Video Article

Imaging Subcellular Structures in the Living Zebrafish Embryo

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Abstract

In vivo imaging provides unprecedented access to the dynamic behavior of cellular and subcellular structures in their natural context. Performing such imaging experiments in higher vertebrates such as mammals generally requires surgical access to the system under study. The optical accessibility of embryonic and larval zebrafish allows such invasive procedures to be circumvented and permits imaging in the intact organism. Indeed the zebrafish is now a well-established model to visualize dynamic cellular behaviors using *in vivo* microscopy in a wide range of developmental contexts from proliferation to migration and differentiation. A more recent development is the increasing use of zebrafish to study subcellular events including mitochondrial trafficking and centrosome dynamics. The relative ease with which these subcellular structures can be genetically labeled by fluorescent proteins and the use of light microscopy techniques to image them is transforming the zebrafish into an *in vivo* model of cell biology. Here we describe methods to generate genetic constructs that fluorescently label organelles, highlighting mitochondria and centrosomes as specific examples. We use the bipartite Gal4-UAS system in multiple configurations to restrict expression to specific cell-types and provide protocols to generate transiently expressing and stable transgenic fish. Finally, we provide guidelines for choosing light microscopy methods that are most suitable for imaging subcellular dynamics.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53456/>

Introduction

In vivo imaging provides direct visualization of cellular behaviors in the most physiological context. The transparency of zebrafish embryos, their rapid and external development and a rich array of genetic tools that permit fluorescent labeling have all contributed to the growing use of *in vivo* microscopy to elucidate the dynamics of key developmental events. Imaging studies of nervous system development in zebrafish have for example greatly expanded our knowledge of the behavior of neural progenitor cells and the fate of their progeny including their subsequent migration, differentiation and circuit integration¹⁻⁸.

The stage is now set to investigate the subcellular dynamics underlying these cellular behaviors. Indeed, zebrafish are already being exploited as tools for *in vivo* cell biology. It is now possible to visualize mitochondria⁹⁻¹¹, centrosomes^{2,8,12-14}, Golgi¹⁵, the microtubule⁴ and actin¹⁶ cytoskeleton, endosomes¹⁷ and components of the apical membrane complex^{1,18}, among other subcellular structures in zebrafish embryos *in vivo*. So far, much of what is known about the function of these organelles comes from studying their behavior in cultured cells. While *in vitro* studies have yielded tremendous insight into cell biology, cells in culture do not fully represent the complexity of the *in vivo* situation and therefore do not necessarily reflect the function and dynamics of subcellular organelles *in vivo*. Zebrafish embryos offer a viable *in vivo* alternative to examining subcellular dynamics.

As vertebrates, zebrafish possess many organ systems (e.g., neural retina) that are homologous to those found in mammalian species. Additionally, zebrafish embryos are increasingly being used to model human diseases^{19,20}, including those related to centrosomal function (e.g., microcephaly²¹ and Leber's congenital amaurosis²²) and to mitochondrial function (e.g., Parkinson's disease²³, tauopathies^{10,24} and Barth syndrome²⁵). *In vivo* imaging at the cellular and subcellular level in these instances will permit a better understanding of the cell biology underlying these pathological states.

The overall goal of the methods described here is to provide a comprehensive guide to investigate organelles and other subcellular structures in zebrafish embryos using *in vivo* light microscopy. The entire work-flow involved in visualizing and tracking subcellular structures *in vivo* is described - from genetic labeling approaches, to generating transiently expressing and stable transgenic fish, and finally to imaging using

wide-field and confocal microscopy. While each of these procedures is used by numerous zebrafish laboratories, the protocols described are optimized and streamlined for investigating the dynamics of subcellular structures. Two specific aspects of the work described here warrant mention: First, the use of the Gal4-UAS expression system in multiple configurations to genetically label organelles in specific cell-types. Second, a direct comparison of wide-field and confocal microscopy to image subcellular structures *in vivo*.

Current strategies to genetically label organelles and other subcellular structures in zebrafish either make use of capped mRNA^{1,4,8} or DNA based constructs where promoter elements directly drive the expression of fusion proteins^{9,14,15}. *In vitro* transcribed capped RNA results in rapid and broad expression, that is not tissue-specific however. Additionally, expression levels diminish over time as the capped RNA is diluted or degraded. Thus the use of RNA based constructs to examine organelle dynamics at later stages in development is limited (usually up to 3 days post-fertilization).

These limitations can be overcome by using DNA constructs, where spatial and temporal control of expression is determined by specific promoter elements. When DNA based constructs are used in the context of the Gal4-UAS system significant improvements to transgene expression levels are observed^{26,27}. In this bipartite expression system, cell-type specific promoter elements drive the expression of a transcriptional activator Gal4, while reporter genes are cloned downstream of the Gal4-binding upstream activating sequence (UAS). By combining UAS reporters with appropriate Gal4 drivers, expression can be restricted to specific cell-types, circumventing the need to clone reporter genes behind different promoters every time a specific expression pattern is desired. Furthermore, the expression of multiple UAS reporter genes can be driven by a single Gal4 activator. The Gal4-UAS system thus provides a versatile and flexible genetic approach for subcellular labeling.

Wide-field and confocal microscopes are the workhorses of most laboratories. Wide-field systems typically use an arc lamp as a light source and detect the emitted light with a sensitive camera that is placed at the end of the light path. This imaging modality is typically restricted to thin samples as out-of focus light obscures in-focus information in thicker samples. Confocal microscopes differ from wide-field systems in that they are built to favor signals that originate from the focal plane over those that originate out of focus (*i.e.*, "optical sectioning")²⁸. To achieve optical sectioning a pinhole is placed in the emission path in a conjugate position to the point light source. Lasers are used as light sources and signals are detected with photomultiplier tubes (PMTs). Practically, a laser beam is swiped over the sample point-by-point and the fluorescence emission at each spot (pixel) is detected by the PMT.

Here we image the very same subcellular structures in living zebrafish embryos using both wide-field and confocal microscopy to provide a direct comparison of both microscopy modalities. The underlying aim of providing such comparisons is to offer guidelines for choosing the most appropriate microscopy technique for the specific question at hand.

