interaction analysis by single molecule localization. (a) Diffraction-limited images of individual fluorescent molecules (left) and localization with nanometer precision by fitting the intensity distribution with a Gaussian function (right). (b) Frame-by-frame localization of interaction partners detected simultaneously in two different channels for co-localization/co-tracking (c) or PICCS (d) analysis. (c) Trajectories obtained by single molecule tracking providing information on the diffusion properties (left). Frame-by-frame co-localization within both channels (right, top) followed by co-tracking (right, bottom) yields the trajectories of protein complexes. (d) Spatial correlation of individual molecules by plotting the cumulative number of molecules B (red) detected in a distance $r$ from molecules A (blue). The fraction of complexes can be estimated from the exponential contribution in the resulting cross-correlation function.
illumination microscopy (SPIM) opens new exciting possibilities for their application to protein interaction analysis in living cells.

**Single molecule localization techniques**

Imaging of individual fluorescent molecules by far-field microscopy yields diffraction-limited intensity distributions, which can be fitted to determine the center of gravity with a precision far beyond the resolution of the image [32] (Figure 3a,b). The localization precision mainly depends on the number of detected photons versus the background signal, yielding typical accuracies within molecular dimensions (5–30 nm) and even below [33]. As a diffusive molecule will continuously change its position, high image acquisition speed is needed for precise localization. Localization of soluble molecules is only possible for relatively large species or within very crowded environments such as the nucleus [34]. For this reason, single molecule localization can discriminate binding of proteins to membranes or other structures of the cell. Thus, binding of cytosolic proteins to interaction partners at membranes can be visualized and the life-time of complexes can be quantified [35].

Since the propensity of statistic co-localization within distances below 100 nm is rather low, the formation of proteins complexes can be quantified by dual-color single molecule imaging [36]. Statistical co-localization of molecules within these dimensions depends on the density — i.e. the concentration — and therefore methods for reliably discriminating protein complexes from statistically co-localized molecules is required. For homogeneously distributed interaction partners, this is possible by simply correcting the observed co-localizations for the theoretical number of statistic co-localizations [37]. Since molecules are often heterogeneously organized within cells, other means have been developed for unambiguously identifying protein complexes. In case of diffusive molecules, this is possible by tracking co-localized molecules over multiple frames [38] (Figure 3c). This method has been very successfully employed to monitor and quantify receptor dimerization in the plasma membrane of living cells [39,40]. While the quantification of complexed versus the free interaction partners allows to directly determine equilibrium dissociation constants, co-tracking also has the potential to assess the dynamics of protein interaction as the length of co-trajectories correspond to the lifetime of a complex. Thus, the dynamic equilibrium of protein complex formation can potentially be fully analysed. Although this strategy has been successfully applied for short-lived complexes ($k_d > 1 \text{s}^{-1}$) [39,40], effective co-tracking is limited by photobleaching and tracking fidelity. Highly photostable fluorescent probes such as quantum dots (QDs) have been demonstrated to allow co-tracking over extended time periods with very high spatial and temporal resolution [41]. For quantitative protein interaction analysis, however, monofunctional QD are crucial [42], in order not to bias complex formation by avidity effects.

While single molecule co-localization and co-tracking is readily applicable up to densities of ~5 molecules/μm², at higher protein concentrations single molecule localization is obstructed by the convolution of the individual signals. Reducing the degree of labeling improves localization and tracking, but at the same time reduces the propensity for co-localization of labeled molecules. This problem can be overcome by combining dual-color co-tracking with local, intensive photobleaching. Labeled proteins and protein complexes diffusing into the photobleached area show conserved stoichiometry of labeling yet are thinned out to a level, which allows precise localization and tracking (‘thinning out clusters while conserving stoichiometry of
labeling" [43]. Using a model system, interaction probabilities down to 2.5% have been shown to be reliably quantified by this method [44].

However, out the large spectrum of available fluorescent proteins, only few are sufficiently bright and photostable for reliable single molecule tracking [45]. Particle image cross-correlation spectroscopy (PICCS) provides an alternative, robust strategy for quantifying proteins complex formation in living cells without the need for tracking individual complexes (Figure 3d). By analysing the cumulative probability of detecting interaction partner B in a distance r of interaction partner A, statistic and interaction-mediated co-localization can be efficiently separated [46]. Thus, the fraction of molecules in complexes and the (local) concentration of interaction partners are readily determined, providing the possibility to determine $K_d$. Though tracking is not required, this method still requires the ability to localize the entire ensemble of both interaction partners, which can be achieved with densities up to 5–10 molecules/μm$^2$. Higher densities require to localize molecules sequentially rather than simultaneously, which can be achieved by photoactivation localization microscopy (PALM) in fixed cells [47]. On this basis, pair-correlation PALM was recently established as a method for quantifying protein co-clustering [48].

