Methods 56 (2012) 55–62

Contents lists available at SciVerse ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Review Article

Recent advances in imaging embryonic myoblast fusion in *Drosophila*

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ARTICLE INFO

Article history:
Available online 18 August 2011

Keywords:
Drosophila
Live imaging
Myoblast fusion
Actin foci
Muscle
Embryo

ABSTRACT

Myoblast fusion in the *Drosophila* embryos is a complex process that includes changes in cell movement, morphology and behavior over time. The advent of fluorescent proteins (FPs) has made it possible to track and image live cells, to capture the process of myoblast fusion, and to carry out quantitative analysis of myoblasts in real time. By tagging proteins with FPs, it is also possible to monitor the subcellular events that accompany the fusion process. Herein, we discuss the recent progress that has been made in imaging myoblast fusion in *Drosophila,* reagents that are now available, and microscopy conditions to consider. Using an Actin-FP fusion protein along with a membrane marker to outline the cells, we show the dynamic formation and breakdown of F-actin foci at sites of fusion. We also describe the methods used successfully to show that these foci are primarily if not wholly present in the fusion-competent myoblasts.

1. Introduction

The muscle fibers that surround the body of the *Drosophila* larva, and are used for its locomotion throughout larval life, are formed in the embryo between embryonic Stages 12 and 16. As in most other organisms, these body wall muscles arise by fusion of myoblasts into multinucleate fibers. Almost all of our knowledge of the cell behaviors, cell movements, and subcellular protein localizations that occur during myoblast fusion has been inferred from analyses of single time points in fixed tissue samples [1–7]. Many studies have established that the process of myoblast fusion in the *Drosophila* embryo begins at approx Stage 12 or 8 h AEL (after egg laying) with the specification of two myoblast subgroups that will fuse with one another, the founder cells and the fusion-competent myoblasts (FCMs). These fusion events are highly orchestrated in both time and space, and specific muscle fibers with unique orientations and attachment sites become apparent as fusion proceeds. Cell–cell fusion continues for approximately 5 h and is virtually complete by the end of Stage 15, after which time muscle contraction becomes evident.

By the time of hatching, 30 distinct muscle fibers are present in mirror-image duplication in each abdominal hemisegment, each with a distinct size, shape and location [8–11]. This pattern of muscles is dictated by founder myoblasts that are located in characteristic and reproducible position in the embryo, with one founder cell for each muscle fiber. At their earliest stage, the founder myoblasts appear to contain information that specifies the unique features of each muscle fiber and to convey this information to the remaining FCMs as they "seed" the fusion process. Thus the FCMs take on the identity of the founder cell with which they fuse. The unique differentiation program of each founder cell is dictated by expression of a specific combination of muscle identity genes, a topic that has been discussed in several excellent reviews [12,13]. Following the initial fusion event, FCMs continue to fuse with the developing myotube until it has achieved its normal size. Thus, this process is inherently asymmetric, and represents a fusion event between two distinct types of cells: founder cell: FCM or syncitia: FCM. Most notably, a combination of live imaging, differential expression and electron microscopy has established both the morphological and molecular asymmetry of the sites of fusion [7,14].

Recognition and adhesion between founder cells and FCMs are critical prerequisites to myoblast fusion and are initiated by the cell surface receptors Kirre or Rst in the founder cells and primarily Sns in the FCMs [15–17]. These receptors, in turn, relay signals to downstream components that converge on F-actin nucleation promoting factors. Vrp1/WASp is present in the FCMs and subsequent syncitia, but not the founder cells, while HEM/SCAR are present in both cell populations. Both pathways result in Arp 2/3 mediated F-actin polymerization at the adhesion site [5,18–24]. The accumulation of F-actin at cell–cell contact sites has been imaged extensively in both fixed and live muscle tissue, revealing a tight intense focus in the FCMs and a thin sheath in the founder cells. Recent data has also established that the FCM protrudes into the founder cell/syncitia at this site of F-actin polymerization [7,14]. Fusion may then initiate at a single pore that zippers out between cells, a model supported by EM analysis of samples prepared by

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1046-2023/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2011.08.008

high pressure freezing to preserve membranes [14]. An alternative view, in which the membrane breaks down at multiple points by vesiculation, derives from EM analysis of samples prepared by conventional chemical fixation [1,6]. Herein, we provide an example of our live imaging data that reveals that the F-actin foci is formed only in the FCMs at its site of contact with the myotube and also reveals the depolymerization of F-actin coincident with membrane breakdown and cell–cell fusion ([7]; Fig. 2).

2. Description of methods

In the Sections below, we touch upon many of the issues one must consider to maximize the chances of success for live imaging in general, but focus primarily on our experience imaging myogenesis. We note several excellent technical reports of live imaging in *Drosophila* embryos from which the reader may obtain additional information [25–28].