Using the approaches described here we demonstrate Gal4-UAS based genetic labeling of mitochondria and centrosomes. These organelles are imaged in different cell-types of the nervous system and in muscle cells using wide-field and confocal microscopy to demonstrate the suitability of each imaging modality. The methods described here can easily be adapted for investigating other organelles and subcellular structures in the living zebrafish embryo.

Protocol

All animal experiments were performed in accordance with local regulations of the government of Upper Bavaria (Munich, Germany).

1. Labeling Organelles and Other Subcellular Structures

NOTE: Here genetic reporter constructs that fluorescently tag centrosomes, mitochondria and cell membranes are described.

1. Use conventional cloning methods²⁹ to generate fusion proteins that fluorescently label centrosomes and mitochondria. Clone the coding sequence of zebrafish centrin4 (cetrn4) in-frame with a fluorescent protein² (FP) such as Yellow fluorescent protein to generate cetrn4-YFP. Clone the mitochondrial targeting sequence of subunit VIII of cytochrome c oxidase in-frame with an FP such as Cyan fluorescent protein to generate mitoCFP⁹⁻¹¹.
2. To restrict expression of the fusion proteins (cetrn4-YFP or mitoCFP) to specific cells of the zebrafish embryo (*e.g.* neurons or muscle cells) use the bipartite Gal4-UAS expression system^{26,27}. First generate a UAS reporter construct. Use conventional methods to clone the fusion protein in a UAS expression vector, downstream of the Gal4-binding UAS cassette (variants range from 1x to 14x UAS, see Discussion)^{26,27,30} and a minimal promoter (E1b basal promoter from the carp β actin gene), and generate UAS:cetrn4-YFP and UAS:mitoCFP.
NOTE: To prepare UAS reporter constructs for stable transgenic line generation additional elements need to be cloned (see 3 below)
3. To visualize the cellular context in which centrosomes or mitochondria are labeled, generate UAS reporter constructs in which cell membranes are labeled by FPs. Clone the first twenty amino acids of zebrafish Gap43 (containing palmitoylation sites) in-frame with an FP to target that FP to the cell membrane (memFP)^{31,32}.
4. Obtain driver constructs or transgenic lines in which cell-type specific promoter elements drive the expression of the transcriptional activator Gal4-VP16²⁷, Gal4FF³³ or GalTA4³⁰.
5. Combine the UAS reporter constructs with an appropriate driver construct to restrict expression to the desired cell-type of interest (*e.g.*, in neurons or muscle cells). To do this co-inject Gal4 driver and UAS reporter constructs at the one-cell stage of fertilized eggs (for detailed instructions see 2 below) to generate transiently expressing fish. Alternatively, generate a UAS reporter stable transgenic line (for detailed instructions see 3 below) and cross to a Gal4 driver stable transgenic line to generate offspring bearing both transgenes.
NOTE: It is also possible to inject UAS reporter construct/s into fertilized eggs from a Gal4 driver stable transgenic line or a Gal4 driver construct into fertilized eggs from a UAS reporter stable transgenic line.
6. To co-express multiple distinct fusion proteins using the Gal4-UAS system, use a Gal4 driver and UAS reporter/s in one of the following configurations: A. Multiple, separate UAS reporter constructs (*e.g.*, UAS:mitoCFP and UAS:memYFP co-injected with a Gal4 driver

construct)¹⁰. B. Multiple UAS cassettes on a single reporter (e.g., UAS:cetn4-YFP, UAS:memCerulean)¹² C. Bi-directional UAS reporter (e.g. UAS:memYFP,mitoCFP)^{2,10,24} (Figure 1).

Figure 1. Strategies to co-express fusion proteins using the Gal4-UAS system.

Co-expression of multiple fusion proteins can be achieved by using UAS reporter cassettes in various configurations: (A) multiple, separate UAS-driven constructs, (B) multiple UAS cassettes on a single construct or (C) a bidirectional UAS cassette on a single construct. [Please click here to view a larger version of this figure.](#)

2. Generate Transiently Expressing Fish

NOTE: The following is an adaptation of previously published protocols^{34,35}. A basic injection setup should include a stereomicroscope with a magnification range up to 12x, a micromanipulator with a micropipette holder and a source of air pressure. Prepare 2.1-2.5 ahead of time:

- To prepare egg microinjection chambers, prepare a solution of 1.5 % (w/v) agarose in water. Add agarose powder to water and microwave until the agarose is completely dissolved. Fill a petri dish (100 x 15 mm) with this solution.
 - Allow the agarose solution to cool to approximately 45 °C before placing a plastic microinjection mold (40 mm x 66 mm, with 6x 50 mm long ridges that are 1.5 mm wide, 1 mm deep and spaced 3 mm apart) on the surface of the molten agarose, taking care not to introduce bubbles at the interface.
 - After the agarose sets, store at 4 °C O/N. Carefully remove the mold using a spatula. Prepare multiple microinjection chambers at a time, store at 4 °C and use repeatedly over several weeks.
- Use a micropipette puller to generate glass injection capillaries with a long shank³⁶.

NOTE: The specific settings used to generate injection capillaries should be determined empirically. Injection capillaries can be pulled ahead of time and stored prior to use.
- Measure the concentration of plasmid DNA (Gal4 driver and UAS reporter constructs) for microinjection using a spectrophotometer according to the manufacturer's manual. Measure the ratio of absorbance at 260 nm and 280 nm (A260/280). Dilute the DNA to a final concentration of 100 ng/μl in nuclease-free water.

NOTE: A A260/280 ratio of ~1.8 signifies pure DNA. Consider using a commercial kit (based on silica-based binding matrix) to purify the plasmid DNA to be used for injection. Plasmid DNA should be of high quality to prevent toxicity.
- Prepare 10 μl injection-mix by combining DNA (0.5 to 2.5 μl of a 100 ng/μl stock for a final concentration of 5 to 20 ng/μl), Danieau's solution (0.33 μl of 30x stock), Phenol Red (0.25 μl, optional) and nuclease-free water to a 10 μl total volume.

