Imaging the secretory pathway: The past and future impact of live cell optical techniques

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Received 21 December 2004; received in revised form 21 April 2005; accepted 27 April 2005
Available online 16 May 2005

Abstract

Classically, the secretory pathway has been studied using a combination of electron microscopic, biochemical and genetic approaches. In the last 20 years with the arrival of molecular biology and epitope tagging, fluorescence microscopy has become more important than previously. Moreover, with the common availability of Green Fluorescent Protein (GFP) and confocal microscopes in the last 10 years, live cell imaging has become a major experimental approach. This review highlights the impact of the recent introduction of single-cell quantitative time-lapse imaging and photobleach techniques on the study of the secretory pathway, and the potential impact of those optical techniques which may play a significant future role in the study of the Golgi apparatus and the secretory pathway. Particular attention is paid to techniques (Fluorescence Resonance Energy Transfer, Fluorescence Correlation Spectroscopy) which can monitor protein–protein interactions in living cells.

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Keywords: Golgi apparatus; Green fluorescent protein; Microscopy; Fluorescence

1. Introduction

The major organelles of the secretory pathway were initially defined by George Palade, Keith Porter, Albert Claude and contemporaries in studies based in large part on electron microscopy and membrane fractionation (see [1] and references therein). Later work by James Rothman, Randy Schekman and many others defined much of the biochemical basis of the transport steps between these organelles using a combination of yeast genetics and in vitro systems (reviewed in [2–5]).

With the advent of GFP [6], time-lapse imaging of living cells has made a major contribution to our knowledge of trafficking between the Golgi apparatus and other intracellular organelles. In particular, live cell imaging has emphasized the important roles of cytoskeleton in trafficking between organelles (e.g., [7–10]), and the importance of large pleiomorphic transport carriers for some transport steps (reviewed in [11], see also Rodriguez-Boulan and Müsch, this issue). What follows is intended to highlight both the past use of live cell optical techniques in studies...
of the secretory pathway and the potential future role of new optical techniques (Table 1). Descriptions of commonly used wide-field, confocal and deconvolution systems and methodologies as used in most studies to date are readily available (see [12] and references therein) and will not be repeated. Particular emphasis will be placed on techniques capable of directly monitoring protein–protein interactions in living cells. It is hoped that application of these perhaps more exotic techniques may help to bridge the gap between the primarily morphological information obtained to date from direct visualization and the detailed base of biochemical knowledge we now possess from other sources.

2. Use of time lapse imaging to visualize cargo transfer between compartments of the secretory pathway

Time lapse imaging of living cells has been widely utilized in those subfields of cell biology where appropriate fluorescent probes could be easily introduced (as in studies of the endocytic pathway or of calcium signaling) or where

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Major optical techniques applicable to the secretory pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Technique</strong></td>
<td><strong>Primary uses</strong></td>
</tr>
<tr>
<td><strong>Techniques generally used by cell biologists</strong></td>
<td></td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>Visualization of protein distribution in fixed cells. Usually nonquantitative.</td>
</tr>
<tr>
<td>Directly labeled protein</td>
<td>Visualization of protein distribution in fixed or living cells.</td>
</tr>
<tr>
<td>(GFP fusions or in vitro fluorescent derivatives)</td>
<td>Quantification is less difficult than that of antibody binding as epitope accessibility is not an issue.</td>
</tr>
<tr>
<td>Time-lapse imaging (non-quantitative)</td>
<td>Change in location of a protein in a living cell over time. Motion/dynamics of visible organelles and structures in living cells.</td>
</tr>
<tr>
<td>Time-lapse imaging (quantitative)</td>
<td>Change in location of a protein in a single living cell over time. Typically used to determine kinetics of transfer of a bolus of protein between organelles. Varying degrees of sophistication can be applied to this analysis ranging from determination of general timescale to fitting mathematical models based on systems of differential equations.</td>
</tr>
<tr>
<td>Fluorescence Recovery after Photobleaching (FRAP)</td>
<td>Often used to determine ability of a protein to diffuse within a single membrane or within cytosol, effectively a probe of local environment. Also commonly used to visualize kinetics of exchange of a protein between two compartments in cases where the trafficking of the protein cannot be effectively synchronized. The two compartments can be organelles of the secretory pathway or an organelle and cytosol.</td>
</tr>
<tr>
<td>Fluorescence Resonance Energy Transfer (FRET)</td>
<td>Used to locate particular protein–protein interactions inside cells. Requires a fluorescent tag on each of the proteins. FRET is sensitive to the exact distance between these fluorescent tags and will fail if they are too far apart.</td>
</tr>
<tr>
<td><strong>Techniques generally requiring detailed biophysical background</strong></td>
<td></td>
</tr>
<tr>
<td>Fluorescence Correlation Spectroscopy (FCS)</td>
<td>FCS can determine diffusion coefficients, degree of clustering of a molecule and molecular concentrations. It can have advantages over FRAP for fast-diffusing (i.e., cytosolic) proteins and when the fluorescent protein is a mix of fast-diffusing and slow-diffusing species. Requires a specialized apparatus. Data analysis non-trivial.</td>
</tr>
<tr>
<td>Fluorescence Cross-Correlation Spectroscopy (FCCS)</td>
<td>FCS variant used to detect complexes between two proteins each carrying a different fluorescent tag. Unlike FRET the technique is insensitive to the precise location of the fluorophores on the interacting proteins.</td>
</tr>
<tr>
<td>Image Correlation Spectroscopy (ICS)</td>
<td>Same uses as FCS but can be implemented with standard confocal microscope capable of rapid image acquisition. Can give some spatial resolution as well as directional information in the case of protein flow. However less capable than FCS of analyzing fast (i.e., cytoplasmic) diffusion.</td>
</tr>
<tr>
<td>Image Cross-Correlation Spectroscopy (ICCS)</td>
<td>ICS-based technique to detect interaction between two proteins tagged with different fluorophores. Requires a confocal microscope capable of rapid simultaneous acquisition of two channels. Current generation confocal microscopes are generally capable of this. However specialized mathematical expertise and custom computer software is needed.</td>
</tr>
<tr>
<td>Fluorescence Lifetime Imaging Microscopy</td>
<td>Primarily used as a sensitive technique to detect FRET. Requires specialized equipment that is not yet generally available.</td>
</tr>
<tr>
<td>Total Internal Reflection Microscopy (TIRF)</td>
<td>Used to detect events occurring very close to the cell surface (e.g. vesicle fusion). Can also be used to visualize single fluorescent molecules on or very near the cell surface.</td>
</tr>
<tr>
<td>I^® M, 4Pi, STED</td>
<td>Experimental techniques for ultra-high resolution imaging.</td>
</tr>
</tbody>
</table>
fluorescent probes were not needed (e.g., cell motility). Thus, equipment and methodologies for time-lapse imaging already existed when GFP-tagging was introduced to study the secretory pathway. Of greatest importance were the ready availability of computer-controlled charge-coupled diode (CCD) and confocal microscope systems capable of automatically acquiring time-lapse series of up to several hundred images with intervals between images of one to several seconds. These image intervals proved sufficient in practice to track individual transport intermediates.