2.1. Methods of tagging proteins for live imaging

Fluorescently tagged proteins (FPs) have made it possible to examine single living cells in real time. The advent of derivatives and spectral variants of green fluorescent protein (GFP) (see Section 2.2) provides the ability to visualize multiple fluorescent proteins at the same time within the same cell. These reporters allow cell movement, cell morphology and/or organelles to be tracked over time [29]. For imaging in the muscle cells, fluorescent reporters are available to mark the nucleus and cell membrane, and fusion proteins are available for tracking F-actin, WASp, Vrp1/Sltr and Blown fuse (Table 1). Reporters tagged with a nuclear localization signal (*NLS-mCherry*, *H2B-YFP*, *NLS-EGFP*; Table 1) permit the tracking of individual cell movements, and reporters tagged with membrane targeting sequences such as *gap-GFP* (Table 1) have been particularly valuable in visualizing cell fusion (see Fig. 2). Moreover, transgenic flies harboring fluorescent fusion proteins that mark a variety of subcellular organelles (Golgi, ER, mitochondria, vesicles, etc.) have been used extensively in non-muscle cells and are readily available (http://flystocks.bio.indiana.edu/Browse/misc-browse/gfp.html). As noted with the F-actin, Blown fuse, WASp and Vrp1/Sltr examples above, one can also fuse the protein of interest to an FP and track it over time or carry out in-depth quantitative analysis using fluorescence.

In principle, FPs can be added to either the N- or C-terminal end of the target protein, though one position may be preferable to the other. Protein processing, such as cleavage of the signal transfer sequence at the N terminus of a transmembrane receptor must also be considered. While addition of a fluorescent tag does not perturb the function of the original protein in most cases, we have observed examples in which the FP fusion interferes with protein stability and/or its ability to rescue a mutant embryo. Thus it may be desirable to carry out functional assays for new fusion proteins before extensive use in imaging studies to ensure that the protein is behaving as its untagged counterpart. Nonfunctional proteins are often mis-localized and turn over quite rapidly in the cell, a problem that is readily apparent. Examining expression of the fusion protein in *Drosophila* S2 cells can also be quite useful before generating transgenic animals. One resolution for this problem may be the addition of a 4–8 amino acid 'linker' in between the protein of interest and the fluorescent tag. As with other types of tags, this linker may allow protein domains to assume their most stable conformations and engage in protein–protein interactions without steric hindrance. Other important issues to consider are whether the fusion protein can cause a dominant phenotype when overexpressed or whether the presence of the fluorescent tag induces aggregation of the protein of interest. Rigorous studies might seek to confirm localization data with that collected by other methods, such as in situ hybridization or immunohistochemical staining. These issues are easily addressed in the embryonic mesoderm by expression of the fluorescently tagged transgene with an appropriate Gal4 driver (Table 1), and the resulting musculature compared to its wild-type counterpart.

2.2. Choice of fluorescent protein

Several factors must be considered for the successful use of an FP before the start of an imaging experiment. If using a new FP for the first time in *Drosophila*, it is best to check codon usage for efficient translation. As noted above, one must also ensure that the FP does not cause toxicity in the musculature, is expressed efficiently, and is sufficiently bright that the signal is easily detected above any autofluorescence. This issue is particularly challenging in the mesoderm, where membranes are not as distinct as in epithelia. Robust signal strength can therefore be very useful in permitting analysis with a higher level of resolution. The limitations of low signal strength may be overcome by using multiple copies of a transgene.

Another important factor in live imaging is the photostability of the FP, so that it can be reliably imaged for the desired time frame. We and others (Table 1) have found that EGFP, mVenus, and mCherry are the brightest and most photostable in their respective spectral class. One should also carefully consider the desired time frame for visualization. There is a clear lag inherent in the Gal4 system, since the Gal4 protein must be made before the fluorescent protein can be transcribed (see also Section 2.3). The length of time needed for the fluorescent protein to fold can also be a critical factor in selection of the FP, since some FPs require proper folding for fluorescence. If the FP is being used to tag a protein of interest, it should not be an obligate oligomer. Thus, though the tandem dimer Tomato (tdTomato) is considerably bright and photostable, it is not the FP of choice for a fusion protein. DsRed also forms oligomers, though the monomer version has recently become available. Finally, when visualizing several tagged proteins, the individual FPs used should have minimal overlap in their excitation and emission spectra. The combination of Venus and EGFP should be avoided for this reason. As for any fluorescence-based assay, the FPs should be optimized to work with the available

Table 1
Fluorescent proteins available for live imaging myoblast fusion in *Drosophila*.

	Reference
FPs used in Drosophila muscle	
UAS-NLS-EGFP	[41], Bloomington Stock Center
UAS-H2B-YFP	[42]
sns-NLS-mCherry	[7]
UAS-gap-GFP	[7,43], Bloomington Stock Center
UAS-EGFP	[44], Bloomington Stock Center
UAS-GFP-WASp	[45]
UAS-Blow-mCherry	[45]
UAS-Sltr-mCherry	[45]
UAS-GFP-Actin	[14,20,46], Bloomington Stock Center
UAS-Actin-mCherry	[7,47]
UAS-GFP-Moesin	[48]
UAS-mCherry-Moesin	[49]
UAS-Lifeact-GFP	Abmayr lab, unpublished; Bloomington Stock Center
twi-GFP-actin	[29]
Drivers	
twi-Gal4	[7,14,20,45,50], Bloomington Stock Center
mef2-Gal4	[51], Bloomington Stock Center
24B-Gal4	[52], Bloomington Stock Center
SG30-Gal4	[53]
rp298-Gal4	[14,35]
sns-Gal4	[14,33]