NOTE: Empirically determine the concentration of DNA that yields suitable expression. Gal4 driver and UAS reporter plasmids need to be co-injected. No expression will ensue when only the Gal4 driver or UAS reporter plasmid is injected into wild-type fertilized eggs. If however the fertilized eggs are from a Gal4 driver line then injection of one or several UAS reporter plasmids suffices. Conversely, if microinjecting into a UAS reporter line, only the Gal4 driver plasmid needs to be injected. Finally, while Phenol Red can greatly help to visualize injections, it is also a fluorescent compound itself. Therefore, Phenol Red could obscure visualizing FPs if imaging is planned before 24 hr post-fertilization (hpf) as it may not be sufficiently diluted by this time.
- The evening before injections are planned, set up several pairs (typically 5 to 10) of male and female zebrafish for breeding. Use breeding tanks with an insert containing a gridded bottom and a removable divider to separate the male and female fish.

NOTE: Male and female zebrafish can generally be distinguished by their body shape. Males tend to be slender while females tend to exhibit a ventrally protruding abdomen. Males additionally tend to have a yellowish-red coloring on their ventral abdominal area.
- Immediately prior to DNA injections, remove the dividers to allow the male and female to mate. Approximately 15 to 30 min after removing the divider, check for eggs at the bottom of the tank. Transfer the adult fish using a fishing-net to a new breeding tank.
- Pour the water, containing the newly-laid fertilized eggs, out of the breeding tank into a sieve (e.g., a plastic tea-strainer). Wash the eggs in the sieve using a spray bottle containing Danieau's solution. Invert the sieve onto a petri dish and use a spray bottle with Danieau's solution to recover all the eggs (typically 50 to 200 eggs per breeding pair of adult fish).

8. Using a microloader pipette, back-fill a pulled injection capillary with the DNA injection-mix. Mount the injection capillary into the holder of a micromanipulator and trim the tip, under the visual control of a stereomicroscope, using forceps to create a micropipette that can penetrate the chorion and cell cytoplasm while being able to deliver a suitable volume of the DNA.
NOTE: The degree to which the tip of the injection capillary is trimmed should be determined empirically and can best be judged when injecting eggs.
9. To determine the volume of DNA for injection, discharge a drop of the injection solution into mineral oil. Measure the radius (r) of the drop using a calibration micrometer and calculate its volume ($4/3 \pi r^3$). Modulate the volume by adjusting the injection pressure and/or the duration of each injection pulse.
10. Using a plastic pipette, transfer all the collected eggs (typically 50-200, from 2.7 above) into the trenches of a microinjection chamber. Under the visual control of a stereomicroscope, use forceps to orient the eggs so that the cell cytoplasm (transparent) and yolk (dense, non-transparent) can be readily identified.
11. Under the visual control of a stereomicroscope, inject DNA into the cell cytoplasm of the fertilized egg, taking care to ensure that the injected volume (1 to 2 nl) corresponds to approximately 10% of the cell's volume.
NOTE: Phenol red in the DNA solution aids in the visualization of the volume injected.
12. After injection, loosen the eggs from the trenches of the injection mold by flushing them with a spray bottle containing Danieau's solution. Subsequently transfer eggs into a fresh petri dish using a plastic transfer pipette and maintain in a 28.5 °C incubator.
13. Maintain un-injected eggs as controls to determine whether injections contribute to high mortality rates, either as a result of physical damage sustained during micropipette penetration or the DNA mix.
NOTE: High mortality rates following injections could be due to numerous factors, including excessively high DNA concentration or injection volumes and/or plasmids with contaminants. To prevent toxicity from low quality DNA preps, commercial kits (based on silica-based binding matrix) can be used.
14. At regular intervals following injections, use a stereomicroscope to screen for unfertilized eggs and dead or malformed embryos and discard these.
NOTE: Deem the eggs that appear to be at the one-cell stage several hours after fertilization, as unfertilized. Identify malformed embryos by gross anatomical defects such as a compromised anterior-posterior body axes. Amorphous material within a chorion suggests that the embryo did not proceed through developmental stages and died. To ascertain if embryos that were microinjected undergo a normal course of development consult anatomical atlases³⁷.
15. Transfer the embryos using a plastic transfer pipette to Danieau's solution containing 1x 1-phenyl 2-thiourea (1xPTU) between 10 and 24 hpf to prevent pigment formation. Maintain the embryos in Danieau's solution containing PTU for the duration of the experiment.
NOTE: In addition to melanophores, iridophores on the surface of the skin can be problematic for imaging. While PTU does not inhibit iridophore formation, mutant lines with reduced numbers of iridophores such as roy orbison (roy) exist and can be used³⁸.
16. After embryos hatch (usually on the second or third day post-fertilization, 2 or 3 dpf), discard the chorions and exchange the Danieau's solution containing PTU.
NOTE: These steps are important to ensure the quality of the embryo medium and the viability of the healthy embryos.

3. Generate Stable Transgenic Lines

NOTE: Stable transgenic lines can be efficiently generated using the Tol2 transposon system. A Tol2 transposon vector containing the transgene of interest is co-injected along with mRNA encoding the transposase enzyme into fertilized eggs at the one-cell stage. Transposase protein derived from the injected mRNA catalyzes the excision of the transgene cassette from the transposon vector and its integration into the genome³⁹.

