Techniques for Studying Protein Trafficking and Molecular Motors in Neurons

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This review focused on techniques that facilitated the visualization of protein trafficking. In the mid-1990s the cloning of GFP allowed fluorescently tagged proteins to be expressed in cells and then visualized in real time. This advance allowed a glimpse, for the first time, of the complex system within cells for distributing proteins. It quickly became apparent, however, that time-lapse sequences of exogenously expressed GFP-labeled proteins can be difficult to interpret. Reasons for this include the relatively low signal that comes from moving proteins and high background rates from stationary proteins and other sources, as well as the difficulty of identifying the origins and destinations of specific vesicular carriers. In this review a range of techniques that have overcome these issues to varying degrees was reviewed and the insights into protein trafficking that they have enabled were discussed. Concentration will be on neurons, as they are highly polarized and, thus, their trafficking systems tend to be accessible for study.

Key Words: protein trafficking; kinesin; myosin; vesicle; neuron

Introduction

Shortly after GFP was cloned [Prasher et al., 1992; Chalfie et al., 1994], it was used as a tag to visualize proteins in living cells [Marshall et al., 1995; Olson et al., 1995; Rizzuto et al., 1995]. These initial experiments observed protein in relatively static distributions and served mainly to corroborate results obtained using immunocytochemistry on fixed cells. Later experiments identified wholesale shifts in protein localization that occurred as a result of changes in the physiological state of the cell. For instance, GFP-tagged myosin was observed to concentrate within the cleavage furrow during cytokinesis or in the posterior cortex in migrating cells [Moores et al., 1996]. In these early studies using GFP, however, evidence of vesicular movement, such as the presence of discrete, mobile fluorescent puncta, was missing. This absence was likely due to the fact that signals from moving vesicles tend to be much weaker than those from static proteins. Subsequent studies where vesicle movement was visualized benefited from technical advances in microscopy, such as the advent of more sensitive CCD cameras [Burack et al., 2000]. In addition, experimental manipulations such as synchronizing protein movement through temperature manipulation [Presley et al., 1997] or eliminating background using Fluorescence Recovery after Photobleaching (FRAP) [Nakata et al., 1998] highlighted moving proteins and allowed them to be distinguished from static protein. As the study of protein trafficking has progressed increasingly sophisticated and effective techniques have been developed to increase signal, decrease background and to isolate aspects of protein trafficking. In the following review we will highlight these techniques and review some of the insights into protein trafficking that they have enabled.

Temperature Manipulation for Synchronizing Vesicle Movement

The vast majority of most proteins is stationary at any given time [Lindsey and Ellisman, 1985], and thus when GFP-tagged proteins are expressed the signal from stationary protein tends to obscure the relatively small signal associated with protein that is present in transport vesicles. The first study to overcome this problem visualized vesicle movement in the initial steps of the secretory pathway [Presley et al., 1997]. In this study newly synthesized transmembrane protein was sequestered in the endoplasmic reticulum, and then released suddenly and simultaneously, allowing transport vesicles moving between the endoplasmic reticulum and the Golgi to be visualized. This paradigm exploited the peculiar properties of a temperature-sensitive mutant of the membrane glycoprotein of the vesicular stomatis virus (VSVG-ts045), which is a trimer at regular temperature, but is monomeric at elevated temperatures.

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In its monomeric form VSVG is prevented from leaving the endoplasmic reticulum (ER), whereas multimeric VSVG freely moves down the secretory pathway [Kreis and Lodish, 1986]. Accordingly, VSVG-ts045-GFP was expressed in cultured cells for 24 hours at elevated temperature causing it to accumulate in the ER. Following a sudden switch to physiological temperature, VSVG-ts045-GFP was simultaneously released from the ER, causing the abrupt release of a relatively large quantity of protein within vesicles that move between the ER and ER-Golgi intermediate compartment (ERGIC), and allowing those vesicles to be clearly visualized (Fig. 1A).

