Three new *Drosophila* markers of intracellular membranes

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The need for cellular markers that permit a quick and accurate evaluation of a protein's subcellular localization has increased with the surge of new data generated by the Drosophila genome project. In this report, we present three ubiquitously expressed Drosophila transgenes that expressed a green fluorescent protein variant (enhanced yellow fluorescent protein) that has been targeted to different intracellular membrane targets: the Golgi apparatus, mitochondria, and endoplasmic reticulum. These markers serve as an internal standard for characterizing a protein's subcellular localization or as a means of tracking the dynamics of intracellular organelles during normal or abnormal cellular or developmental processes. We have also examined fixation artifacts using these constructs to illustrate the effects that fixation and permeabilization have on intracellular membrane organization.

INTRODUCTION

With the completion of the *Drosophila* genome project, there has been a trend in *Drosophila* research toward assessing gene function at the cellular level. As the field of functional genomics races toward a comprehensive understanding of gene function, a need arises for intracellular standards that permit a quick and accurate evaluation of a novel protein's localization within the cell. Fusion proteins to green fluorescent protein (GFP) and its variants have been used to detect a target protein's subcellular localization in both living and fixed cells (1–3). Here we describe three *Drosophila* transgenes that ubiquitously express a GFP variant, enhanced yellow fluorescent protein (EYFP), which has been targeted to different intracellular membrane-bound compartments: the Golgi apparatus, the endoplasmic reticulum, and the mitochondria. While extensive work has been performed on fixation and fixation artifacts in electron microscopy, there is little or no literature dealing with such topics in fluorescent light microscopy, especially regarding fixation of whole tissues in a multicellular model organism. Because EYFP can be examined in living and fixed preparations, we took the opportunity to examine the effects of fixation and permeabilization on the expression and localization of the targeted EYFP moiety.

MATERIALS AND METHODS

Microscopy

All images (both live and fixed) were captured using a fluorescein isothiocyanate (FITC) filter (Chroma High Q filter; Chroma Technology, Rockingham, VT, USA) and a 60× UPlanFl oil-immersion objective [numerical aperture (NA), 1.25] with oil on an Olympus BX51 compound fluorescent microscope (Olympus America, Melville, NY, USA) equipped with a Cool Snap fx charge-coupled device (CCD) digital camera (Photometrics, Tucson, AZ, USA). The camera and microscope were controlled using Image Pro® Software (Media Cybernetics, Silver Spring, MD, USA). Captured images were imported into Microsoft® PowerPoint® for presentation.

Sample Preparation

Tissue from wandering third instar larvae was dissected in Shields and Sang M3 Insect tissue culture media (Sigma, St. Louis, MO, USA). Living tissue was mounted on a glass microscope slide in a wet mount of 20 µL cell culture media under a 22-mm coverslip prepared with clay feet to prevent damage to the tissue. Fixed tissue was mounted using the ProLong® Antifade Kit (Molecular Probes, Eugene,

OR, USA) and examined within 24 h of preparation because formaldehyde-based fixes tend to degrade. We observed little difference in tissue quality within 24 h of fixation.

Cloning and P-Element Germline Transformation

The membrane-targeted moieties were PCR-amplified from mammalian expression vectors pEYFP-Mito, pEYFP-ER, and pEYFP-Golgi (for the mitochondria, endoplasmic reticulum, and Golgi apparatus, respectively; BD Biosciences Clontech, Palo Alto, CA, USA) and cloned into a *Drosophila* spaghetti squash (*sqh*) CASPER 4 P-element transformation vector (gift of D. Kiehart, Duke University, Department of Biology, Durham, NC, USA) (Figure 1). EYFP is a brighter, red-shifted variant of the original jellyfish GFP. The *sqh* promoter expresses ubiquitously, permitting examination in a wide variety of tissue types without induction. P-element-mediated germline transformation was performed as previously described (3).