2.1. ER to Golgi trafficking

Electron microscopic studies of cells [13] and permeabilized cell systems [14] showed that cargo proteins accumulated in complex membranous structures immediately after exit from ER. These were termed vesicular–tubular complexes (VTCs) or intermediate compartment. On the basis of an immunofluorescence pulse-chase study, Saraste and Svenson proposed that VTCs moved on microtubules to carry cargo to the Golgi apparatus [15]. However, many in the field considered the VTCs to be a stable compartment from which COPI vesicles transported cargo to Golgi (see [16,17] for contemporaneous reviews).

The introduction of GFP permitted direct visualization of the VTCs in living cells [7,9]. Transport intermediates with the characteristics of VTCs and containing a GFP-tagged cargo protein were seen to move as intact units from the cell periphery to the Golgi apparatus (see Fig. 1). This movement depended on microtubules [7,9] and on dynactin/dynein [9]. These structures had many of the properties of VTCs and colocalized sequentially with the coat protein COPII and COPI [7]. More recent light microscopic studies have emphasized that transport carriers are produced repeatedly from specialized ER exit sites that persist over time [18,19].

These studies were taken at the time as strong evidence that VTCs themselves were the ER to Golgi transport carriers [20]. However, proof that the pre-Golgi intermediates described in these light microscopic studies were indeed VTCs required correlative EM (i.e., the identification of the same structure in the same cell by both live cell fluorescent imaging and electron microscopy). This was reported recently by Mironov et al. [21].

Live cell imaging therefore has made a major contribution to our knowledge of the means by which proteins are transported from the ER to the Golgi, allowing the identification of the transport intermediates as VTCs and their interactions with cytoskeleton. Due to the small size of these intermediates, some important questions including the sorting and recycling of COPI interacting proteins [22] have proved difficult to address by light microscopy, although progress has been possible [23].

2.2. Golgi to cell surface trafficking

Similar studies identified post-Golgi transport carriers filled with VSVG or other cargoes and targeted towards the cell surface [24,25]. These structures could be large (sometimes several microns), making them readily detectable in live cell imaging experiments. Toomre et al. [25] described two varieties of structures (elongated tubules and punctate structures). Hirschberg et al. [24] were able to identify both the detachment of elongated tubular carriers from the Golgi apparatus and their fusion with the plasma membrane. The kinetics of this final fusion step were analyzed in detail by Toomre and Schmoranzer [26,27]. Kreitzer demonstrated that basolateral transport intermediates fused only to the lateral surface of polarized cells and that cytoskeletal interactions were important in this targeting [8].

In addition to Golgi/cell surface trafficking, it proved possible to visualize trafficking pathways leading from the Golgi apparatus to endosomes. Live-cell studies in which clathrin or the clathrin vesicle-associated protein GGA was visualized along with the mannose-6-phosphate receptor revealed tubular structures leaving the Golgi apparatus [28,29]. These structures moved away from the Golgi apparatus and underwent apparent transient “kiss-and-run” interactions with endosomes. Adaptor Protein 1 (AP-1)
containing transport intermediates emerging from the Golgi apparatus also could be seen to merge with endosomes [30]. Tubules containing multiple clathrin-coated pits were described previously from tomographic reconstructions of trans-Golgi membranes, but it was assumed from these reconstructions that the transport intermediate was a coated vesicle that budded from the tubule [31]. These light microscopic studies implied the presence of Golgi-to-endosome transport intermediates with both coated and uncoated membranes on the same intermediate. It is unknown whether the targeting information on the intermediate (i.e., motor, tether and SNARE proteins) is associated with clathrin.