An additional property of the secretory pathway, that protein cannot leave the ERGIC at 15°C [Kuismanen and Saraste, 1989], was exploited to allow visualization of vesicles as they left the ERGIC and entered the Golgi apparatus (Fig. 1B). These techniques were then used to show that transport vesicles within the secretory pathway move along microtubules and to implicate Dynein in the movement between the ERGIC and the Golgi. This study showed the potential of GFP-tagged proteins for studying vesicular transport, and of the strategy of inducing the sudden release of protein from the ER, which inspired future techniques that allowed the study of vesicular trafficking of a range of proteins (see below).

Reducing Background Signal with FRAP and Photoactivation

In the first study to examine vesicular trafficking of proteins in neurons [Nakata et al., 1998] background signal from stationary proteins was reduced using FRAP. Originally developed as a method for measuring lateral diffusion within membranes [Edidin, 1994], FRAP uses cycles of photobleaching of a specific fluorophore, followed by fluorescence imaging of that fluorophore in the bleached area (Fig. 2A). Photobleaching has the effect of eliminating fluorescence from all tagged protein (including stationary protein) in a designated area, allowing the visualization of vesicles that move into that area without background labeling [Nakata et al., 1998]. Cultured neurons collected from the dorsal root ganglia of adult mice were infected with adenovirus encoding the neuronal proteins GAP-43, SV2, SNAP-25, and Synaptophysin. For approximately 3 minutes after photobleaching the bleached area was free from background and transport vesicles could be observed moving into it. Additional photobleaching allowed the vesicles to be followed beyond this initial window. This study showed that transport vesicles carrying GFP-tagged proteins could be observed in cultured neurons. Also, correlative electron microscopy revealed that these moving fluorescent puncta were, in fact, the same transport vesicles that had previously been observed using electron microscopy alone [Lindsey and Ellisman, 1985].

A major drawback to using FRAP for visualizing protein movement is that photobleaching leads to the creation of free radicals, which causes damage to cells [Stephens and Allan, 2003]. To limit this damage the areas where photobleaching is performed tend to be relatively small, limiting the area of the cell in which the vesicles are visible. Despite this precaution, photodamage in the form of blebbed membranes and irregular cellular morphology is clearly visible in neurons that have been exposed to photobleaching [Nakata et al., 1998].

Fig. 1. Temperature manipulation for synchronizing vesicle movement. (A) Transfected cells are incubated at 40°C for 24 h (left). The incubation temperature is then switched to 32°C, allowing vesicles secreted from the ER to be visualized (right). (B) Transfected cells are incubated at 15°C for 2 h (left). The incubation temperature is then switched to 20°C, allowing vesicles secreted from the ERGIC to be visualized.

Fig. 2. FRAP and photoactivation. (A) A neuron expressing a GFP-tagged protein undergoes photobleaching of a selected area (white rectangle). The bleached area is free from background and transport vesicles can be observed moving within it. (B) A neuron expressing pgFP-tagged protein is photoactivated within a selected area (dark gray rectangle), increasing its brightness greater than 50 fold compared with its ground state. Vesicles from the photoactivated area can be observed as they enter adjacent regions where there is no background fluorescence.
et al., 1998]. One solution to the problem of how to illuminate a spatially defined subset of proteins while limiting photodamage is to activate fluorescence rather than quench it. Fortunately, there is a photoswitchable form of GFP (paGFP), which is very dimly fluorescent in its ground state, but becomes greater than 50 fold brighter following illumination with 400 nm light [Patterson and Lippincott-Schwartz, 2002; Patterson and Lippincott-Schwartz, 2004]. This methodology allows fluorescently tagged proteins to be activated within a circumscribed area and then observed as they leave that area and move into regions where there is no background fluorescence (Fig. 2B). A particularly good example of the power of photoactivation is found in a study examining the transport within the axon of cytosolic proteins that are not only associated with vesicles, but also belong to the “Slow component b” fraction [Scott et al., 2011]. In particular, two proteins within this slow component, CAMKIIa and Synapsin 1A were tagged with paGFP and photoactivated within discrete regions in the axons of transfected hippocampal neurons. Using this technique the authors found that Synapsin 1A was present in two different pools: a fast moving pool associated with transport vesicles and a slower pool that is made up mostly of proteins. This latter pool moves in an anterograde direction in a manner that is dependent on motor proteins. This study was the first to visualize the movement of proteins within the slow fraction, and to provide evidence that they are conveyed, either directly or indirectly, by molecular motors.