Initial Characterization of EYFP Constructs in Insect Cells

Because the EYFP constructs were designed for expression in mammalian tissue culture cells, we first determined whether the same organelles were labeled in *Drosophila* cells. Although the localization of each construct appeared to be similar in both human HeLa tissue culture cells and *Drosophila* Schneider line 2 tissue culture cells, we confirmed the localization in *Drosophila* tissue culture cells by double labeling the transfected cells with a fluorescent dye that specifically labeled each organelle, using MitoTracker® Orange CMTM-Ros for the mitochondria, NBDC₆-ceramide for the Golgi apparatus, and ER-Tracker™ Blue-White DPX for the endoplasmic reticulum (all from Molecular Probes). In each case, we found that the signals overlapped perfectly, suggesting that our constructs labeled the intended structures in *Drosophila* cells (data not shown). Three independent insertions of each construct were characterized, and each displayed subcellular patterns of localization to specific compartments that we interpreted

as analogous to the structures we identified in the tissue culture.

Buffer Preparation

Buffer compositions are as follows: phosphate-buffered saline (PBS); phosphate lysine periodate (PLP), 0.037 M sodium phosphate, 0.075 M lysine, 0.01 M sodium periodate, pH 7.2; and PIPES/EGTA/magnesium (PEM), 0.1 M PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.9. An 8% paraformaldehyde stock solution was prepared by dissolving 80 g of paraformaldehyde in 80 mL of warmed water and 10 μ L of 10 M NaOH and heated to 55°C for 2–3 h, distilled water was added to 100 mL, and the solution was vacuum-filtered to remove the flocculent insoluble fraction. To make fixatives, an appropriate amount of this paraformaldehyde stock solution was diluted in the desired concentrated buffer solution. In 1 $\times$ PEM and 1 $\times$ PBS buffers, a concentration of either 2% or 4% of paraformaldehyde was used; PLP fixative was made with 2% paraformaldehyde. Samples were fixed for 20 min at either 4°C or 21°C. To test for the effects of permeabilization, a 30-min wash of 1 $\times$ PBS, 1% bovine serum albumin (BSA), and 0.1% Triton® X-100 (Sigma), called PBT, was performed on fixed samples.

RESULTS

We examined the expression of each construct in several different tissues, including muscles, neuronal cells, and epithelial cells (imaginal discs, guts, and salivary glands). In general, *Drosophila* cells are small; thus, in this report, we used the giant epithelial cells of the third instar larval salivary gland to demonstrate our results. These cells are large (approximately 100 μ m in diameter) and permit easy observation of the subcellular localization of each of the organellar/intracellular membrane-targeted EYFP constructs. Considering that all immunological protocols involve a fixation step and that EYFP can be visualized in both living and fixed tissues, we examined the effects of fixation on the organization and distribution of intracellular membrane structure. We tested two concentrations of paraformaldehyde-based fixative (2% or 4%) in three buffer solutions commonly used in *Drosophila* research: PBS, PEM, and PLP, which has been reported to work well for membrane-associated epitopes (4,5). Not surprisingly, in all cases, we observed some degree of alteration of the subcellular EYFP patterning after fixation, regardless of the buffer used. We also examined the effects of fixation at two dif-

ferent temperatures [room temperature and on ice (4°C)] but saw no difference in results between these conditions (data not shown).

EYFP-Golgi showed a punctate distribution through the cytoplasm of a wide range of cells types, including salivary gland (Figure 2A), neuron, muscles, and intestinal cells (data not shown). While fixation does not alter the overall distribution of the EYFP-Golgi punctate-labeled bodies, we observed subtle differences in the morphology of these structures after fixation, especially in the PLP buffer (compare Figure 2, A and D). In this buffer, we consistently observed finer distribution of Golgi throughout the cell. We do not know the basis for this observation. We also observed alteration in the EYFP-Golgi body organization as a function of paraformaldehyde concentration. Living EYFP-Golgi are composed of irregularly shaped, lobular structures (Figure 3A, inset). Fixation in 2% paraformaldehyde in PBS buffer resulted in a bloated, swollen appearance to these structures, perhaps as a consequence of incomplete fixation (Figure 3B, inset). Fixation in 4% paraformaldehyde fixative in a PBS buffer resulting in a rounded, smaller, more regularly shaped appearance (Figure 3C, inset).