In neurons, a great variety of structures were seen with punctate or tubular morphologies. These structures could be visualized traveling down axons at rates (1–5 μm/s) consistent with the known rate of microtubule motor-mediated fast axonal trafficking [32]. Long tubules carried the protein GAP-43, while punctate structures apparently below the resolving power of light microscopy (and thus potentially classical coat-mediated transport vesicles) carried the synaptic vesicle protein synaptophysin [32]. Importantly, many of these structures were observed to bud directly from the Golgi apparatus in the cell body. This is not true for all structures visualized, thus it cannot be ruled out that some of these proteins originate from endosomal membranes or cell surface rather than directly from Golgi.

Proteins targeted to neuronal spines [33], to axonal growth cones [34], and to synaptic vesicles [32] seem to use transport intermediates associated with different kinesins. Thus, in neurons, there may be more post-Golgi transport intermediates than the two minimally postulated in polarized epithelial cells (see Enrique-Boulan and Musch, this issue).

Thus, live cell imaging has been especially fruitful in analyzing the relationship of post-Golgi intermediates with cytoskeleton. This relationship is often cell-type and morphology specific, as the microtubule and actin cytoskeletons will be organized very differently in different types of cells (e.g., polarized epithelial cells, neurons, muscle cells or fibroblasts). Most work has been restricted to flat almost two-dimensional tissue culture cells since repetitive acquisition of a single image plane at intervals of 1 s or better are easily achieved with most current imaging equipment. Acquisition of complete three-dimensional data sets at rapid (<10 second) time intervals is required for time-lapse imaging of some cell types such as polarized epithelial cells. This rapid 3-D imaging has proven possible although it has required the development of special methodologies [8,35].

2.3. Retrograde (Golgi to ER trafficking)

A retrograde pathway from the Golgi to the ER was proposed initially to explain the retention of ER proteins in the early secretory pathway. The sequence KDEL was found on many soluble ER-retained proteins, and a KDEL-interacting protein termed the KDEL receptor was shown to be Golgi-localized [36,37]. It was proposed that the KDEL receptor could bind escaped KDEL-containing proteins and retrieve them to the Golgi apparatus. (reviewed in [17]). The rapid redistribution of Golgi enzymes to the ER by the drug brefeldin A showed that Golgi proteins could be transferred to ER [38]. Experiments with toxins provided direct evidence that such a retrograde pathway could function constitutively [39].

Various lines of evidence indicated that this retrograde pathway was mediated by COPI vesicles. Later, the -KKXX sequence was postulated to be a retrieval sequence targeting Golgi proteins into ER-targeted COPI vesicles [40–42]. Since brefeldin A induced the loss of COPI [43] and the formation of microtubule/kinesin-dependent tubules from the Golgi apparatus [44], its effects were difficult to explain by this model except as a nonphysiological pharmacologically-induced phenomenon.

Time-lapse imaging of a GFP-tagged version of the KDEL receptor was reported in HeLa cells [45]. Sciajk et al. observed tubules extending from Golgi and containing a GFP-tagged version of the KDEL receptor (KDELr–GFP) in non-drug-treated living cells. These tubules resembled the BFA-induced tubules but detached from the Golgi apparatus prior to moving into the periphery. KDELr–GFP tubules were not observed in nocodazole-treated cells, implying a microtubule requirement also found for the BFA-induced tubules [45]. Rates of movement of KDELr–GFP containing tubules were fast (>1 μm/s) [45] indicating a possible role for microtubule motors such as kinesin.

White et al. observed similar tubules containing GFP-tagged KDEL receptors [46]. They could also image tubules which contained both GFP-tagged Rab6 and shiga toxin. Microinjection of antibody against COPI blocked the recycling of KDEL receptor from the Golgi to the ER but had no effect on the cycling of a GFP-tagged Golgi enzyme [47]. These results were interpreted as evidence that at least two Golgi to ER pathways exist, one pathway dependent on COPI and one pathway that involved Rab6 but did not depend on COPI. Rab6 could participate in tubule formation by recruiting a kinesin [48]. This would be consistent with previous experiments in which it was found that microinjection of an anti-kinesin antibody blocked formation of BFA-induced tubes [44].

While the Golgi to ER movement of COPI vesicles would be difficult to observe by light microscopy due to their small size and due to the short distance needed to reach ER, their appearance would be unlikely to be that of elongated tubules. Therefore, live cell imaging experiments indicate small COPI vesicles do not provide the sole means of retrograde trafficking from the Golgi to the ER. However, a major (even majority) role for COPI vesicles cannot currently be excluded on the basis of the microscopic data. No careful quantitative analysis has been done which both measures the flux between the ER and Golgi of a GFP-
tagged protein and attempts to account for this flux in visible tubular transport intermediates. While an attempt to quantitate the fraction of cargo carried by visible transport intermediates would be difficult, it is important to place bounds on the proportion of the total retrograde traffic that goes by short-distance pathways not detectable by light microscopy.

2.4. Intra-Golgi trafficking

While cargo proteins are known to enter the Golgi apparatus at the cis face and exit at the trans face, the means by which these proteins traverse the Golgi is still controversial. In one class of models, the cargo remains in a single Golgi cisterna which matures and progresses towards the trans end while Golgi enzymes are relocalized via COP1 vesicles [49] or by diffusion through tubular connections (see Mironov et al. this issue). Alternatively, the cargo is transferred between static cisternae by shuttle vesicles [50].