1. Clone the transgene reporter cassette (e.g., UAS:mitoCFP) between Tol2 inverted terminal repeats in one of the Tol2 vectors currently used in the zebrafish community as described⁴⁰.
NOTE: Tol2 vectors containing a selectable cassette in which promoter elements drive FP expression in organ systems unrelated to the area of interest (e.g., heart⁴¹ or lens⁴²) are useful for screening UAS reporter transgenic lines in the absence of Gal4 transactivation.
2. Using a commercial kit (based on silica-based binding matrix), purify the plasmid DNA to be used for injection. Ensure that the plasmid DNA is of high quality to prevent toxicity.
3. Transcribe Tol2 transposase-encoding mRNA using an appropriate transcription vector such as PCS-TP⁴³. Briefly, linearize PCS-TP and use a commercial kit to generate capped mRNA and follow the manufacturer's protocol. Take care to avoid contamination with RNases (e.g., use RNase-free pipette tips and tubes). Aliquot the RNA, at a concentration of 100 ng/μl, for single-use and store at -80 °C.
4. On the morning of the injections, thaw an aliquot of transposase mRNA on ice. Mix the transposon DNA vector (2 μl of 100 ng/μl) and transposase mRNA (2 μl of 100 ng/μl) in a 1:1 ratio, and add 6 μl of RNase-free water to bring the total volume to 10 μl.
NOTE: A concentration of 20 ng/μl is recommended for each but the optimal concentration should be determined empirically. Use RNase-free water for dilution. Use gloves when handling the DNA-RNA injection mix and keep it on ice for the entire period of injections to prevent degradation of the mRNA.
5. Using the detailed instructions in **section 2** above, inject the DNA-RNA injection mix into fertilized eggs at the one-cell stage. Under visual control of a stereomicroscope target the cell cytoplasm at the one-cell stage for the highest rates of transgenesis efficiency. Maintain the injected embryos at 28.5 °C until ready to screen for transgene expression.
NOTE: PCR can be done to check for efficiency of the Tol2 transposase as previously described⁴⁴.
6. Screen embryos in a petri dish for transgenesis using the oculars of a fluorescence dissecting microscope.
NOTE: Use the appropriate filter sets of the microscope to visualize FP expression of the selectable cassette (i.e., fluorescence in the heart or lens) at 2 or 3 dpf. Identify potential transgenic embryos by expression (in heart or lens) and raise these embryos to adulthood (F_0 generation) using standard protocols⁴⁵. Alternatively, identify potential transgenic embryos by PCR as described in⁴⁶.
7. Once the UAS reporter F_0 fish are 2.5 - 3 months of age, cross them (see **2.5** for details) to non-transgenic wild-type fish to acquire eggs to establish an F_1 generation. Alternatively, cross the UAS reporter F_0 fish to a Gal4 driver line to establish an F_1 generation. In the latter case, the UAS-driven transgene can be directly monitored in the embryos obtained from the cross.
8. Use a fluorescence dissecting microscope to screen F_1 embryos for expression of the transgene or selectable cassette.

NOTE: When expression is confirmed then the transgene can be said to be germline transmitted. Transgenesis is non-Mendelian at the F_0 stage. The number of embryos expressing the transgene may vary from a small number to the vast majority in individual clutches. Transposon integration in different F_0 fish may vary greatly, resulting in different expression patterns. Therefore maintain F_1 fish from different F_0 founders as separate sub-lines. Transposon integrations in F_1 fish are stable and are passed onto subsequent generations in a Mendelian fashion.

9. Maintain each F_0 fish in separate tanks to re-identify transgene carriers.

4. Prepare Embryos for Imaging on an Upright Microscope

NOTE: The procedures described here have been optimized for imaging on upright microscopes with long-working distance water-dipping-cone objectives.

1. Use a fluorescence dissecting microscope to screen embryos for transgene expression.
NOTE: Expression of a UAS reporter transgene will depend on the specific Gal4 driver line used. Select embryos for imaging experiments based on desired expression pattern.
2. Transfer the selected embryos using a plastic pipette to a separate petri dish containing Danieau's buffer with 1x PTU and 1x Tricaine.
NOTE: When labeling is sparse (only a few cells in an organ system) mount the embryos in agarose (see 4.3-4.7 below) and screen for transgene expression using a wide-field microscope with high magnification objectives (see 5.1 below). Tricaine anesthetizes the fish and should be effective within seconds. If fish are not immobilized it is likely that the Tricaine has degraded. Possible reasons for this degradation include poor storage of Tricaine, which is light-sensitive⁴⁷.
3. Prepare the agarose to embed the fish by dissolving low-melting agarose powder in Danieau's buffer to a final concentration of 0.7%. Aliquot (1 ml) and keep in a heat-block at 40 °C until ready to use.
NOTE: Higher concentrations of agarose (upto 1.5%) can also be used to embed fish.
4. Add 50 μ l of a 20x stock of Tricaine and 20 μ l of a 50x stock solution of PTU to the 1 ml aliquot of low-melting agarose and mix well by tapping the side of the tube. Return the tube to the heat-block. Using a plastic transfer pipette, gently pipette a few (1-10) anesthetized embryos into the agarose, transferring as little liquid as possible to avoid diluting the agarose.
5. Using a plastic pipette transfer all the embryos with a small amount of agarose to a glass bottom petri dish.
6. Working relatively fast, use forceps to position the embryos in the desired orientation, depending on the structure to be imaged.
NOTE: Orient embryos on their side if the retina or Rohon-Beard (RB) sensory neurons should be imaged.
7. Allow the agarose to solidify for at least 15 mins. Add Danieau's buffer containing 1xPTU and 1xTricaine to cover the embryos embedded in agarose. Place the dish containing agarose-embedded embryos in an incubator at 28.5 °C until ready to image.

5. Imaging Cellular and Subcellular Structures using Wide-field or Confocal Microscopy

NOTE: Wide-field microscopy and point-scanning confocal microscopy are the most widely used modalities to image fluorescently labeled zebrafish embryos. **Table 1** summarizes the main advantages and disadvantages of both systems. For both forms of microscopy, embryos are mounted in agarose as described in 4 above, and maintained at 28.5 °C throughout the imaging experiment by using a heating chamber on the stage of the microscope. Importantly, the dish with agarose-embedded embryos is allowed to equilibrate to 28.5 °C before imaging commences as temperature fluctuations cause drift in the z-dimension. When conducting long-term imaging experiments (over several hours), place a Plexiglas cover over the petri-dish to reduce evaporation of the buffer.