EYFP-Mito was also distributed in punctate structures throughout the cytoplasm in salivary gland cells (Figure 2I). Furthermore, we noticed that EYFP-Mito also had a strong localization at cell cortex (Figure 2I, arrowhead) and an apical localization in the salivary gland epithelium basal to the adherens junction region, as determined by the localization of Merlin (data not shown; D.R. LaJeunesse, unpublished observations). The fixation of EYFP-Mito in all buffer conditions used resulted in loss of the cortical mitochondrial localization, while overall, the punctate distribution of the mitochondria seemed relatively unperturbed (compare Figure 2I with J, K, and L).

In living salivary gland epithelial cells, EYFP-ER labeled a reticular network found throughout the cytoplasm (Figure 2E). This reticular pattern was observed in different tissue types in varying degrees, ranging from completely filling the cytoplasm to a fine diaphanous network (data not shown). In this case, the pattern resembled that

Figure 1. *Drosophila* spaghetti squash enhanced yellow fluorescent protein (EYFP) membrane-targeting constructs. Mammalian expression vectors pEYFP-ER, pEYFP-Golgi, and pEYFP-Mito were used to target sequence for the endoplasmic reticulum, Golgi apparatus, and the mitochondria, respectively. The arrows mark the start of transcription. SV40, simian virus 40; UTR, untranslated region.

of a GFP fusion with an endoplasmic reticulum-specific protein disulfide isomerase (6). Interestingly, while the Golgi and mitochondria maintained some of the structural integrity that was observed in living tissue after fixation with a paraformaldehyde-based fixative, fixation of EYFP-ER resulted in complete loss of all observable structure. The result is an amorphous mass that occupies the cytoplasm and that is completely devoid of any discernable reticulum organization (compare Figure 2E with F, G, and H).

Since many immunological localization techniques contain a permeabilization step that usually involves a wash with a buffered solution containing a detergent, we wished to examine the effects on the organization and appearance of our labeled structures by treating the fixed tissue with a detergent wash. As our detergent, we used standard concentration 0.1% of a common reagent, Triton X-100. After fixation, we exposed the samples (either EYFP-Golgi or EYFP-Mito salivary glands) to a 30-min incubation/wash with PBT. This step is similar

in both composition and duration to the blocking and incubations steps found in many common immunohistological protocols used in *Drosophila* research. As in the previous experiments, we fixed under several different buffered conditions and used either a 2% or 4% concentration of paraformaldehyde. These results are shown in Figure 3. As stated before, fixation with paraformaldehyde resulted in alterations of Golgi morphology (compare Figure 3A and inset with B and C). Furthermore, poorer preservation translated into a complete loss of label after incubation with buffer containing 0.1% Triton X-100 (compare Figure 3, B and D), while stronger fixation preserved the Golgi morphology better (compare Figure 3, C and E).

DISCUSSION

Subcellular localization is a powerful aid in determining gene function.

Here we report the characterization of three new EYFP intracellular membrane markers for use in the *Drosophila* model system (*Sqh::EYFP-Golgi*, *Sqh::EYFP-ER*, and *Sqh::EYFP-Mito*) that label the appropriate intracellular organelles in living and fixed tissues and that permit the assessment of subcellular structures in living and fixed tissues and cells. These constructs will be of great use to the *Drosophila* research community, and stocks containing these constructs have been deposited at the Bloomington *Drosophila* Stock Center at Indiana University (Bloomington, IN, USA). These tools offer a unique opportunity for the researcher studying intracellular organization and organelle structure. Although there are antibody markers and other GFP fusion proteins to endogenous endoplasmic reticular, mitochondrial, and Golgi-targeted proteins for use in *Drosophila* research (6–9), the new intracellular membrane markers reported in this report have distinct advantages over these