**Inducing the Interaction of Proteins Using FKBP/FRB/Rapamycin**

Another set of techniques that has been extremely useful in the study of protein trafficking involves inducing the interaction of proteins in complexes using the FKBP/FRB/Rapamycin system. This system consists of the immunosuppressive drug Rapamycin [Vezina et al., 1975], and two proteins to which it binds simultaneously [Banaszynski et al., 2005]: FKBP12, a 12 kDa protein, and an 11 kDa domain within mammalian Target of Rapamycin (mTOR) protein, known as FRB. These two proteins do not bind directly to each other [Bierer et al., 1990], however, in the presence of Rapamycin the two proteins are incorporated into the same complex, causing them to interact indirectly with one another [Erdjument-Bromage et al., 1994]. By fusing an arbitrary protein to FKBP and a second to FRB it is possible to make virtually any two proteins interact in a rapamycin-dependent fashion [Muthuswamy et al., 1999]. This property of the FKBP/FRB/Rapamycin complex has recently been exploited to attach molecular motors onto labeled organelles, allowing the properties of those motors and the cytoskeletal elements on which they move to be probed (Fig. 3A). Note that many researchers use rapalog, a homolog of Rapamycin that does not interact with endogenous FKBP and is less likely to induce off-target effects, instead of Rapamycin [Clackson et al., 1998].

![Diagram](image)

**Fig. 3. Inducing the cargo-motor interaction.** (A) Cargo fused with GFP and FKBP interacts with a motor domain of kinesin fused to FRB in a Rapamycin-dependent manner. Note that a Rapamycin analog such as rapalog can also be substitute for Rapamycin. (B) Cargo is linked with GFP and the LOV domain fused to a peptide. The motor domain of kinesin (with the tail domain truncated) is fused to the PDZ domain that recognizes the peptide fused to the LOV domain. Blue light causes uncoiling of the LOV domain, removing steric hindrance and allowing the peptide to interact with the PDZ domain, which, in turn, causes the motor domain to interact with the cargo.

A protein consisting of FKBP fused to the PEX domain, a peroxisomal targeting signal, and RFP can fluorescently label peroxisomes and allow them to be inductively attached to motor proteins fused to FRB [Kapitein et al., 2010a]. Labeled peroxisomes are easily visualized and do not move appreciably in the absence of Rapamycin [Kapitein et al., 2010b], which suggests that they are not actively transported by motor proteins. When Rapamycin, or one of its analogs is added, the labeled peroxisomes interact with it and, indirectly, with the motor protein fused to FRB. Subsequent movements of labeled peroxisomes thus represent movements driven by the motor protein. Observation of the peroxisomes in the presence of Rapamycin reveals information about the motor protein that is fused to FRB. For instance, peroxisomes that were induced to interact with Dynine did not enter the axon and were sequestered in dendrites. Thus, it can be concluded that dynein movement has the effect of driving cargo out of the axon [Kapitein et al., 2010a]. In addition, results of this assay can be combined with known properties of the motor proteins involved to yield information about the underlying cytoskeletal elements on which the motors are moving. For instance, the fact that peroxisomes moved in reverse along the axon and in both directions in the dendrites is consistent with microtubules in the axon being oriented in parallel with their plus ends facing distally and with microtubules in the dendrites facing in both directions, confirming the results of classic experiments [Baas et al., 1988].
The ability of the peroxisome/FKBP/FRB system to probe the cytoskeletal elements upon which motor proteins move was exploited to investigate the arrangement of actin filaments in the axon versus the dendrites [Watanabe et al., 2012]. Although the relative orientations of microtubules in the axon and dendrites has been known for decades, much less is known about the orientation of actin filaments. Actin tends to be unstable, particularly upon fixation, and there is no technique for determining the orientation of actin filaments that is comparable to the “hooking” technique that was used to determine the orientation of microtubules [Baas et al., 1988]. By inducing the attachment of Myosin motors to labeled peroxisomes, it was determined that the actin filaments in the proximal axon are predominantly oriented with their plus ends facing the cell body, whereas in dendrites, equal numbers of actin filaments point in either direction [Watanabe et al., 2012]. This observation is consistent with the presence of a vesicle filter in the proximal axon that prevents vesicles carrying dendritic proteins from moving into the axon [Arnold, 2009; Al-Bassam et al., 2012]. Thus, by inducing the interaction of motor proteins with labeled peroxisomes it is possible to probe the cytoskeletal structures that mediate protein trafficking.