In principle, given sufficient resolving power, cisternal maturation could be distinguished from other models by direct visualization of trafficking through the Golgi apparatus. Unfortunately, the interior of this organelle provides a major challenge to light microscopy. Although the Golgi is an extended organelle, it consists of a ribbon of stacks of approximately 50 nm-thick cisternae. A stack might contain 10 cisternae if it is very thick, giving in the best case a thickness of not much more than 0.5 μm. Thus, it is marginally possible to resolve the separation between cis and trans markers. Light microscopy, even of a living cell, has not given to date a clear answer as to whether a cargo protein is moving through the stack in a maturing cisterna or via other means. Microscopy-based studies attempting to address this issue have in general utilized electron microscopy as well (e.g., [51]).

3. The use of photobleaching and photoactivation to probe local environment

Under typical imaging conditions total GFP fluorescence is a linear function of the illumination intensity, the detector efficiency and the number of molecules. Thus, changes in the relative number of GFP molecules in a sufficiently large organelle like the Golgi apparatus can be measured over time [24,52]. For such a change to be detected, the total fluorescence pattern must start in a non-equilibrium state. This can be done using the temperature-sensitive ts045 variant of VSVG in combination with temperature blocks as previously described. Pharmacological perturbations (such as brefeldin A) can also be used.

Such “tricks” tend to be non-general, that is, they are specialized to specific model systems. Also, they do not provide the means to visualize motility of a protein within a single large membrane (e.g., plasma membrane or ER). The related techniques of photobleaching and photoactivation do provide general methods to create non-equilibrium states in which microscopic motions can be analyzed.

3.1. Fluorescence recovery after photobleaching

Photobleaching followed by analysis of recovery kinetics can be used to detect and analyze molecular motions within a single membrane [53] or flux between different intracellular compartments [24] provided the motion is over distances or between structures resolvable by light microscopy.

Photobleaching techniques (often referred to as fluorescence recovery after photobleaching (FRAP) or fluorescence photobleaching and recovery (FPR)) have a history that well predates GFP. The fluid mosaic model of the plasma membrane [54] originally predicted that integral membrane proteins could be mobile. Classic experiments by Frye et al. [55] established that when two cells labeled with spectrally distinguishable fluorescent antibodies were fused, the antibodies were initially confined to two hemispheres of the heterokaryon but mixed over a timescale of several minutes [55]. Subsequent to these first experiments, FRAP was used to confirm that a large fraction of many cell-surface proteins were mobile, and to measure their diffusion coefficients. These diffusion coefficients were usually an order of magnitude or slower than expected for unhindered diffusion in a lipid bilayer [56,57], and it was postulated that barriers of various kinds hindered diffusion at the cell surface (reviewed in [58]).

While FRAP of proteins on intracellular membranes was technically extremely difficult in the pre-GFP era, Storrie et al. were able to microinject a fluorescent antibody directed against the cytoplasmic domain of VSVG. They observed a diffusion coefficient of $1 \times 10^{-9}$ cm$^2$/s with a very small fraction of the protein being immobile in the Golgi apparatus [59]. Cole et al. [60] made the first measurements of the mobilities of resident Golgi proteins using GFP tags. They reported that GFP-tagged versions of the KDEL receptor, galactosyl transferase and mannosidase-2 correctly localized to Golgi and could be imaged. FRAP of small Golgi subdomains gave diffusion coefficients of $3 - 5 \times 10^{-9}$ cm$^2$/s consistent with unrestrained diffusion in a lipid bilayer [56], and little or no immobile fraction [60]. The same proteins could be redistributed to the ER if the Golgi apparatus was destroyed by treatment of the cells with brefeldin A. In ER, these proteins showed similar high mobilities to those measured in Golgi [60]. Thus, the membranes of the secretory pathway (ER and Golgi) did not seem to have the same barriers to diffusion as found on the cell surface.

One problem that has been addressed with FRAP experiments is the nature of ER retention of misfolded proteins. VSVG is temperature sensitive and accumulates in the ER at 4 °C due to interactions with calnexin and other chaperonins. The mechanism of this retention was unknown but proposed mechanisms included incorporation of the protein into aggregates containing chaperonins or through
association to chaperonins bound to an ER matrix ([61] and references therein). Nehls et al. [62] used FRAP of VSVG–GFP to explore the mechanisms of retention of this cargo protein under conditions of misfolding. Surprisingly, VSVG–GFP was highly mobile whether it was retained in a misfolded state at its nonpermissive temperature or was retained in a fully-folded state in brefeldin A treated cells at the permissive temperature. These results indicate that interactions with chaperonins sufficient to retain the protein in ER need not lead to immobilization of the misfolded protein. VSVG–GFP could be immobilized however by expression of a dominant-negative BiP mutant lacking GTPase activity [62]. These results were consistent with a role for transient (rather than strong) ER matrix binding in retaining these proteins.

FRAP can also quantitate transient associations of peripheral membrane proteins with membranes. In [63], a GFP-tagged Arf1 was shown to rapidly associate with and dissociate from Golgi membranes as measured by photobleach. In [64], similar techniques were used to show that the coat protein (and Arf1 effector) COPI also associated only transiently with Golgi membranes (residence time 45 s in Chinese hamster ovary cells).

