METHODS & TECHNIQUES

Visualisation of ribosomes in Drosophila axons using Ribo-BiFC
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ABSTRACT

The distribution of assembled, and potentially translating, ribosomes within cells can be visualised in Drosophila by using Bimolecular Fluorescence Complementation (BiFC) to monitor the interaction between tagged pairs of 40S and 60S ribosomal proteins (RPs) that are close neighbours across inter-subunit junctions in the assembled 80S ribosome. Here we describe transgenes expressing two novel RP pairs tagged with Venus-based BiFC fragments that considerably increase the sensitivity of this technique we termed Ribo-BiFC. This improved method should provide a convenient way of monitoring the local distribution of ribosomes in most Drosophila cells and we suggest that it could be implemented in other organisms. We visualised 80S ribosomes in different neurons, particularly photoreceptors in the larva, pupa and adult brain. Assembled ribosomes are most abundant in the various neuronal cell bodies, but they are also present along the full length of axons. They are concentrated in growth cones of developing photoreceptors and are apparent at the terminals of mature larval photoreceptors targeting the larval optical neuropil. Surprisingly, there is relatively less puromycin incorporation in the distal portion of axons in the larval optic stalk, suggesting that some of the ribosomes that have initiated translation may not be engaged in elongation in growing axons.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: BiFC, 80S, Ribosomes, Neurons, Axons, Drosophila

INTRODUCTION

Ribosomes are ubiquitous molecular machines that translate gene sequences into the thousands of different proteins that make and operate every organism, so ribosomal components are some of the most abundant and evolutionarily conserved macromolecular constituents of cells. Each ribosome is made up of two complex ribonucleoprotein subunits – 40S and 60S in eukaryotes – and the joining of these into 80S functional ribosomes is tightly regulated. Even when cells are replete with ribosome subunits there are physiological situations (e.g. during nutrient deprivation or other cell stresses) when relatively few are assembled into protein-physiological situations (e.g. during nutrient deprivation or other cell stresses) when relatively few are assembled into protein-

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Methods & Techniques

Fig. 1. See next page for legend.
we termed Ribo-BiFC. Here we describe an improved version that employs transgenic flies expressing either of two novel RP pairs (RpS18/RpL11 and RpS6/RpL24) - the prefix ‘e’ is for eukaryotic ribosomal proteins without bacterial homologs – that are tagged with BiFC fragments of Venus fluorescent protein (Hudy et al., 2011). These Venus-based reporters greatly improved the sensitivity of the method and revealed clear ribosome signals along the full length of axons and at the axon terminals of both developing and mature neurons. In eye photoreceptor axons, which we examined in most detail, intense ribosome signals are particularly apparent in their growth cones during larval and pupal development. We suggest that these Venus-tagged RP pairs can provide a useful research tool with which to monitor the subcellular localisation and trafficking of assembled ribosomes in most Drosophila cells and tissues.

**RESULTS**

**BiFC-Venus-tagged 80S ribosomes can be detected in axons and growth cones of photoreceptor neurons**

The ribosomal protein pairs RpS18/RpL11 (us13/usL5) and RpS6/RpL24 (es6/el24) span inter-subunit potential contact points, on the surface of the ‘head’ and the ‘foot’, respectively, of the 80S ribosome (Fig. 1A). We generated UAS-driven Drosophila transgenes encoding these proteins that were tagged with complementing fragments of Venus fluorescent protein corresponding to the N-terminal domain (VN, 1-173 aa) and C-terminal domain (VC, 155-238 aa) (Fig. 1B). These yield a brighter and more specific BiFC interaction than YFP constructs (Hudy et al., 2011). Moreover, our characterisation in S2 cells indicated that fluorescence from the inter-subunit Venus BiFC complex might be more stable during translation elongation than the one from the corresponding YFP complex (Al-Jubran et al., 2013).