### Spatial protein redistribution

Rather than trying to analyse the dynamic equilibrium of interaction partners statistically distributed within cellular membranes and the cytoplasm, recent approaches have exploited microtechnological and nanotechnological techniques for redistributing and immobilizing proteins within living cells. This has been achieved by patterning proteins within the plasma membrane using micropatterned functionalized substrates, or by nanoparticles injected into the cytoplasm.

The concept of protein interaction analysis by patterning proteins in the plasma membrane is depicted in Figure 4a. Cells expressing a bait protein in the plasma membrane are cultivated on a support presenting spatially resolved functionalities for capturing the bait protein via its extracellular domain. Thus, the bait protein is enriched and immobilized within a predefined micropattern. Interaction of prey proteins carrying a fluorescent tag thus can be quantified via the fluorescence contrast, preferably by surface-selective imaging using total internal reflection fluorescence microscopy. Moreover, the interaction dynamics of protein complexes can be probed by fluorescence recovery of photobleaching (FRAP) experiments: since the bait protein is immobilized, FRAP in the functionalized zones can be attributed to the exchange of bleached prey protein bound to the micropatterned bait by the non-bleached species in the cytosol, which is typically limited by complex stability (Figure 4b,c). Thus, dissociation rate constants can be readily quantified within a range of $\sim 1 \text{s}^{-1}$ to $10^{-3} \text{s}^{-1}$, which is very relevant for many protein complexes. Pioneering work in this field was carried out based on capturing of the membrane receptors such as CD4 by means of micropatterned antibodies [49]. Spatial reorganization of Lck, a cytosolic, membrane-anchored interaction partner, was observed and the interaction dynamics was further explored by FRAP and single molecule tracking (SMT) [49,50]. This method proved powerful for probing interactions with other transmembrane receptors [51]. Recently, the concept was extended towards more general and multiplexed bait patterning strategies, allowing to monitor signal activation in living cells [52].

Alternatively, spatial rearrangement can be achieved by injection of relatively large, functionalized nanoparticles into the cytosol of living cells. Bait protein can either be attached to the nanoparticle before injection, or complexes are captured directly from the cytoplasm by using suitable capturing methods [53]. In combination with magnetic control of the functionalized nanoparticle, this method was recently established for probing the stability of protein complexes in the cell by FRAP on the nanoparticle [54]. Thus, an in-cell solid-phase binding assay was achieved.

### Concluding remarks

Monitoring and quantifying protein interactions in the living cell remains challenging, as not only demanding and specialized experimental equipment, but also sophisticated data evaluation is required. Out of the four principal, highly complementary approaches I have presented here, the most suitable has to be carefully chosen to address a specific interaction in the cell. Energy transfer techniques are promising for probing relatively small and structurally well-defined protein complex, and quantification is possible at both high and low protein concentrations. Single molecule fluctuation-based techniques can detect and quantify interactions at low and medium expression levels in solution and at membranes, independent on the size and the stoichiometry of complexes. Single molecule localization-based techniques are particularly powerful at very low concentrations and with relatively slowly diffusing complexes. Interactions can be directly visualized, allowing much more intuitive data evaluation compared to correlation techniques. Moreover, the dynamic equilibrium of association and dissociation can potentially be fully characterized. Yet, optimization of labeling and imaging is required, as background and photobleaching is a major problem for single molecule imaging techniques. Importantly, all single molecule-based techniques directly provide concentrations, which is a prerequisite for quantitative analysis. The dynamics of protein complexes can be studied for relatively transient interactions ($<1 \text{s}$). By contrast, protein rearrangement by micropatterning or nanoparticles allows probing the stability of high-affinity
protein complexes in cells. Measurements are relatively simple and intuitive, but currently the required micromaterials and nanomaterials are not commercially available.

All these approaches profit from the rapid development in the field of microscopy techniques and fluorescence probe development. Fast cameras for localization-based super-resolution imaging [55], novel approaches for optical sectioning for three-dimensional single molecule localization and tracking [56] as well as fast confocal imaging beyond the diffraction limit by stimulated emission depletion (STED) [57] will boost protein-interaction analysis on the single molecule level. Maybe even more importantly, next to optomized fluorescent proteins and organic dyes, nanoparticles providing a broad spectrum of photophysical properties are emerging as promising probes for protein interaction analysis. In this context, more efficient postranslational labeling techniques will play a pivotal role, as well as suitable membrane-permeable probes [58]. The application of nanoparticles for unbiased protein interaction analysis requires strategies for efficient conjugation to target proteins in a defined 1:1 stoichiometry without affecting their function [53,59]. On the basis of recent and future developments of nanomaterials as selective, multifunctional probes for protein interactions in combination with in-cell delivery and targeting, novel tools for probing protein interactions in living cells will soon be available. With these powerful emerging techniques, the future of protein interaction analysis in living cells is bright.

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