3.2. Photoactivation

Photoactivatable fluorophores (reviewed in [65]) have been used extensively in monitoring the movement of cytoskeletal components, e.g., [66,67]. In most cases, the fluorophore is “caged” and non-fluorescent but can be uncaged by exposure to long-wavelength ultraviolet light applied to a spatially restricted region of the target cell. Such use typically has required the in vitro labeling of a protein followed by its introduction into a cell.

Recently, an efficient photoactivatable GFP was described [68]. It is initially fluorescent only when excited by short-wavelength light (near 400 nm) and is invisible when imaged with a 488-nm argon laser line. After photoisomerization is induced with intense 400 nm illumination, the excitation spectrum is shifted and the GFP becomes visible with 488 nm illumination. Other recently-engineered GFPs with similar properties also exist [69]. Photoactivation potentially has advantages over photobleaching in situations where the intent is to visualize the fate of a pool of protein originating from a small area or when observations must continue for a sufficiently prolonged period of time that substantial synthesis of new GFP-tagged protein will occur. Such newly-synthesized protein could affect the experimental design if tagged with conventional GFPs, but would be invisible if tagged with a photoactivatable GFP.

3.3. Summary

For each of the membranes described above (ER, Golgi and plasma membrane), initial FRAP experiments were aimed at probing the then unknown membrane environment. Subsequent experiments have attempted to interpret effects on motility of a protein in terms of interactions with other proteins (e.g., potential interactions of misfolded VSVG with chaperonins). FRAP has also been used to monitor the gain or loss of peripheral membrane proteins from membranes with a major focus of interest being the nature of the protein–protein interactions required for the membrane interactions. Selective pharmacological or mutational ablation of hypothetically interacting proteins has proved helpful in some cases (e.g., [62]). A major caveat of the described experiments is that the protein–protein interactions of interest are inferred but not directly shown. The optical techniques used do not directly monitor these protein–protein bindings. Rather what is measured is an increase or decrease in the motility of the monitored protein.

4. High throughput light microscopy

The availability of complete sequenced genomes from multiple organisms of interest to cell biologists combined with “high-throughput” recombinant DNA techniques has made possible large-scale epitope or GFP tagging of unknown or poorly characterized proteins. This has made possible rapid screens in mammalian cells [70] and in yeast [71] for protein localization. The Peperkok group has recently described a functional screen in which multiple GFP-tagged proteins are overexpressed and cells assayed for slow transport of a cargo protein to the cell surface [72]. Microscope-friendly techniques have been devised for transfection of large numbers of siRNAs [73]. Thus rapid and systematic knockout screens are possible using light microscopic functional assays. Recently yeast two-hybrid screens and mass-spectrometry-based approaches have facilitated the rapid determination of binding partners of unknown proteins (reviewed in [74]). However, the data obtained are initially devoid of significant physiological context and may serve only as starting points for further investigation via traditional means. Biochemistry both in whole cell and in vitro systems provides powerful tools for this more detailed characterization but does not easily provide the ability to monitor protein–protein interactions in real physiological contexts (i.e., living cells) with spatial resolution.

5. Emerging light microscopy approaches

Much of the knowledge gained in vitro of the molecular machines that organize transport between organelles of the secretory pathway is defined in terms of protein–protein interactions. Arf1 binds to coat proteins such as COPI, AP-1 and GGA as an essential part of the recruitment of these proteins to membranes (reviewed in [75]). Coat proteins subsequently interact with the cytoplasmic domains of
cargoes [75]. Other sequential interactions involving Rabs, tethers and SNARES are required for membrane docking and fusion (reviewed in [76]). There has been surprisingly little effort at directly monitoring these and other protein–protein interactions of relevance to the secretory pathway in living cells using optical techniques. The combination of time-lapse imaging and in vitro systems is one possible solution (see [33] for an example). This approach has been used frequently in the cytoskeletal community and could be utilized more in studies of the secretory pathway. However, such in vitro approaches are beyond the scope of this review.

Light microscopic techniques capable of reporting molecule–molecule interactions in intact living cells have been extensively used in some fields of cell biology, for example, ligand–receptor binding at the cell surface. Fluorescence Resonance Energy Transfer (FRET) is the most widely-utilized and well-known optical microscopy technique used to detect protein–protein interactions in intact cells. FRET is relatively simple to implement but is very sensitive to the distance between the fluorophores on the interacting proteins and so may not work for all pairs of complexed proteins. Other techniques have recently been developed based on Fluorescence Correlation Spectroscopy (FCS). These techniques are less sensitive to the precise location of the fluorophores on the complexed protein but have the disadvantage of requiring considerable mathematical sophistication to use.

6. The use of fluorescence resonance energy transfer to measure protein–protein interactions

6.1. Fluorescence resonance energy transfer

Fluorescence Resonance Energy Transfer (FRET) occurs when an excited fluorophore transfers its energy to another fluorophore via a quantum tunneling phenomenon rather than emitting it (Fig. 2A). This phenomenon was first described theoretically by Förster [77] and was verified experimentally by Stryer [78]. FRET between chemical fluorophores has been frequently used to detect interactions between proteins in vitro, on the cell surface or inside cells. Thus, FRET between GFP variants would seem ideal to spatially and temporally localize protein–protein interactions important to the operation of the secretory pathway. However, technical limitations related to the maximum distance over which FRET can be easily detected have limited its use (see Fig. 2).