We tested the new transgenes in the Drosophila larval visual system, which is an excellent model for microscopic visualisation of the axonal projections of neurons. The eye is made up of about 750 ommatidia, each having eight photoreceptor neurons (the R-cells: R1-R8). R1–R6 axons project to a synaptic layer of the brain optic lobe termed the lamina plexus, and R7 and R8 axons pass through the lamina and end in a deeper brain region termed the medulla (Fig. 1C) (Mencarelli and Pichaud, 2015). Expression of either of our BiFC-Venus RP pairs in developing eye by using the GMR-GAL4 driver (Freeman, 1996) results in a strong signal. Within the growing photoreceptors, this is brightest in the cell bodies located in the developing eye, but it is apparent along the entire length of the photoreceptor axons, both in R1-R6 (ending in the lamina) and in R7 and R8 (ending in the medulla) (Fig. 1D; panel I, RpS18/RpL11; Panel II, RpS6/RpL24). The RpS18/RpL11 pair was used in the experiments described below.

The signal from the Venus-based reporters is much stronger than from the previous YFP-based RpS18/RpL11 transgene pair, which was only apparent in the cell bodies and proximal regions of the axons (Fig. 1D, panel III). This was despite the fact that substantial amounts of conventional GFP- or RFP-tagged versions of RpS18 and RpL11, which report the distributions of free ribosomal subunits as well as assembled ribosomes, are abundantly present throughout the axons when expressed with GMR-GAL4 (Fig. S1A). Although the expression levels of the tagged proteins could not be directly assessed in photoreceptors, as these make up only a small fraction of the cells in the tissue, our previous western blotting analysis of salivary glands indicates that these tagged proteins are at a substantially lower level than the endogenous counterparts, even when expressed in salivary glands with a strong GAL4 driver that results in a BiFC signal much brighter than that detected in the photoreceptors (Al-Jubran et al., 2013). Moreover, there is no evidence of proteins being considerably toxic when expressed with GMR-GAL4 since the eye develops as expected, except for a very mild glossy eye phenotype (Fig. S2B).

The neuronal distribution of the signal is confirmed by immunostaining with mAb24B10, which specifically recognises chaoptin, a GPI-linked cell surface glycoprotein that is present only on photoreceptor neurons and their axons (Fig. 1E) (Reinke et al., 1988; Zipursky et al., 1985). There is also intense 80S ribosome signal in enlarged foci at the tips of the R7 and R8 axons in the medulla region (Fig. 1E), which is probably in growth cones (Prokop and Meinertzhagen, 2006). Strong signals in photoreceptor growth cones are also apparent during pupal development (Fig. S2A). By comparing the pattern of the 80S signal with that of chaoptin, which mostly stains the periphery of the growth cones (compare insets in Fig. 1E), it is clear that the most intense ribosome signal is inside the growth cones. Comparison of the 80S signal with that of mCD8-GFP, another plasma membrane marker (Lee and Luo, 1999), which is evenly distributed along the axon (Fig. S1B, panel I versus panel II), also supports the conclusion that the whole interior of the growth cones must be replete with 80S ribosomes.

We also found signals in the axons of functional adult fly photoreceptors (Fig. S2A). Although the Ribo-BiFC signal is weaker than in developing photoreceptor axons, the reduction is probably a consequence of reduced expression of the GMR-GAL4 expression in adult flies, as this is also apparent when expressing mCD8-GFP alone (unpublished data). To test further whether ribosomes are present in the axons of mature neurons, we examined the Bolwig’s organ. This is the organ of sight/light-sensation of the larva. It consists of a bilateral bundle of 12 photoreceptors near the mouth-hook at the anterior of the animal, which projects their axons in a nerve that joins with the optical stalk of the eye-disc before entering the brain optic lobe and terminates in a distinctive small region of the medulla representing the larval optical neuropil in each
brain hemisphere (Fig. 1C) (Hofbauer and Campos-Ortega, 1990; Larderet et al., 2017). Within the neuropil, synapses are formed with the lateral neurons required for the circadian behaviour of the larva as well as the other neurons comprising the larval optical system (Helfrich-Förster et al., 2002; Keene et al., 2011; Larderet et al., 2017). We detected clear Ribo-BiFC signals along the Bolwig’s nerve and at its terminals in larval optical neuropil (Fig. 2A).