	Wide-field	Point-scanning confocal
Cost	Relatively inexpensive	Expensive (approx. 5x more)
Photo-bleaching	Low	High. The sample is exposed to laser light above and below the focal plane.
Photo-toxicity	Low	High (see Photo-bleaching above)
Acquisition speed	Fast	Slow
Resolution in xy	Abbe's law	Abbe's law (can be improved by app. 40% using a very small pinhole; however, in most settings this has little practical application)
Optical sectioning	Poor	Yes (can be adjusted by pinhole size)
Tissue penetration	Limited to superficial structures (e.g., Rohon-Beard cells or muscle fibers)	Limited to < 100 μ m from the surface of the embryo

Table 1. General comparison of wide-field and point-scanning confocal microscopy

1. **Wide-field microscopy**
NOTE: Here guidelines are provided for imaging zebrafish embryos using an upright wide-field microscope with long-working distance water-dipping-cone objectives. The microscope is equipped with a cooled CCD camera, and filter sets for visualizing different fluorophores mounted on an automated filter wheel for rapid acquisition of multiple channels. Image acquisition is controlled by μ Manager, an open source microscopy software package⁴⁸. A specific example for imaging mitochondria in RB sensory neurons is provided. RB cells are genetically labeled using membrane targeted YFP and their mitochondria are CFP-tagged (see 1.1 above).
 1. Mount embryos on their side in low-melting agarose as described in 4 above.
 2. Allow the dish with mounted fish to equilibrate to 28.5 °C in a heating chamber on the stage of the microscope before commencing imaging.

3. Use a low magnification water-dipping-cone objective and look through the oculars of the microscope to choose an area on the surface of the embryo to image.
NOTE: If the stable transgenic *MitoFish* reporter line, Tg(UAS-E1b:mYFP,mitoCFP)^{mdc6}, is used in conjunction with a driver line such as HuC:Gal4 then most RB neurons are labeled, and appear as a dense mesh-work of neurite arbors on the surface of the embryo. If microinjection of DNA constructs is used to achieve sparse labeling (e.g., Sensory:Gal4-VP16 with UAS:mitoCFP and UAS:MA-YFP), screen embryos to identify double-labeled cells.
4. Open the microscope software (μ Manager 1.4) and click on "Illumination" under "Configuration settings" to define the correct filter sets for the wavelength of interest (YFP or CFP for the current example). Under "Camera settings" enter the exposure time required to acquire a suitable image.
NOTE: The "Illumination Settings" is pre-set by the user upon installation of the software and is loaded when opening μ Manager. If the software is not configured to control the filter wheel, manually move the filter wheel in the turret to the appropriate position.
5. Change to a long-working distance water-dipping-cone objective ranging from 40-100x magnification. Choose the objective that has the highest numerical aperture (NA) and is chromatically corrected (apochromat).
6. Click on "Live" to choose the field of view: In the case of the RB neuron, image mitochondrial transport at the stem axon, emanating from the cell body, or in the peripheral arbor.
NOTE: The peripheral arbors of RB neurons in the caudal fin fold of the embryo provide an ideal opportunity to image a large field of view that is flat and can therefore be captured on a single frame with a wide-field microscope. It is therefore important to mount the embryo as flatly as possible on its side, so that the caudal fin fold is parallel to the bottom of the petri dish.
7. After choosing a field of view, click on the "rectangular selection" button in the ImageJ menu, define a region of interest (ROI) and click on "ROI" in the μ Manager window. After selecting the ROI for imaging, click "Stop" and "Save" to take an image of the YFP channel to record the local morphology of the neurite/s.
8. To image mitochondrial transport click on the "Multi-D Acq." button. Observe an additional window ("Multi-dimensional Acquisition") and select the number of time-points as well as the interval between time-points on this window. Use frame rates of 0.3-1 Hz. Carry out recordings for at least 10 min to collect as many data-points as possible.
9. In the "Multi-dimensional Acquisition" window, click on "Channels", add and define the wavelength for imaging (CFP for mitochondria in this example) and the time of exposure. Keep exposure times to below 400 msec for imaging mitochondrial transport.
10. Click on the option "Save Images" in the "Multi-dimensional Acquisition" window, to automatically save the files in a specific folder outlined in the 'Directory root'. Click on "Acquire" at the upper right corner to start time-lapse imaging.
11. Manually readjust the focus while time-lapse recording to compensate for any drift in the z dimension.
NOTE: Using these parameters mitochondrial transport in RB neurons can be imaged for as long as 4 hr.

2. Confocal microscopy

NOTE: Here guidelines are provided for imaging with an upright Olympus FV1000 confocal microscope and long-working distance water-dipping-cone objectives. The microscope is equipped with multiple laser lines and several detectors (conventional PMTs and more sensitive gallium arsenide phosphide detectors) that allow for multichannel imaging. Specific reference is made to Olympus Fluoview software. However, the imaging parameters described here should be easily transferable to other confocal systems.