To create conditions in which FRET can occur, two proteins are labeled with distinct fluorophores such that the emission spectrum of one fluorophore (the donor) overlaps with the excitation spectrum of the other. Given these conditions, energy transfer from the donor to the acceptor can occur over short distances, resulting in emission at the acceptor’s normal emission wavelength. Since this process competes with direct emission of a photon from the donor, the time the donor spends in an excited state is also reduced.

FRET can thus be detected by many means (e.g., monitoring a shift in wavelength of the emitted light, by detecting dequenching of the donor after photobleaching the acceptor, by measuring directly the time the donor spends in the excited state or by measuring depolarization of emitted light). A comprehensive recent summary of techniques for detecting FRET is found in [79]. The most commonly used methods for implementing FRET, along with needed controls are detailed in [80,81].

Energy transfer between donor and acceptor depends on dipole–dipole interactions between the two molecules and therefore falls off sharply (inverse sixth power) with distance (see Fig. 2B). FRET efficiency between molecules can be described by the distance at which 50% energy transfer occurs, usually denoted as $R_0$. $R_0$ for FRET between the most commonly used fluorophores is usually in the range from 2 to 8 nm and is less than the size of many protein molecules. Tables of $R_0$ for many common dyes are found in [80]. $R_{05}$ between GFP variants can be found in [82].

Several GFP variants (e.g., CFP/YFP) make good FRET pairs with $R_0$ of up to 4–5 nm (4.92 nm for CFP/YFP) [82]. This is comparable to the $R_0$ of 6.0 nm between Cy3 and Cy5 [83]. In the case of fluorescent protein (FP) FRET, a substantial portion of this distance (>2 nm) is spent traversing the two FP molecules. Fig. 2C shows graphically the relationship between $R_0$ and the size of typical GFP-tagged protein molecules. It should be apparent from inspection of this figure that GFP–GFP FRET between two interacting fusion proteins will depend critically on the placement of the GFPs on the protein molecules for proteins large with respect to $R_0$. Since many of the coats, SNAREs, tethers and other proteins of interest in the secretory pathway are significantly greater than 5 nm in size, this limited $R_0$ may be an important factor limiting the use of FRET in studies of the secretory pathway.

Despite technical problems, there are reports of successful use of GFP–GFP FRET to monitor protein–protein interactions in the secretory pathway. There are several studies of oligomerization of specific subunits of protein complexes in the ER or Golgi apparatus (e.g. [84–87]). Sato et al. [88] have used FRET in vitro to monitor the interaction of the SNARE protein Bet1p with the COPII component Sec24p. Majoul et al. have used FRET to investigate the interactions between COPI, Arf1 and the Arf–GAP in the formation of COPI-coated vesicles. In this study FRET was shown between GFP variants of Arf1, the Arf–GAP, COPI and cargo and it was concluded that COPI vesicles contain Arf1 its GAP and COPI in a single tripartate complex [89]. These studies demonstrate that FRET is a viable technique for monitoring protein–protein interactions within the secretory pathway although whether energy transfer is detectable may depend critically on the...
location of the fluorescent tag on particular proteins (see Fig. 2C).

6.2. Fluorescence lifetime imaging microscopy

After absorbing a photon a fluorophore enters an excited state rather than instantly re-emitting. The excited state typically decays exponentially over a timescale of nanoseconds and the emission photon is emitted when this excited state decays. The decay time of the excited state differs substantially between fluorophores. The fluorescence lifetime for Texas Red is 4.21 ns and for Cy3 is 1.36 ns [90]. GFP variants also have variation in fluorescence lifetimes (e.g., 1.32 ns for CFP and 3.69 ns for the YFP5 variant [91]). Fluorescence Lifetime Imaging Microscopy (FLIM) is a technique in which the lag between excitation with a pulsed or rapidly varying light source and arrival of emission photons is measured (see Bastiaens and Squire [92] and references therein for a more complete description). FRET competes with photon emission and thus shortens the duration of the excited state of the donor (see [92]). Multiple exponential components can be fitted to a decay curve. Thus, it is possible in principle to distinguish efficient FRET from a small population of molecules from inefficient FRET from the entire population. Fluorescence lifetimes may also be measured from photobleach kinetics since the photobleach rate is inversely proportional to fluorescence lifetime. A time series of images is acquired during the photobleach process and rate constants determined by fitting to the typically exponential decay of photobleaching.
decay curves. The decreased time in the excited state resulting from FRET results in slower photobleach kinetics. This approach, known as pbFRET (discussed in [79] and references therein), has the advantage of not requiring special equipment. Unlike FLIM, the measurement is destructive since the fluorophore of interest must be photobleached. Also, the sample cannot move during the photobleaching process. For this reason, FLIM, though requiring specialized equipment, is more suitable for living cells.