Fig. 2. Visualisation of Ribo-BiFC signals in mature axons. (A) Distribution of the RpS18VN-RpL11VC reporter signals (grey) in the developing photoreceptors axons in one of the larval brain’s optical lobes and in mature axons of the Bolwig’s nerve (arrow), as well as at the Bolwig’s nerve terminals in the larval optic neuropil (arrowhead). (B) Visualisation of the Ribo-BiFC signal in specific mature neurons of different thoracic (T 2-3) and abdominal (A 1-8) segments of the larval ventral nerve cord demarcated by the expression of D42-GAL4 (panel I), dDC-GAL4 (panel II) and CCAP-GAL4 (panel III). Yellow arrowheads indicate some of the neuronal projections and red arrows indicate cell bodies of some individual neurons in the ventral nerve cord.
Fig. 3. See next page for legend.
Fig. 3. Distal regions of growing photoreceptor axons incorporate relatively less puromycin. (A) Immunostaining of puromycin incorporation (red signal, panel I) in tissues expressing RpS18VN-Rpl11VC in the photoreceptors via GMR-GAL4 (yellow, panel II), DAPI staining (blue, panel III) shows the individual nuclei and highlights a monolayer of cells (white arrows), probably glia, surrounding the optic stalk (OS) (yellow arrow); the merged multicolour image highlights the overlap between the puromycin and 80S signals in different regions of the photoreceptors (panel IV); the yellow arrow indicates the position of the optic stalk after which there is a reduced puromycin signal compared to more proximal regions; the BIFC RpS18VN-Rpl11VC signal is shown in green instead of yellow in the merged image for better contrast. (B) Immunostaining of puromycin incorporation (red, panel I) in tissues expressing GMR-GAL4 driven mCD8-GFP (grey, panel II), DAPI staining shows cell nuclei (blue, panel III); the merged image (panel IV) highlights the relatively more intense green colour in the distal segments of the optic stalk; and the mCD8-GFP signal is shown in green instead of grey for better contrast.

![Image](image_url)

We also examined the distribution of 80S ribosomes in other types of neurons by expressing the reporters using different GAL4 drivers (see Materials and Methods): D42-GAL4 is expressed in motor neurons (Fig. 2B, panel I); and DicGAL4 and CCAP-GAL4 drive expression in pairs of laterally located neurons that are present in each segment of the brain ventral nerve cord, the axons/dendrites of which project to the midline (Fig. 2B, panel II and III, respectively). As in photoreceptor neurons, the Ribo-BiFC signals from 80S ribosomes are brighter in the cell bodies, but are apparent along the full length of the axons.

Ribosomes in the distal regions of photoreceptor axons incorporate less puromycin

The classic way to assay for translation is to monitor ribosome-catalysed incorporation of puromycin into the C-terminal of nascent peptides, either radiochemically (Nathans, 1964), or more recently by immunostaining (David et al., 2012; Schmidt et al., 2009). When we incubated salivary glands with puromycin briefly to minimise diffusion of puromycinylated peptides away from translation sites, as previously discussed (McLeod et al., 2014), we saw a good correlation between the 80S BiFC and puromycin signals (Al-Jubran et al., 2013). Puromycin immunostaining has also been recently used to visualise local translation in growth cones of photoreceptors (see Materials and Methods). These, together with the previously described UAS transgenes encoding individual GFP or RFP-tagged RPs, should provide useful tools to distinguish between inactive ribosomal subunits and assembled and actively translating ribosomes in Drosophila neurons (Rugie et al., 2013). However, we propose that our Ribo-BiFC technique provides a method to visualise changes in the subcellular distribution of ribosomes during different stages of Drosophila development and physiological states that is technically more straightforward than others recently developed (Lee et al., 2016). We detected a correlation between the presence of assembled ribosomes and puromycin incorporation, but some of the ribosomes in distal regions of axons seemed not to incorporate puromycin. These may correspond to ribosomes that are either paused on mRNAs after translation initiation or have significantly lower elongation rates. Ribosome pausing has been proposed to be an evolutionarily conserved mechanism to regulate protein synthesis (Darnell et al., 2018). Perhaps a similar regulatory mechanism operates on ribosome-loaded mRNAs present in axons of photoreceptors that are still growing and not yet active in the larval stage (Mencarelli and Pichaud, 2015).