The FKBP/FRB/Rapamycin system can also be used to identify which motors are responsible for carrying particular cargos. Although 45 different kinesin molecules have been identified, individual kinesin motors have been matched with relatively few cargos [Miki et al., 2003]. Reasons for this include the difficulty of obtaining sufficient material to perform biochemical manipulations such as pull-downs on transport vesicles. Also, it is difficult to visualize both the motor protein and the transport vesicle in living cells. To avoid these difficulties a new paradigm was developed that takes advantage of a specific property of the motor domain of the kinesin Kif5C: when expressed by itself in neurons, this domain, moves to the axon with high specificity [Nakata and Hirokawa, 2003; Jacobson et al., 2006]. In this paradigm a labeled transmembrane protein, such as the Transferrin receptor, is co-expressed along with the cargo binding domain (C-terminus) of a specific kinesin motor fused to the FRB domain (e.g., Kif13) and the motor domain of Kif5C fused with FKBP [Jenkins et al., 2012; Bentley et al., 2015]. The addition of Rapamycin induces the indirect interaction of FKBP and FRB, causing vesicles associated Kif13 to interact with the motor domain of Kif5C, which pulls them into the axon. If labeled vesicles, which contain Transferrin, are pulled into the axon when rapamycin is added, then one can assume that both Transferrin and Kif13 are associated with the same vesicles, and that Transferrin and Kif13 form a cargo/kinesin pair. In the same way any transmembrane domain protein/kinesin pair can be tested to determine whether they are associated with the same vesicles. Using a similar paradigm that involved Rab, it was determined that Kif13A, Kif13B, and Kif1A are associated with dendritic vesicle populations. This paradigm was also used to show that Kif13A and Kif13B interact with early endosomes, whereas Kif1A and Kif1B interact with late endosomes [Bentley et al., 2015].

Although FKBP/FRB/Rapamycin system allows molecular motors to be inducibly attached to other proteins and/or organelles with high temporal precision, it does not allow for precise control of where this interaction takes place. To overcome this limitation, an optogenetic system for protein interaction was adapted to mediate the association of molecular motors with other proteins and with organelles in a light-dependent manner [van Bergeijk et al., 2015]. The TULIP (tunable light-controlled interacting protein) system that was used in this study has two components: a PDZ domain, and its cognate C-terminal peptide fused to the light-oxygen-voltage-sensing (LOV2) domain of Phototropin 1 from Avena sativa (Fig. 3B). Under dark conditions the LOV domain is coiled in a compact state, which causes it to sterically hinder interaction between the peptide and the PDZ domain [Strickland et al., 2012]. When exposed to blue light the LOV domain uncoils, removing its hindrance of the peptide/PDZ domain interaction. Using this system it was shown that causing recycling endosomes to leave axonal growth cones by attaching dynein caused a reduction in axonal growth, whereas causing them to enter growth cones by attaching kinesin motors increased axonal growth [van Bergeijk et al., 2015].