Figure 2. Living versus fixed: *Drosophila* salivary gland epithelial cells expressing membrane-targeted enhanced yellow fluorescent protein (EYFP) fixed under different buffer conditions. Living salivary gland epithelial cells expressing ubiquitously expressed *Drosophila* transgenes (A–D) EYFP-Golgi, (E–H) EYFP-ER, and (I–L) EYFP-Mito. (B, F, and J) Cells fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS). (C, G, and K) Cells fixed with 2% paraformaldehyde in PIPES/EGTA/magnesium (PEM) buffer. (D, H, and L) Cells fixed with 2% paraformaldehyde in phosphate lysine periodate (PLP) buffer. Fixation under a variety of buffer conditions results in little alteration to the overall distribution of EYFP-Golgi localization (compare A and B, C and D). However, we observed consistent reduction in the size of EYFP-Golgi when fixed in the PLP buffer. In living larval salivary gland epithelial cells, EYFP-ER localizes to a large cytoplasmic network (E), but fixation destroyed all discernable structure of the label (F, G, and H). EYFP-Mito localization remains generally unaltered after fixation, however, the cortical localization of EYFP-Mito is lost with fixation (I) to cells borders as defined by arrowheads in (J, K, and L).

tools. Since these constructs are merely EYFP moieties targeted to a specific organelle, they will therefore be less likely to affect the structure and organization of these organelles than ectopic overexpression of an endogenous protein. Moreover, the targeted EYFP constructs described in this paper are ubiquitously expressed and are not constructed in the bipartite UAS/GAL system (10); therefore, observation of the EYFP does not require the addition of further genetic elements, thus expediting mutant analysis and phenotypic characterization.

Using these constructs, we also addressed the topic of fixation artifact. An overwhelming majority of information regarding protein subcellular localization has been determined by common immunohistochemical techniques in which a tissue is fixed (usually with a formaldehyde-based fixative), incubated with a primary antibody specific to the target protein, and then labeled with secondary antibodies for detection (11). However, astonishingly little work has been published regarding the extent and role that fixation artifact plays in this process, although differences between living and fixed tissue

have been alluded to in the literature and anecdotally. Traditionally, fixation artifact has been of greater concern in electron microscopy, and through the development of some advanced procedures such as freeze substitution, a few of these obstacles have been overcome (12). In most immunohistochemical procedures, there is a conflict between the fixation and preservation of the epitope. Generally, longer and stronger fixations preserve more of the overall cellular and intracellular structure, but the cost is the loss of the sensitive epitopes that are recognized by the primary antibodies. As shown with immunoelectron microscopy, the fixation of membrane-bound organelles with chemical cross-linking agents such as paraformaldehyde presents an unusual problem because biological membranes are not entirely composed of proteins; moreover, it has been suggested that fixation may even alter the apparent membrane structure through aberrant chemical cross-linking (12).

Here we show that fixation alters the morphology of intracellular organelle structure. However, the extent of artifact was dependent on a fixative as well as on organelle. Some structures fixed well

under a variety of conditions (Golgi and mitochondria), while the endoplasmic reticulum was extremely labile to any fixation protocol. In all procedures, we observed differences between fixed and living specimens. Some features such as the intracellular organization of EYFP-Golgi and the intracellular distribution of EYFP-Mito changed after fixation. However, the most striking results were the effects that a short wash with a buffered detergent solution had on our EYFP markers. Since most immunohistological techniques that are used to investigate intracellular antigens involve a permeabilization step to allow the antibody access to the target protein inside the cell, we examined the effects this procedure has on the localization of our membrane tags. In every case that a permeabilization/wash step was used after fixation with a 2% paraformaldehyde in any buffer formulation, all EYFP expression was lost. This artifact was solely dependent on paraformaldehyde concentration as the use of a 4% solution in any buffer resulted in the preservation of EYFP. The point of these experiments was not to define the conditions of fixation of a given organelle but to provide experimental caveats for future investigation and demonstrate the artifacts that such procedures generate. In general, we found that a 4% paraformaldehyde fixative in a PBS buffer preserved most of the structure and integrity of EYFP-Golgi and EYFP-Mito localization even after a PBT wash, while EYFP-ER fixed poorly under all conditions. However, these fixation conditions might not be appropriate for all epitopes found in an intracellular membrane compartment, and therefore it is appropriate to test a variety of different fixation conditions.