1. Mount embryos in low-melting agarose in an orientation appropriate for the organ/structure to be imaged, as described in 4 above.
2. Allow the dish with mounted fish to equilibrate to 28.5 °C in a heating chamber on the stage of the microscope before commencing imaging.
3. Use long-working distance water-dipping-cone objectives for confocal microscopes in an upright configuration. Choose objectives with the highest possible NA to maximize the amount of fluorescent signals that can be collected and have the best resolving power. When collecting images from multiple channels choose chromatically corrected objectives (apochromat).
4. Open the microscope software and click on "Trans Lamp" or "Epi Lamp" to use either transmitted or fluorescence light respectively to identify the region of interest via the oculars of the microscope.
5. Use the software to set up the following scanning parameters to acquire image stacks:
 1. In the "Acquisition Setting" window verify that the objective chosen for imaging matches the objective that appears on the drop-down menu of available objectives.
NOTE: This is to ensure that any pre-calculated parameters for a particular objective (e.g., lateral and axial resolution, size of the confocal aperture etc.) hold true and can be reliably used.
 2. Select the appropriate laser line/s, and excitation and emission dichroic filters to image specific fluorescent protein(s). Do this by clicking on the "Dye list" button in the "Image Acquisition Control" window and choose the appropriate fluorophore/s. Alternatively, click on the "Light path and dyes" button to set these parameters manually.
NOTE: When the "Dye List" button option is chosen, the software automatically selects the appropriate laser lines, excitation and emission dichroic filters and adjusts the size of the confocal aperture appropriately.
 3. In the "Acquisition Setting" window, adjust the "Zoom" factor and "Size aspect ratio" (i.e., 512x512, 1024x1024 etc.) of the scanned image to obtain the pixel size necessary to best resolve the structures being imaged. Set the pixel size to be about half the theoretical resolution of the objective, thereby following Nyquist sampling criteria. To determine the pixel size of the acquired image click on the button with the symbol for information ("i") in the "Image Acquisition Control" window.
 4. Set the scan speed to the fastest possible (2 μ s/pixel) in the "Acquisition Setting" window.
 5. In the "Image Acquisition Control" window click on Kalman line averaging to reduce noise. A factor of 2 to 3 usually suffices.
 6. Select a sequential scanning mode when imaging fluorophores with overlapping spectra. To do this, click on "Sequential" and "Line" in the "Image Acquisition Control" window.
 7. Adjust the power output of the relevant laser line/s (typically below 5%). The specific values need to be determined empirically.
 8. Click on "XY Repeat" or the "Focus x2" or "Focus x4" button to continuously scan the selected region while adjusting the detector settings for each channel. Adjust "HV", "Gain" and "Offset" for each channel to acquire images that have the highest dynamic range of grey values.
NOTE: The specific values for these settings need to be determined empirically. "HV" adjusts the voltage on the detector to change its sensitivity, "Offset" adjusts the output signal of the detector and "Gain" multiplies the output signal of the detector by a constant factor.

9. Press Ctrl + H to visualize the acquired images via a look-up table (HiLo) that identifies under-saturated (appear blue) and over-saturated pixels (appear red), both of which should generally be avoided. Re-evaluate the power output of the relevant laser line/s and the detector settings.
NOTE: Using high laser power and high levels of the "HV" of the detector will lead to more signal. Higher laser power will however lead to increased bleaching and photo-toxicity. Increasing the "HV" will lead to increased noise. Therefore, a compromise needs to be found when setting laser power and "HV" to acquire images with acceptable levels of noise while preventing photo-damage (see also **Table 1**).
10. Collect images from a defined volume by focusing on the upper and lower limits of the area of interest. In the "Acquisition Setting" window click on the "End Set" and "Start Set" set buttons of the z-stack window at the upper and lower limits of the volume to be imaged. Select a step size that is half of the z-resolution for the given objective (e.g., if z resolution is 2 μm choose 1 μm). To determine the z-resolution of the objective click on the button with the symbol for information ("i") in the "Image Acquisition Control" window.
11. Set up a time series to collect z-stacks at a temporal frequency that is appropriate for the dynamics of the subcellular structure being imaged. In the "TimeScan" sub-window enter the frequency at which z-stacks should be acquired ("Interval") and the number of times the images should be acquired ("Num").
12. Click on the "Depth" and "Time" buttons in the "Image Acquisition Control" window to confirm that a z-stack and time-series will be acquired. Finally click on the "XYZT" button to begin acquiring time-lapse images.
13. After completion of image acquisition, observe "Series Done" on the software interface. Click on it and save the images in "oib" format to record the images and metadata associated with them.
NOTE: Images obtained on the wide-field microscope and confocal microscope can be visualized and analyzed using software such as FIJI, a free public domain image processing program.

Representative Results

Here the use of wide-field and confocal microscopy to image mitochondria and centrosomes in living zebrafish embryos is directly compared and contrasted. Depending on the location of the cells in which organelle dynamics are to be examined and the inherent frequency of the specific subcellular events, generally either wide-field or confocal microscopy is the better choice. We imaged organelles in RB neurons located on the surface of the embryo and in retinal cells located deeper. The superficial location of RB neurons together with their two dimensional geometry make them good candidates for being imaged by both wide-field and confocal microscopy (**Figure 2**). Acquiring images using confocal microscopy is however significantly slower and can lead to an underestimation of the dynamics of specific subcellular events (e.g., movement of mitochondria, **Figure 2C,D**). Images of retinal cells acquired by wide-field microscopy suffer from poor contrast as fluorescence signals from a large volume of tissue obscures detail in the focal plane. Here, the optical sectioning capability of confocal microscopy results in noticeably improved contrast (**Figure 3**).

To enable concurrent visualization of organelles and cellular membranes we used the Gal4-UAS system in three different configurations. First, the UAS *MitoFish* reporter line Tg(UAS-E1b:mYFP,mitoCFP)^{mde6} was used. Here, a bidirectional UAS permits concomitant expression of mitochondrially-targeted CFP and membrane targeted YFP. In combination with appropriate Gal4 driver lines, *MitoFish* label the mitochondria and cell membranes of RB sensory neurons (**Figure 2A-C**) or retinal cells (**Figure 3A,B**) with CFP and YFP respectively. Second, two UAS constructs (UAS:mitoCFP and UAS:MA-YFP) were combined with a Gal4 driver construct (Sensory:Gal4-VP16, sensory neuron-specific enhancer elements from the islet-1 gene)⁷ in transient injections. The mosaic expression that generally results from these injections permitted the tracking of individual cells over days (**Figure 2E**). A third approach employed the use of two UAS cassettes, each driving the expression of a different fusion protein, on a single contiguous construct. This strategy was used to generate *CentrinFish* Tg(UAS:ctn4-YFP,UAS:MA-Cerulean)^{tum1}, in which a centrin4-YFP fusion labels centrosomes and Cerulean is targeted to cell membranes. In combination with specific Gal4 driver lines the centrosomes and cell membranes of retinal cells (**Figure 3C,D**) or muscle fibers (**Figure 4**) are labeled.

Figure 2: Imaging mitochondria *in vivo* in RB sensory neurons of embryonic zebrafish.