Fluorescent Chemical Tags as Alternatives to GFP

Although the majority of studies examining protein trafficking have used genetically encoded fluorophores such as GFP, various chemical tags have also proven to be very useful for visualizing proteins in living cells [O’Hare et al., 2007]. To facilitate the binding of chemical fluorophores to exogenously expressed proteins, the protein of interest is fused with a protein or a peptide tag that binds with high efficiency to cell-permeable synthetic dyes that are administered extracellularly, allowing non-invasive labeling of the target protein. Compared with fluorescent protein labeling, synthetic dye labeling technique has several advantages: (1) chemical tags are relatively smaller in size, and presumably are less likely to perturb the normal function of the target protein; (2) synthetic fluorophores tend to be brighter with increased photostability [Shaner et al., 2005]; (3) synthetic dyes can be easily manipulated. For instance, the same target protein can be labeled with different dyes at different time points [Gautier et al., 2008], allowing for pulse/chase type protein trafficking experiments similar to those using FRAP or photoactivation.

The SNAP-tag is perhaps the most popular chemical tag. It is derived from the human O6-alkylguanine-DNA alkyltransferase (hAGT), which binds to O6-benzylguanine (BG) [Kepler et al., 2003] (Fig. 4). Directed evolution
based on phage display was performed to improve the stability of the hAGT, as well as the specificity and efficiency of its interaction with BG [Juillerat et al., 2003]. The molecularly engineered hAGT (SNAP-tag) is fused with the protein of interest, and cell-permeable BG derivatives conjugated to chemical dyes are used to covalently label the SNAP-tag fused protein. To minimize the background signal caused by the endogenous hAGT, an inhibitor has been developed to specifically block the reaction between the endogenous hAGT and BG derivatives [Juillerat et al., 2005].

The SNAP-tag has been used to investigate protein dynamics and trafficking. In one study, the SNAP-tag was fused to the N-terminus of Na,K-ATPase α subunit, and tetramethylrhodamine-conjugated BG was used as the fluorescent label. In combination with a pulse/chase protocol, the post-synthetic trafficking of Na,K-ATPase was visualized in polarized epithelial cells and found to be different from that of other membrane proteins, suggesting that multiple routes exist for transport from the Golgi to the basolateral membrane in these cells [Farr et al., 2009]. In another study, the SNAP-tag was used to quantify the internalization of G protein-coupled receptors following agonist binding [Calebiro et al., 2013]. When compared with the traditional antibody recognition, the SNAP-tag has a sensitivity that is up to ten-fold greater [Ward et al., 2011]. Based on the success of the SNAP-tag, researchers have developed another hAGT-based tag, named CLIP-tag, which specifically recognizes O2-benzylcytosine (BC) derivatives [Gautier et al., 2008]. Since the SNAP-tag and the CLIP-tag possess orthogonal substrate specificities, they can be used to label two different proteins simultaneously in living cells, which further broadens the use of hAGT-based chemical tags in live cell imaging. Thus, although chemical tags are more cumbersome than GFP in that they require dyes to be added extracellularly, their unique properties make them ideal for specific applications.

Visualizing Endogenous Proteins Using Intrabodies

All of the methods for protein visualization discussed thus far can only be applied to exogenously expressed proteins, because the protein to be visualized must either be fused to a fluorescent protein or to an adaptor that binds to a non-protein fluorophore. Disadvantages to visualizing exogenously expressed proteins include that such proteins often do not localize in the same manner as their endogenous counterparts. In addition, overexpressing such proteins can lead to gain of function phenotypes [El-Husseini et al., 2000]. Finally, overexpressed proteins do not report the levels of endogenous proteins, but rather just report the levels at which they exogenously expressed. A solution to this problem was suggested that used recombinant antibody-like proteins known as “intrabodies,” which are generated using phage display in such a way that both the intrabody protein and the gene that encodes it are co-purified [Nizak et al., 2003]. Using this approach an intrabody was generated that binds specifically to active, but not inactive, forms of the small G protein Rab6. When expressed in cells, the intrabody-GFP fusion labeled activated Rab6, allowing its movements to be visualized. This study demonstrated that it is possible to label endogenous proteins in specific conformations in living cells.

Despite the potential of the intrabody approach for visualizing proteins in vivo, its original implementation has some limitations. For instance, there is no way of regulating expression of intrabodies, which are continuously produced following transfection into cells leading to background.

Fig. 4. SNAP-tag labeling method. The protein of interest (POI) is fused with the SNAP-tag. The dye-labeled benzyl group of the BG derivative can be irreversibly transferred to one of the cysteine residues of the SNAP-tag, allowing the POI to be covalently labeled noninvasively.

