

PERSPECTIVE

Validating the Location of Fluorescent Protein Fusions in the Endomembrane System

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Assessment of gene function generally requires knowledge of the sites of action of gene products. Several experimental approaches can provide relevant information, but all have their limitations and the potential for experimental artifact. In this article we focus on the endomembrane organelles and on the methods that can be used to validate the location of fluorescent protein fusions. We discuss the utility of redundant localization techniques, complementation of mutant phenotypes, and integration of localization data with expected biological function as methods to achieve consensus. We argue that no single piece of evidence is sufficient to address the issue, and that all approaches can reveal useful information about the true steady state location of a protein or about other aspects of its transport and dynamics. As ever, the critical point is the subjective interpretation one puts on each observation in light of the experimental conditions and other pertinent data. We illustrate these points with some successes and failures in our own work.

INTRODUCTION

Accurate assessment of protein localization is crucial to a complete understanding of protein function. An accompanying article (Millar et al., 2009) considers protein localization approaches with respect to the nucleus, chloroplasts, mitochondria, and peroxisomes. Here, we focus on the endomembrane organelles and methods that can be used to validate the location of fluorescent protein fusions. Although the considerations involved in localizing proteins to endomembrane organelles are in principle the same as those for any organelle, the nature of the endomembrane system makes some of these considerations more acute. First, as the organelles that comprise the endomembrane system regularly exchange membrane constituents, many of the proteins of interest are distributed between two or more compartments or may cycle itinerantly between them, so a simple localization pattern cannot be assumed. Second, there are at least four features of the system that increase the potential for tagging-induced artifacts: (1) the luminal sides of some endomembrane compartments are not conducive to yellow fluorescent protein (YFP) or green fluorescent protein (GFP) fluorescence; (2) misfolded or misassembled proteins are retained in the endoplasmic reticulum (ER) or are sorted to the vacuole (Trombetta and Parodi, 2003; Castelli and Vitale, 2005; Vitale and Pedrazzini, 2005; Foresti et al., 2008); (3) almost all proteins of the system must traffic through at least one other organelle (and often several) to reach their final destination; and (4) much of the downstream targeting involves saturable trans-

port and signaling systems, increasing the likelihood that fluorescent protein fusions (FPFs) will be missorted in one or more of the endomembrane compartments.

Currently, FPFs are perhaps the most commonly used protein localization tool. The advantages of FPF visualization over traditional methods of antibody production or epitope tagging are, first, the ease with which they can be generated and detected and, second, the opportunity to study localization in live cells. Coupled with widening access to confocal microscopes, FPFs allow clear images of the location and dynamics of a protein of interest deep within living tissues. They also open the possibility of studying turnover, transport, and molecular interactions using techniques such as Förster resonance energy transfer, fluorescence lifetime imaging, bimolecular fluorescence complementation, fluorescence recovery after photobleaching, and photoactivation, all of which have been reviewed extensively (e.g., Fricker et al., 2006; Fernández-Suárez and Ting, 2008). The principal disadvantage is that the tag may alter the location (and interactions) of the native protein and may even alter the condition of the wider system through dominant effects. Furthermore, unless the fusion is expressed at native levels in a null mutant background, there is inevitably a degree of overexpression. Despite their small size, the same caveats apply to fusions using small epitope tags. Given these considerations, we explore below how the patterns observed with an FPF might be validated, qualified, or falsified. Like the authors of the accompanying article, we argue against a dogmatic approach. Using examples from our own work, we show how multiple approaches often may be necessary to arrive at a more accurate picture of protein localization and function.

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COMPLEMENTATION OF MUTANT PHENOTYPES

Perhaps the simplest verification of FPF functionality occurs when the phenotype of a loss-of-function mutation in the gene of interest can be completely rescued by transgenic expression of the FPF. This is most compelling when (1) the fusion is driven by the native expression signals (upstream and downstream intergenic regions plus introns) rather than a native or heterologous promoter and (2) the distribution of the FPF signal is consistent with gene expression visualized via *in situ* RNA hybridization. However, position effect variation can still result in individual transgenic lines that express substantially more fusion protein than the native endogenous locus. In the absence of a suitable activity assay, the best option is confirmation by immunoblotting that the FPF has a similar abundance to the wild-type protein and is not present in knockout mutants, if available.