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Figure 3. Effect of permeabilization on enhanced yellow fluorescent protein (EYFP)-Golgi in *Drosophila* larval salivary epithelial cells. Living larval salivary gland epithelial cells with (A) EYFP-Golgi; inset, the EYFP-Golgi structures are not round but are composed of multiple lobed structures. (B) Larval salivary gland epithelial cells fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) buffer; inset, amorphous, swollen appearance of Golgi bodies. (C) Larval salivary gland epithelial cells fixed with 4% paraformaldehyde in PBS buffer; inset, small, more discrete appearance of EYFP-labeled structures. (D) Cells fixed with 2% paraformaldehyde and then treated with 30 min incubation with 1× PBS, 1% bovine serum albumin (BSA), and 0.1% Triton X-100 (PBT); inset, no Golgi labeling. (E) Cells fixed with 4% paraformaldehyde in PBS buffer and then treated with 30 min incubation with PBT; inset, preservation of some Golgi staining.

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Microarray results improve significantly as hybridization approaches equilibrium

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Dual-channel long oligonucleotide microarrays are in widespread use. Although much attention has been given to proper experimental design and analysis regarding long oligonucleotide microarrays, relatively little information is available concerning the optimization of protocols. We carried out a series of microarray experiments designed to investigate the effects of different levels of target concentration and hybridization times using a long oligonucleotide library. Based on principles developed from nucleic acid renaturation kinetics studies, we show that increasing the time of hybridization from 18 h to 42 h and 66 h, especially when lower than optimal concentrations of target were used, significantly improved the quality of the microarray results. Longer hybridization times significantly increased the number of spots detected, signal-to-noise ratios, and the number of differentially expressed genes and correlations among replicate arrays. We conclude that at 18 h of incubation, target-to-probe hybridization has not reached equilibrium and that a relatively high proportion of nonspecific hybridization occurs. This result is striking, given that most, if not all, published microarray protocols stipulate 8–24 h for hybridization. Using shorter than optimal hybridization times (i.e., not allowing hybridization to reach equilibrium) has the consequence of underestimating the fold change of differentially expressed genes and of missing less represented sequences.

INTRODUCTION

Major parameters that affect nucleic acid hybridization equilibrium include labeled target concentration and the incubation time allowed for hybridization, both of which are critical because maximum specificity and sensitivity are reached with equilibrium. Typical protocols propose hybridization times of 8–24 h (1) and 16–20 h (2); however, there is little or no experimental data in the literature to justify a given incubation time.

Recent reports have examined different parameters that affect hybridization equilibrium for microarrays. Using a long oligonucleotide platform (60-mer), it was shown that with high complexity targets (i.e., a highly diverse population of RNA), specific target-to-probe hybridization equilibrium took much longer to attain than did nonspecific hybridization equilibrium (3). Increasing the incubation time to approach maximum hybridization substantially improved fluorescent intensities for specific targets. However, other critical criteria that help determine the quality of microarray results, such as differential expression,

signal-to-noise ratios, and correlations among replicates, were not directly assessed. In other studies, using short oligonucleotide platforms (20–30 bases), it was shown that a considerable amount of cross-hybridization occurred under various conditions (4) and that longer hybridization times improved accuracy (5). Our experimental results are in complete agreement with and expand on the findings of these earlier studies (4,5). Using a long oligonucleotide platform, we applied multiple criteria to assess the quality of the microarrays that were carried out under laboratory conditions. We show that as hybridization equilibrium is approached by allowing longer incubation periods and/or approached more rapidly by increasing the concentration of labeled target, microarray quality is significantly improved.

MATERIALS AND METHODS

Microarray Hybridization

Total RNA isolated from mouse (strain Balb/c) heart was purchased