DISCUSSION

Ribosome activation can be directly visualised by the fluorescence emitted as a result of the interaction between pairs of RPs in different subunits that: (a) are tagged with complementary parts of a BiFC-compatible fluorescent protein; and (b) are brought into close contact across the junction between subunits when a ribosome assembles. This technique, here named Ribo-BiFC, was previously used to visualise translating ribosomes in Drosophila S2 cells and salivary glands (Al-Jubran et al., 2013).

Although our previously described technique was not sensitive enough to visualise ribosomes in all neurons, here we described an improved version of this technique. Ribo-BiFC employs UAS-regulated transgenes that express pairs of neighbouring RPs (RpS18/RpL11 and RpS6/RpL24) tagged with BiFC-compatible complementary fragments of Venus fluorescent protein. These new transgenes allow a straightforward and sensitive visualisation of 80S ribosomes in Drosophila neurons and clearly detect assembled ribosomes in the axons and growth cones of developing photoreceptors, as well as in the axons of mature neurons, including larval photoreceptors. Here ribosome signals are also detected at the terminals located in the optical neuropil where synapses are formed with other neurons of the larval visual circuit (Larderet et al., 2017). We predict that the sensitivity of this method could be further increased by genetically combining multiple copies of the transgenes we generated (several P-element inserts are available; see Materials and Methods). These, together with the previously described UAS transgenes encoding individual GFP or RFP-tagged RPs, should provide useful tools to distinguish between inactive ribosomal subunits and assembled and actively translating ribosomes in Drosophila neurons (Rugie et al., 2013). As the Venus BiFC complex is very stable and possibly the key determinant of the Ribo-BiFC high sensitivity, it is not suitable for monitoring rapid changes in translation (Al-Jubran et al., 2013). However, we propose that our Ribo-BiFC technique provides a method to visualise changes in the subcellular distribution of ribosomes during different stages of Drosophila development and physiological states that is technically more straightforward than others recently developed (Lee et al., 2016). We detected a correlation between the presence of assembled ribosomes and puromycin incorporation, but some of the ribosomes in distal regions of axons seemed not to incorporate puromycin. These may correspond to ribosomes that are either paused on mRNAs after translation initiation or have significantly lower elongation rates. Ribosome pausing has been proposed to be an evolutionarily conserved mechanism to regulate protein synthesis (Darnell et al., 2018). Perhaps a similar regulatory mechanism operates on ribosome-loaded mRNAs present in axons of photoreceptors that are still growing and not yet active in the larval stage (Mencarelli and Pichaud, 2015).

MATERIALS AND METHODS

FLY STOCKS

Generation of the transgenes expressing the YN and YC YFP BiFC fragments or simply GFP or RFP tagged ribosomal proteins (RPs) has been previously
described (Al-Junbran et al., 2013; Ruggeiz et al., 2013). The constructs expressing the RPs tagged with either the VN (1–173) and VC (155–238) fragments were similarly generated, cloned in the pUAST vector (Brand and Perrimon, 1993), and transgenic flies produced by P element-mediated transformation of standard yeast strain (Bestgene). The Fkh-GAL4 transgene was used to drive expression in salivary glands (Henderson and Andrew, 2000), GMR-GAL4 expresses in the differentiated cells of the developing and mature eye including photoreceptors (Freeman, 1996), D2-GALA expresses in motor neurons (Vonhoff et al., 2013), ACD-GAL4 and CCAP-GAL4 express in different groups of neurons in brain ventral cord (Vömel and Wegener, 2008).

PBST three times and incubated with anti-mouse-Cy3 secondary antibody (Zipursky et al., 1985). Tissues were washed with antibody (David et al., 2012) (5B12, 1:500) overnight at 4°C. The mouse anti-Al-Jubran, K., Wen, J., Abdullahi, A., Roy Chaudhury, S., Li, M., Ramanathan, P., Matina, A., De, S., Piechocki, K., Ruggeiz, K. N. et al. (2013). Visualization of the joining of ribosomal subunits reveals the presence of 80S ribosomes in the nucleus. RNA 19, 1669-1683. doi:10.1261/mia038356.113

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