6.3. Variations on FRET

FRET between two fluorophores on a single molecule can be used in environmental probes. Such environmental probes include the calmodulin-based cameleon probes in which CFP/YFP FRET provides a measure of calcium concentration [93] and Raichu which is a chimeric protein that provides a means of assaying the GTP-binding state of Ras-family proteins in living cells [94]. The cameleon probes contain two FP domains separated by a domain derived from calmodulin which undergoes conformational changes on binding calcium. These calcium-induced conformational changes alter the relative positions of the FPs with resulting FRET. Raichu is a probe in which CFP and YFP are separated by a spacer containing H-Ras and a domain from Raf that binds Ras-GTP but not Ras-GDP. The probe is designed in such a way that binding of Raf to Ras-GTP brings the FP tags sufficiently close together so that FRET can occur. Bioluminescence Resonance Energy Transfer (BRET) is a variation on FRET in which energy is transferred to a single acceptor fluorophore from a luciferase, thus eliminating the need for externally supplied excitation light [95]. Because there is no excitation light, autofluorescent background is eliminated which decreases a source of noise. Thus, the technique is sensitive even at low efficiencies of energy transfer. However, the number of photons produced per fluorescent molecule is greatly reduced compared to standard FRET making BRET microscopy significantly more difficult. For this reason, BRET has primarily been used spectroscopically with bulk samples.

7. Fluorescence correlation spectroscopy and related techniques

7.1. Fluorescence correlation spectroscopy

Fluorescence Correlation Spectroscopy (FCS; [96,97]) and its variant Image Correlation Spectroscopy (ICS; [98]) are new techniques which have promise for studies of the secretory pathway. FCS is a technique for extracting information from thermal fluctuations in molecule concentration. It therefore requires measurement with extremely high temporal resolution of fluorescence emission from a small confocal or two-photon volume. When the molecules are mobile and the number of molecules within the volume is on average small (<100), the fluorescence detected from within the volume will fluctuate over time. This is because molecules are randomly entering and exiting the volume. From an analysis of these fluctuations, it is possible to calculate the diffusion coefficient of the fluorescent molecules, the concentration of the fluorescent molecules and also their degree of clustering [99]. Importantly, multiple diffusion coefficients can be obtained in multi-component systems (same fluorophore with a range of different diffusibilities) more easily than by photobleach methods [99].

The mathematical methods involved in FCS are well tested in vitro but non-trivial. There have been concerns that FCS data could be more difficult to analyze in cells, in which the behavior of the fluorescent protein could potentially be messy [99]. There is not yet a large base of experience to assess the technique’s performance in living cells. However, Elsner et al. were able to measure diffusion coefficients in Chinese Hamster Ovary cells for Arf1 and COPI of 15 and 0.5 μm²/s, respectively, which are reasonably close to values estimated by FRAP in the same study [100]. FCS and its use in living cells is reviewed at greater length by Hausten and Schwille [101]. A detailed description of the theory and implementation of FCS is given in [99].

7.2. Fluorescence cross-correlation spectroscopy

While FCS has the potential to be a useful technique for measuring protein concentration, mobility and clustering, its “killer application” has not been identified. FCS can measure the motility of proteins but cannot by itself identify which protein–protein interactions may have changed this motility. Fluorescence cross-correlation spectroscopy (FCCS; [102,103]) is an extension of FCS which potentially provides a powerful means of detecting protein–protein interactions in living cells even when the clustered fluorophores are too separated for FRET. Temporal fluctuations in fluorescence from two fluorophores in the same small focal volume are analyzed for correlation. As in standard FCS, a small number of particles (<100) is required in each channel to be easily analyzed. If the fluctuations in intensity are due to arrival or departure of fluorophores on separate particles (i.e., protein complexes), the fluctuations in the two channels will not be correlated, but if the two fluorophores are in part associated, the fraction of each fluorophore associated with the other can be calculated.

An advantage of FCCS over FRET is that the relative localization of fluorophores on two proteins is of little importance so long as the total size of the complex is less than the size of the spot being analyzed (i.e., <200 nm). All that matters is that the fluorophores move together as a unit into the focal spot or not. This can also be a disadvantage as trapping of two proteins together in a much larger structure such as a transport vesicle may be difficult to distinguish
from binding of the two proteins [104]. Unlike FRET, only moving molecules can be analyzed. If the molecules are immobilized, fluctuations in concentration will not occur.

FCCS can give incorrectly low cross-correlations if the two dyes are not imaged from exactly the same volume, while spurious cross-correlations can be induced if the proteins are photobleached faster than they can diffuse [104]. However, detecting and interpreting FCCS between two channels is a simpler proposition than interpreting FCS data (discussed in [101,104]). Thus, FCCS is in principle a viable competitor to FRET for detecting binding interactions between polypeptide chains in living cells.

Most uses of FCCS to date have been in vitro analyses of fluorophores in solution (e.g., [102,105]). The Schwille group showed recently that FCCS could be used to obtain interpretable data from living cells. They concluded from an FCCS study that the A and B chains of cholera toxin are associated together at the cell surface and remain associated in endosomes but separate from each other in the Golgi [104]. The same process has been similarly studied using FRET with similar results [106]. This correspondence of results would argue strongly for further development and testing of FCCS as a tool to detect protein–protein interactions inside cells since FCCS has potential to work in situations where FRET is difficult or not possible.

7.3. ICS/ICCS

Currently, FCS requires a specialized apparatus [99] which is extremely expensive. Thus, cost has limited the use of this technique in living cells. ICS is a variation of the technique in which fluctuations in fluorescence over both time and space are analyzed from rapidly acquired microscope images [98]. Since ICS requires a time-series of images, temporal resolution depends on the image acquisition rate of the microscope and is poorer than temporal resolution of FCS [107]. As a consequence, very fast diffusion such as diffusion of a small protein in cytoplasm may not be measurable with many microscope systems. This is because fast diffusion leads to fluctuations in fluorescence intensity that decay on a timescale much faster than the timescale for image acquisition. However, ICS has been used to obtain diffusion coefficients and detect flow of cell-surface integral membrane proteins [108]. Diffusion of even a fast-diffusing integral membrane protein should be measurable if images are acquired at video rate (30 frames/s) or better [107]. As compensation for its poorer temporal resolution, ICS can be applied to multiple spatial regions of a cell simultaneously and can provide some information not available by FCS including local direction of flow in the case of directed movement [108].