If suitable antisera cannot be made, the general practice is to collect images from several transgenic lines accumulating the FPF at a range of levels and to establish the pattern that is observed when the fusion is at the limits of detection. However, it is important to bear in mind that the normal abundance of the endogenous protein may or may not be above the detection limit of the FPF. An example is provided by the ADP-ribosylation factor-guanadine exchange factor GNOM, which is seen on endosomes, but has been shown by genetic analysis to also act at the Golgi (Richter et al., 2007). The FPF detection limit will vary depending on the quantum efficiency of the fluorescent protein used (Shaner et al., 2007) and the microscope's sensitivity and configuration (e.g., microscope optics, image integration time on a CCD device, and line averaging versus summing on a point scanning instrument). Although weakly expressing FPF lines rarely generate the most aesthetically pleasing images, they are likely to yield the most reliable data, and lines with stronger expression levels should be used only if it is clear that the FPF localization is unchanged. In this respect, results obtained with transient overexpression of FPFs can be particularly difficult to interpret. If the case is to be made that the localizations observed in strongly expressing transformed cells are identical or similar to those seen in weakly expressing cells, a set of images acquired from a weakly expressing line might be legitimate material for supplemental data.

It could be argued that complementation at endogenous abundance is a sufficient criterion for validation of FPF localization and is necessary wherever possible. We would argue that it is neither strictly necessary nor sufficient. First, complementation simply shows that, under the conditions tested, a sufficient portion of the FPF is active at the appropriate site(s) of action. It cannot demonstrate that this is equivalent to the major steady state location of the FPF. Given that artificial microRNA data frequently show that proteins must be knocked down to below 30% of their native levels before loss-of-function phenotypes are observed (Hanikenne et al., 2008), the corollary is that >70% of a fusion protein may be mislocalized in a fully complemented line. This figure is likely to be even higher for proteins whose activity is

highly regulated and for those that are not normally very abundant. An example is the overexpression of the tonoplast AVP1 H⁺-pyrophosphatase, which results in ectopic localization to additional endomembrane compartments and increased abundance of H⁺-ATPases at the plasma membrane (Li et al., 2005). The logical conclusion is that visualizations of strongly expressed AVP1-FPFs in endomembrane structures other than the tonoplast are suspect.

It is also possible that sufficient functional FPF may be present in some cell types to rescue a mutant phenotype but is not detectable. An example of this is seen in our efforts to visualize the ABCB19 transporter that mediates basipetal auxin transport along the embryonic axis and in stamen filaments (reviewed in Titapiwatanakun and Murphy, 2008). *ABCB19* normally is expressed in vascular and cortical tissues as well as in epidermal cells at the root apex and in anther filaments, as judged by promoter reporter fusions, *in situ* RNA hybridization, and quantitative RT-PCR analyses (Blakeslee et al., 2007; Titapiwatanakun et al., 2009). An FPF-ABCB19 fusion that fully restores all mutant phenotypes does not produce a detectable signal in epidermal cells (Wu et al., 2007; Titapiwatanakun et al., 2009), whereas another ABCB19-FPF construct that is detected in epidermal cells produces a discernable ectopic root waving phenotype (Mravec et al., 2008; Titapiwatanakun and Murphy, 2008). This example additionally illustrates differences that may arise from the positioning of the fluorescent tag in an FPF (N-terminal, C-terminal, or internal) and suggests that no single construct can necessarily be interpreted as fully correct. The degree of confidence one attributes to the localization of a complementing FPF will be influenced by the particular circumstances of the experiment but is best not taken dogmatically as sufficient proof of endogenous localization. Similarly, if the complementation is partial, we would argue that the fusion protein may have utility, but the interpretations must be qualified accordingly.