RB sensory neurons in the caudal part of the fin fold of 2 dpf *MitoFish* were imaged using an apochromat 40x water-dipping-cone objective with an NA of 0.80, either by wide-field (**A**) or confocal (**B**) microscopy. Panels to the right show detail of the region outlined. **C** Wide-field time-lapse images of a small region of interest in the peripheral arbor of an RB neuron in 2 dpf *MitoFish*. The movement of a single mitochondrion (arrow in 0") was tracked over 100 sec; six time-points are displayed. The position of the mitochondrion in the previous time-point is outlined in magenta. **D** The position of the moving mitochondrion in **C** is plotted over the 100 sec time-window at a sampling frequency of 1 Hz (light pink). The position of the very same mitochondrion was additionally plotted with an artificially down-sampled temporal resolution of 0.05 Hz to mimic the typical acquisition speed of a confocal microscope (magenta). The magenta kymograph does not reveal the fine non-directed movements of the tracked mitochondrion, which would lead to an underestimation of its dynamics. **E** Confocal images of an RB sensory neuron at 2 and 3 dpf. Labeling of individual RB cells and their mitochondria was achieved by co-injecting a sensory neuron-specific Gal4 driver construct together with two UAS reporter constructs, UAS:mitoCFP and UAS:MA-YFP, at the one-cell stage and screening for isolated double-labeled cells. The image is contrast inverted and individual mitochondria are depicted schematically as magenta dots. Scale bar **A,B** 20 μ m, **C** 2 μ m, **E** 50 μ m. Mitochondrially targeted CFP (mitoCFP), membrane targeted YFP (MA-YFP). [Please click here to view a larger version of this figure.](#)

Figure 3: Comparison of wide-field and confocal microscopy to image organelles *in vivo* in the embryonic zebrafish retina.

Otx2 promoter elements drive the expression of CFP in mitochondria and YFP in cell membranes (A and B) or YFP in centrosomes and Cerulean in cell membranes (C and D) in the retina of 2 dpf zebrafish embryos. To achieve this labeling pattern, Otx2:Gal4 transgenic fish were either crossed to *MitoFish* (A and B) or *CentrinFish* (C and D). A 40x water-dipping-cone apochromat objective (NA 0.80) was used to acquire images of the same region in each retina using wide-field (A, C) and confocal (B, D) microscopy. Insets in A and B show detail of a region of the inner nuclear layer, insets in C and D show a cell in M-phase. Scale bar 20 μ m. Mitochondrially targeted CFP (mitoCFP), membrane targeted YFP (MA-YFP), centrin4-YFP (cetn4-YFP), membrane targeted Cerulean (MA-Cerulean). [Please click here to view a larger version of this figure.](#)

Figure 4: Comparison of wide-field and confocal microscopy to image centrosomes *in vivo*.

A single muscle fiber with fluorescently tagged cell membranes and centrosomes (Otx2:Gal4; *CentrinFish*) was imaged using wide-field (A) and confocal (B) microscopy. In both cases a 40x water-dipping-cone apochromat objective (NA 0.80) was used. Scale bar 10 μ m. Centrin4-YFP (cetn4-YFP), membrane targeted Cerulean (MA-Cerulean) Please click here to view a larger version of this figure.

Discussion

Here, we demonstrate the versatility of the Gal4-UAS expression system to fluorescently tag mitochondria, centrosomes and the cellular membranes of specific cell-types *in vivo* in zebrafish embryos. Many fluorescent fusion proteins that label other organelles or subcellular structures can be found in the published literature and can be obtained from the respective laboratory, commercial sources or non-commercial plasmid depositories (e.g., Addgene). To design a new fluorescent fusion protein, several parameters need to be considered, including which FP to use and whether to fuse the FP at the amino or carboxy terminus of the protein of interest. For a more thorough discussion about generating fluorescent fusion proteins, readers are referred to in-depth articles about the subject⁴⁹⁻⁵¹.

We highlight three strategies for achieving co-expression of fusion proteins using UAS-driven transgenes. Multiple, separate UAS reporter constructs allow for combining different existing reporter constructs without the need for additional cloning. Reporter expression levels can also be titrated independently. However, higher levels of co-expression are achieved when either multiple UAS cassettes or a bidirectional UAS cassette on a single construct are used. Since FPs are used as tags it is relatively easy to verify co-expression. It should be noted that other methods for multi-cistronic expression also exist, including the use of internal ribosomal entry sites⁴⁰ and viral 2A peptides^{52,53}. As an alternative to DNA based constructs, *in vitro* transcribed capped mRNA can be used to express fluorescent fusion proteins to label subcellular structures^{1,4,8}. The caveat of this approach is however that expression is limited to the first few days of development and is not cell or tissue specific. Irrespective of the method chosen to express fusion proteins, it is critical to ensure that expression levels do not lead to non-specific labeling and compromise the physiological state of the cells. In this regard, the amplification of reporter gene expression inherent in the Gal4-UAS system²⁷ can be problematic, manifesting for example as cytosolic labeling even when the FP is targeted to a specific organelle. When available, antibodies should be used to verify that the expression patterns obtained by fusion proteins reflect the endogenous situation. In some instances, injection of low(er) DNA concentrations could mitigate the problem of mis-localization of the fusion protein. Alternatively, vectors with a low number of UAS repeats could help to titrate down transgene expression levels³⁰. Similarly, for the activator Gal4-VP16, modified versions exist, which are reported to drive expression comparatively weakly and are therefore less toxic^{30,33}. If mis-localization of the fusion protein persists despite efforts to titrate down transgene expression levels, it may be necessary to forgo the Gal4-UAS system and drive expression by promoter elements directly.

The stable transgenic lines, *MitoFish*¹⁰ and *CentrinFish*¹², we describe in this manuscript were made using 14xUAS cassettes. We maintain these lines in the background of Gal4 driver lines to detect alterations in expression over generations, since reporter lines with multiple UAS repeats have been reported to be prone to silencing⁵⁴. We have not seen evidence of silencing over the 3 generations we have propagated the *CentrinFish*. We have however seen variegated expression in *MitoFish* and therefore stringently select embryos with 'full', strong levels of expression to propagate for future generations. The current literature suggests that expression vectors with 5xUAS repeats could be an alternative for generating stable transgenic lines - the reporter is driven to high enough levels³⁰ and the comparatively low number of UAS repeats may make it less prone to transgene silencing, although non-repetitive UAS repeats are reportedly even less susceptible⁵⁴.