ICCS is a variant of FCCS in which rapidly acquired image sequences are analyzed for correlations in spatial or temporal fluctuations in fluorescence between the two channels [107,109]. Like ICS, ICCS can be implemented with any confocal microscope that can scan a region rapidly, provided that it is possible to acquire from two channels simultaneously [107]. Like ICS, ICCS cannot analyze fluctuations that occur on a timescale faster than the rate of image acquisition. Thus, fluctuations resulting from diffusion of cytoplasmic proteins could be invisible to ICCS although easily detected by FCCS. ICCS has been applied to cell-surface membrane proteins and could potentially be used to detect protein–protein interactions in ER or Golgi membranes. Analysis of FCCS and ICCS data is still largely “do-it-yourself” and requires a fair degree of mathematical sophistication (see [99,107,108] for more information). Given that FCCS/ICCS may be able to monitor protein/protein interactions in a very general way, including interactions not detectable by FRET, it is likely there will be more development and application of this technique in the future to studies of the secretary pathway. An important open question is the extent that data analysis and interpretation can be reliably carried out by mathematically unsophisticated users perhaps with the help of computer programs.

8. Specialized techniques

8.1. Total internal reflection microscopy

Total internal reflection (TIRF) or evanescent wave microscopy is a fluorescence microscopy technique in which excitation light from the objective intersects the surface of a coverslip at a very shallow angle such that it is reflected from the sample/coverslip interface without passing through the sample [110]. For quantum mechanical reasons, photons can still be absorbed tens to hundreds of nm from the coverslip interface depending on the angle of incidence. As fluorescence is obtained exclusively from a thin interface next to the coverslip, TIRF is well suited to visualizing processes that occur on or very near the plasma membrane of cells. Thus, TIRF has been used to observe the fusion of secretory granules [111] and post-Golgi transport intermediates [26,27]. Due to high signal-to-noise (since autofluorescence from within the cell is simply not seen) TIRF has also been used to track single fluorescent molecules on or very near the cell surface [112]. Unfortunately, TIRF is not useful for observing processes in the interior of cells (past the first 200 nm) but it could potentially be used with in vitro Golgi preparations.

8.2. Single-particle tracking

Probes of a variety of sizes from 500 nm latex beads to 20 nm gold particles have been used for single-particle tracking of cell surface receptors [113]. Many fluorescent molecules have been used for this purpose including phycoerythrin [114] or GFP [115]. It has proven possible even to track phycoerythrin molecules diffusing in cytoplasm [116]. Thus, there are no barriers in principle to...
tracking individual molecules on ER or Golgi membranes. A major technical difficulty is that because extremely intense illumination is required, individual molecules are often rapidly photobleached, usually on a timescale of several seconds or less.

Quantum dots [117] supply a new extremely bright and photostable reagent for single-molecule tagging (reviewed in [118]). While more photoresistant than chemical fluoroophores, rapid flickering over millisecond timescales must be taken into account in experimental designs. Quantum dots conjugated to Fab fragments have been used to track individual molecules of NMDA receptor into synapses [119], demonstrating they can be used practically.

8.3. Ultrahigh resolution imaging

Light microscopy is limited in resolution due to diffraction with a maximum horizontal resolution of about 200 nm even with the best optics. Many structures of interest to cell biologists are smaller. Golgi cisternae are only 50 nm thick. A COPI or COPII coated pit is roughly 70 nm in diameter. Many tubules found in the Golgi region are roughly 50 nm in diameter. There have been recent developments which may allow light microscopic resolution in this range (reviewed in [120]).

In near field optical microscopy (reviewed in [121]), a needle with a small hole is scanned physically over the sample. Excitation light emerges from the needle and emission light is collected through the same hole. The size of the needle tip determines resolution and this resolution can be <50 nm. The need to physically scan the needle makes this technique both slow and unsuitable for use in the cell interior. It might be applicable to in vitro systems however.

Some other techniques permit the use of a more conventional fluorescence microscope or confocal system. In two related techniques, I³ M [122] and 4Pi [123], the sample is imaged with a double objective lens, which increases numerical aperture and dramatically increases axial (vertical) resolution. 4Pi has been used to image the location of a GFP-tagged enzyme within the Golgi apparatus [124]. Extension to multi-label imaging is ongoing [124]. Stimulated emission depletion microscopy (STED) is another very exciting high-resolution technique. STED differs from the above-mentioned techniques in that it uses nonlinear optical effects to achieve both vertical and lateral nanometer-scale resolution in cells [125]. Should it be possible to achieve light microscopic resolution of less than 50 nm in a living cell, the impact on our view of the dynamics and structure of the Golgi apparatus would likely be dramatic.

Acknowledgements

I wish to acknowledge financial support from the Canadian Institutes for Health Research (MOP-49590; G203261). The manuscript was critically read by Mark Terasaki, Robert Lodge, Brian Storrie and three anonymous reviewers and has benefited greatly from their suggestions.

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