Fig. 5. Visualizing endogenous proteins using FingRs. (A) A fusion of the FingR gene with the gene encoding GFP are transfected into cells. Following expression, the tagged FingR binds with the endogenous POI, allowing it to be visualized. (B) The FingR is fused to a zinc finger DNA binding domain (ZF) and a KRAB(A) transcriptional repressor domain. In addition, a ZF DNA binding site (ZFBS) is inserted upstream of the promoter. When the binding of FingRs to the POI is not saturated, 100% of newly made FingRs bind to the endogenous POI. When the POI is saturated, newly made FingRs move to the nucleus because of the nuclear localization signal within the ZF. The ZF binds to the ZFBS and the KRAB(A) represses the transcription. Thus, the level of the FingR is matched to the level of the endogenous POI.
labeling from unbound intrabody that could obscure signal from bound intrabody. Furthermore, the scFv backbone that was used is prone to instability when expressed intracellularly due to the inability of disulfide bonds to form [Goto and Hamaguchi, 1979; Goto et al., 1987; Proba et al., 1998]. To overcome these limitations a novel class of intrabodies known as FingRs (Fibronectin intrabody generated with mRNA display) was generated [Gross et al., 2013], which have a scaffold based on the 10FnIII domain of human fibronectin, which does not have disulfide bonds [Batori et al., 2002] (Fig. 5A). In addition, a novel transcriptional control system regulates the expression of FingRs so that sufficient protein is generated to bind all of the target, but with minimal unbound protein [Gross et al., 2013] (Fig. 5B). FingRs have been generated against PSD95, Gephyrin and Ca2+/Calmodulin Kinase II (CaM-KII). The Gephyrin FingR was used to visualize the movement of transport vesicles within both axons and dendrites of dissociated pyramidal neurons [Gross et al., 2013]. Using the CaMKII.FingR, novel clusters of CaMKII were visualized within the cell bodies of dissociated cortical neurons [Mora et al., 2013]. Unlike other clusters visualized with CaMKII-GFP fusion proteins, which formed in response to prolonged Ca2+ influx and ischemia [Aronowski et al., 1992], these clusters rapidly disappeared with Ca2+ influx. These studies showed that the intrabody approach can be used to accurately monitor the trafficking of endogenous proteins without producing off-target effects and can reveal features of protein trafficking that are not apparent using overexpressed, tagged proteins.

Conclusions

Although methods for studying protein trafficking have come a long way from the initial attempts to visualize GFP fusion proteins, numerous technical challenges remain. Perhaps the most limiting aspect of using GFP to study trafficking is still its high rate of bleaching. For this reason, movies of GFP-tagged protein are rarely longer than 100 frames. A fluorescent protein that is more resistant to bleaching would enable protein trafficking to be visualized over longer periods of time and at higher time resolution allowing a much richer picture of protein trafficking to be obtained. A second challenge is to provide context to protein trafficking by visualizing more than one or two proteins at a time. This will require the generation of fluorescent proteins with good optical qualities that absorb and emit at wavelengths beyond the traditional red and green ranges. Although numerous non-green fluorescent proteins have been developed that work at different wavelengths from blue to infrared [Calebiro et al., 2013], none of these has optical properties that approach that of GFP and its variants.

A final challenge is to visualize protein trafficking in the context of living organisms. Vesicle trafficking has been observed in vivo in Caenorhabditis elegans [Maeder et al., 2014] and drosophila larvae, however, there are few, if any, reports of protein trafficking done in intact mammalian organisms. Although extremely difficult technically, visualizing protein trafficking in an intact animal would provide context that is missing from experiments in culture. For instance, how does the metabolic state of the organism—during sleep, for instance—change protein trafficking in specific neurons? Perhaps with the maturation of recently developed imaging modalities such as selective plane illumination microscopy (SPIM) [Huisken et al., 2004], which permits imaging of thick samples with minimal photobleaching, there will be an opportunity to explore such questions and begin to understand protein trafficking in a broader, more physiological context.

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