It should also be recognized that certain FPF locations raise more serious causes for concern than others. Accumulation partially or exclusively in the ER might indicate overexpression artifacts, particularly in transient expression, or tag-induced misfolding and retention by quality control mechanisms. Additionally, accumulation at terminal membranes, such as the plasma membrane or the tonoplast, may indicate either saturation of a trafficking pathway for retrieval or targeting or a failure to recycle to the cytosol. For example, in a study of the *Arabidopsis* small GTPase RAB-A2a (Chow et al., 2008), it could not be determined whether the native protein normally recycles off the membrane before or after it arrives at the plasma membrane. Although endogenous protein and FPFs to the wild type and GTP-bound mutant forms could be detected at the plasma membrane, it could be argued that this occurred only in circumstances where normal rates of recycling to the cytosol were inhibited; because the plasma membrane is the end of the line, this is where unrecycled protein will accumulate.

PERSPECTIVE

INTEGRATING LOCALIZATION AND EXPECTED BIOLOGICAL FUNCTION

A clue to the validity of FPF localization can be obtained by asking whether the FPF exhibits the expected biological behavior, which will depend on the extent of a priori knowledge of the protein and its biology. In some cases, certain genetic backgrounds may be expected to shift the location of an FPF in a predictable way. Alternatively, mutant forms of the FPF may also give predictable shifts in localization. For example, in the case of small GTPases, it is reassuring if mutations that are expected to alter membrane localization, protein interactions, or nucleotide binding status each shift the location of the protein in ways that are consistent with its known or presumed biological activity (Chow et al., 2008). Inhibitor treatments can also be used to assess FPF functionality, especially when the protein is predicted to be secreted, endocytosed, associated with the cytoskeleton, influenced by inositol signaling, or associated with a discrete set of membrane lipids.

Given that the many endomembrane compartments that link the ER, plasma membrane, and tonoplast appear as subresolution dots in the light microscope (particularly after fixation), a simple demonstration that an FPF is associated with distinct punctate structures is insufficient. Validation of the FPF distribution in live cells requires the use of a faithful colocalizing marker, generally another FPF that emits at a wavelength that can be distinguished from the FPF of interest (e.g., a red FPF that can be visually distinguished from a green FPF). With the ready availability of FPF markers for most known organelles and of suitable primary antibodies developed against marker proteins from diverse organisms, it is increasingly possible to find a suitable FPF marker and to use either its intrinsic fluorescence or a double immunofluorescence protocol for colocalization with the protein of interest. However, such efforts become more difficult when the FPF of interest is transiently distributed across multiple subcellular compartments.

COMPARISON OF FPF AND IMMUNOLocalIZATIONS

When an antibody of sufficient specificity is available, the distribution (but not intensity) of an FPF signal can be compared with immunolocalization signals obtained for the endogenous corresponding protein in specific cell types. As the antibody will most probably also recognize the FPF, this normally requires the endogenous localization to be determined in separate specimens that lack the FPF construct. If the FPF can be shown to colocalize reproducibly and at a consistent comparative signal strength with another antigenically unrelated marker (immunological or FPF), this marker can then be used as an internal control when localizing the endogenous signal. If an FPF of interest is distributed across more than one organelle, it may prove difficult to find a suitable marker with the same quantitative distribution. A solution to this problem may be presented if the protein of interest is encoded by a small gene family. If two or

more members of the family exhibit identical localization as FPFs (determined using spectrally distinct FPFs in colocalization studies), it may then be possible to raise antisera that are specific to one isoform, for example, through use of synthetic peptide antigens. These antibodies may then be used to determine the endogenous localization of one isoform in cells that also express the FPF to another isoform, which acts as an internal control. This will also control for changes to FPF localization that may occur under conditions of immunofluorescence (see below). This strategy was adopted recently to validate the localizations of FPFs to a family of Rab GTPases in *Arabidopsis* (Chow et al., 2008).