The palette of FPs currently available to tag organelles or other subcellular structures is very broad⁵⁵. When choosing a particular FP as a tag, consideration should be given to the following parameters: First, the excitation and emission spectra of the FP to determine the specific laser line as well as the excitation and emission filters required for its visualization. Second, the brightness and photo-stability of the FP. Third, the speed with which the FP matures following translation. Fourth, whether the FP has a tendency to aggregate in fusions. Regarding the last parameter, care should be exercised and control experiments should be done to test the suitability of each FP for specific experiments. For example, while the red FPs mCherry and DsRed are widely used, they reportedly form aggregates in certain fusions⁵⁶. We have found TagRFP-T⁵⁷, a more photo-stable variant of TagRFP, to perform well when targeted to mitochondria and to cellular membranes (unpublished observations). When multiple organelles need to be concomitantly visualized, FPs with non-overlapping spectra should be used. We have successfully used combinations of cyan FPs (CFP and Cerulean) and YFP (Figures 2-4). By exploiting the entire spectral range from blue to near infrared, one can

greatly expand the number of subcellular structures that can be concurrently visualized². In addition to the conventional FPs, photo-activatable FPs are particularly useful to probe organelle dynamics, e.g., the use of the FP Kaede to track the fate of individual mitochondria⁵⁸.

Which microscopy technique - wide-field or confocal - is the most appropriate for imaging depends on a number of factors, including the location of the cells to be imaged and the speed with which specific subcellular or cellular events are expected to occur. Wide-field microscopy is the preferred modality in superficial locations and sparsely labeled samples. It offers low photo-toxicity and imaging at high speed at a reasonable price. However, if imaging needs to be performed in non-superficial parts of the zebrafish, in densely-labeled samples or to gain three-dimensional information, confocal or another form of optical sectioning microscopy becomes the method of choice.

Irrespective of which cellular or subcellular structure is being monitored, there is often a trade-off between acquiring the best possible image and keeping the sample alive and in good physiological condition for repeated time-lapse imaging. Photo-toxicity can manifest as changes in the behavior of organelles, aberrant morphology of cells or even cell death. Key to reducing photo-bleaching and photo-toxicity for both wide-field and confocal microscopy is to use the lowest possible levels of light to acquire images. In this regard, objectives with the highest possible NA are key to maximize the amount of fluorescent signal that can be collected. For confocal microscopy, a number of parameters can be adjusted to compensate for the use of lower laser power. Detectors with higher quantum efficiency compared to conventional PMTs, e.g., gallium arsenide phosphide detectors, can be used to ensure that emitted photons are more likely to be detected. Alternatively or additionally, higher dynode voltages can be applied in the PMT to increase the sensitivity of the detectors. The confocal aperture (pinhole) can be opened to allow for the collection of more fluorescent signal, albeit with a loss of axial resolution. To expose the samples to as little light as possible, scanning should be done at high speeds such that the dwell time of the laser per pixel is low. Scanning at lower spatial resolution also has the effect of increasing the speed of scanning. Imaging less frequently has the additional benefit of exposing the sample to less light, however should only be considered if it does not compromise the comprehensive sampling of the investigated processes.

As an alternative, spinning disc confocal microscopes and confocal microscopes that are equipped with a so-called 'resonant' scanner offer the ability for fast scanning. Both modalities have the advantage that they are faster and less photo-toxic than 'classical', point-scanning confocal microscopes. However, spinning disc confocal microscopes are more limited in z-resolution and cannot penetrate as deeply into tissue as point-scanning confocal microscopes. Similarly, the application of resonant-scanner confocal microscopes can be limited as the very short pixel dwell time will lead to poorer image quality which could, in turn, preclude the detection of sub-cellular events (e.g., binary images with reduced signal-to-noise).

Lastly, while wide-field and confocal imaging are highlighted here, other forms of light microscopy such as two-photon⁵⁹ and light-sheet microscopy⁶⁰ might be more appropriate for specific questions. Two-photon microscopy is based on the excitation of a fluorophore by the simultaneous absorption of two photons in the infrared range of the light spectrum. In common with confocal microscopy, it uses point scanning to acquire images from the sample and has optical sectioning capabilities by virtue of the non-linearity of two-photon excitation. The use of long wavelength light for fluorophore excitation permits imaging at depths several hundred microns from the surface. Furthermore the restriction of excitation to a small imaging volume prevents photo-bleaching and photo-toxicity outside of the focal plane. However, not all FPs have high multiphoton absorption cross-sections, a problem that is particularly prevalent in red FPs. Furthermore, finding a single infrared wavelength to simultaneously excite multiple distinct FPs is difficult, making multi-channel imaging cumbersome. Light-sheet microscopy uses a thin 'sheet' (typically thickness ranging from 2 to 10 μm) of laser light to excite the sample and detects fluorescence signals from this illuminated focal plane at a perpendicular angle using a sensitive camera. Optical sectioning is achieved by illuminating a single plane at a time. This restriction of excitation light reduces the occurrence of photo-toxicity. Since fluorescence signals from the entire illuminated plane are collected simultaneously, image acquisition is significantly faster than point scanning confocal and multiphoton microscopes. All these characteristics make light-sheet microscopy particularly suited to imaging dynamic cellular events with high spatiotemporal resolution in large volumes of live samples. Indeed, using light-sheet microscopy cell fate in the entire zebrafish embryo was comprehensively tracked during the first 24 hr of development⁶¹. With continued improvements to better imaging penetration^{62,63} and spatial resolution⁶⁴, it is conceivable that light-sheet microscopy will be employed to probe dynamics not only at the cellular but also at the subcellular level in whole zebrafish embryos.

Disclosures

The authors have nothing to disclose.

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