In all cases, interpretation is aided by data derived from independent methods, as each method has its own drawbacks. Although the use of antibodies against endogenous proteins was advocated above, the obvious drawbacks to this approach are that the method depends on epitope accessibility and requires tissue fixation (plus embedding and sectioning in some cases) and there is potential for artifacts inherent in these methods. Most immunofluorescence protocols rely on chemical fixation, which is slow relative to the dynamics of membrane trafficking, so there is ample opportunity for dynamic steady state distributions to be altered. More aesthetically pleasing results achieved with some fixation methodologies may therefore be difficult to reconcile with FPF visualizations. A good example of this is a comparison of the immunofluorescence visualizations of PIN1 with PIN1-FPFs in whole-mount *Arabidopsis* tissues. A very discrete basal localization of the PIN1 signal can be obtained using some tissue fixation techniques (Geldner et al., 2001; Michniewicz et al., 2007), but PIN1-FPF signals tend to be comparatively more diffuse (Heisler et al., 2005; Sieburth et al., 2006), perhaps as a consequence of some degree of overexpression. In some cases, immunogold electron microscopy can be used to enhance the resolution of subcellular protein localization, but similar problems with fixation artifacts are often present with this method (Ripper et al., 2008).

A second example is provided by the study of Chow et al. (2008) referred to above. In this study of the Rab-A2 family members, FPFs driven by native expression signals were each observed at the trans-Golgi network except in the most highly expressing lines where additional labeling of the plasma membrane was seen and attributed to overexpression. In immunofluorescence experiments, however, enhanced plasma membrane labeling was commonly observed for both the endogenous protein and for moderately expressed FPFs in the same cells. With these observations, it is arguable that data obtained from the low-abundance FPFs in living cells is more indicative of the actual biological behavior of the proteins and that the fixation process used in whole-mount immunolocalizations artificially increased some signal at the plasma membrane. On the other hand, it could be argued that immunolocalization detected native proteins where FPFs were not detectable. We suggest instead that all of the data are informative. In this case, additional experiments using mutants that stabilized the protein in its active conformation showed localization exclusively at the

plasma membrane, whereas those that stabilized its inactive conformation were excluded from the plasma membrane at all concentrations. Taking all of the data into account, it was concluded that Rab-A2 proteins are likely to be distributed between the TGN and the plasma membrane, with the latter being a minor or transient site of accumulation unless the system is perturbed by overexpression or fixation (Chow et al., 2008). This is also consistent with a priori expectations about this type of molecule: they are expected to be recruited from the cytosol to the surface of one membrane and after which they may traffic to another membrane before being recycled back to the cytosol.

If antisera are not of sufficient specificity to distinguish between multiple family members, or if the antigenic epitopes do not survive fixation and embedding procedures, they still may be used in membrane fractionation studies to validate FPF subcellular localization. The use of SDS-PAGE often can resolve the endogenous protein from both nonspecific proteins and from the (larger) FPF to provide a good internal control for endogenous distribution. However, this technique has much lower organellar and cellular resolution, as it requires extracts from whole plants or whole organs, and organelle distributions overlap extensively, even with careful selection of density gradients/phase separations and markers (Dunkley et al., 2006). However, biochemical and immunoblotting methods can also be useful in sorting out whether visualized FPF signal represents intact protein, particularly with labile proteins that are subject to rapid turnover. For example, in SDS-PAGE immunoblots prepared from *ProPIN1*: PIN1-GFP transformants (Benková et al., 2003; Heisler et al., 2005), we often find a high proportion of truncated protein detected with GFP antisera in extracts prepared in the presence of an array of protease inhibitors (Titapiwatanakun et al., 2009), raising the concern that some visualized PIN1-GFP intracellular signals may represent truncated proteins.

CONCLUSIONS

Determining protein localization inevitably is an exercise in imperfection. The use of diverse methods and biological tests allows greater confidence to be assigned to a suggested location. If appropriate caveats are used, even unsupported FPF data can provide some clue to the targeting signals within a protein of interest and, thus, to its native location. It is important, however, that such evidence is appropriately cited by future users. The need for confirmation depends on the significance of the location proposed with respect to protein function and on the additional data that support that function. Experience suggests that one does well to avoid dogmatic insistence on any single approach, no matter how well controlled.

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PERSPECTIVE

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