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Genetic tools for multicolor imaging in zebrafish larvae

Thomas Weber *, Reinhard Köster

TU Braunschweig, Zoological Institute, Cell Biology and Cellular Physiology, Spielmannstr. 7, 38106 Braunschweig, Germany

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ABSTRACT

Zebrafish gain increasing popularity as animal model for the study of various aspects of modern cell biology as well as model organism for human diseases. This is owed to the fact that zebrafish represent a cost effective and versatile *in vivo* alternative to *in vitro* cell culture systems and to invertebrate- and classic rodent models as they combine many strengths of each of these systems. Zebrafish with their small size and rapid embryonic development can be maintained at relatively low costs with females giving rise to more than hundred eggs per week, thus allowing for the efficient analysis of cellular and subcellular processes. Moreover, such analysis can be performed using sophisticated imaging techniques, and transgenic zebrafish lines that express any gene of interest can be generated relatively easily. Among other advantages, the powerful genetic tractability of this vertebrate model organism combined with the *in vivo* multicolor imaging options make zebrafish unique for addressing questions of *in vivo* cell biology in vertebrates. In this article we outline these options by reviewing recent advances in zebrafish genetics with focus on the molecular tools and methods that are currently established for the use of zebrafish for multicolor imaging.

1. Introduction

Zebrafish represent an excellent genetically tractable vertebrate model uniquely allowing for the combination of *in vivo* neuroimaging, behavior testing, and compound screening. Zebrafish combine several experimental advantages, owing to their small size, fast development, robustness, high fecundity, easy transgenesis, and the transparency of early larvae. Applications using *in vivo* imaging of zebrafish larvae are numerous and comprise nearly all fields of modern cell biology. Part of this emphasis on *in vivo* imaging is owed to the parallel development of multicolor imaging tools and protocols in the past years, which have been achieved by the coordinated work of many laboratories. These include improvements in fluorophores and in the technical equipment for their detection, as well as the implementation of tissue specific expression systems and vectors for multicolor imaging.

This article is written for newcomers to the field and for scientists interested in multicolor imaging, alike to review the possibilities that the zebrafish model offers. Therefore different aspects relevant for imaging will be covered, grouped into three major topics: (A) Discernible fluorescence emission and detection (sections 2–3). (B) Tissue- or cell-specific expression of fluorophores (section 4). (C) Vectors and expression systems for combinatorial genetics (section 5) and applications (section 6).

Our focus is on the molecular tools (C) that have been optimized over the past years for the simultaneous, non-invasive observation of multiple colored structures *in vivo*. Some recent publications about how these tools can be used to address biological questions will be reviewed in the last section.

2. Fluorophores

2.1. Vital dyes

Fluorescence-based *in vivo* imaging relies on the staining of cells or cellular structures by a fluorophore that can be excited to emit light, usually in the visible range, which is detected by various kinds of microscopy. Today a number of vital dyes that are based on organic fluorophores are available that can be used efficiently for live imaging experiments. Embryos are simply incubated in these membrane-permeable dyes which are taken up by cells. For example BODIPY dyes can serve as versatile vital counterstains in combinations with other fluorophores [17]. Quantum dots are synthetic inorganic fluorophores that are injected into organs or tissues of interest to visualize dynamic processes such as the blood flow. Their advantage is the virtual lack of destruction by photobleaching. The handling of these and similar molecules as well as applications for their use have been described in detail

Abbreviations: PCR, polymerase chain reaction; DAPI, 4′,6-diamidino-2-phenylindole; FP, fluorescent protein; GFP, green fluorescent protein; RFP, red fluorescent protein; IRES, internal ribosomal entry site; Gal4, transcription factor, binds to UAS; UAS, upstream activating sequence, binding site for Gal4; E1b, minimal promoter, part of UAS.

* Corresponding author. Current address: University Medical Center Göttingen, Department of Pediatrics, Robert-Koch-Str. 40, 37075 Göttingen, Germany.
E-mail address: thomas.weber@med.uni-goettingen.de (T. Weber).

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[81,56]. Using subcellularly targeted dyes such as MitoTracker or LysoTracker it is possible to label and observe specific organelle structures or to monitor the physiological state of cells *in vivo* [30,40]. Acridine orange (AO), is a membrane-permeable, aromatic organic dye. AO can be excited by blue–cyan light (436 nm–505 nm) and fluoresces green (525 nm) in the nuclei of living cells when intercalating in double stranded DNA. AO has dichromatic properties and interactions with single-stranded nucleic acids due to dye-base stacking result in red fluorescence (613 nm). The dye accumulates in acidic vesicles such as lysosomes by ionic trapping, labeling these structures in orange/red when observed under low excitation light conditions in cultured cells (dimers or aggregates). Aggregated AO within lysosomes quickly reacts with bright light under standard epifluorescence imaging conditions, thereby inducing a photodynamic reaction that involves lysosomal bursting, the disruption of chromatin integrity and a shift in fluorescence emission from orange/red to green. This correlates with the appearance of intense green nuclear fluorescence due to intercalation of AO in dsDNA [22]. That is, AO can be measured in two emission-wavelength reflecting interactions with nucleic acids and aggregation in acidic compartments that correlate with the living state of the cell. These properties have been exploited to distinguish between healthy (green) cells and compromised cells in AO-stained cells in culture e.g. [80]. Nuclei of necrotic or apoptotic cells appear orange, indicating the presence of denatured ssDNA or nuclear acidification. When staining living zebrafish embryos with AO, which occurs at concentrations that are 2–5 times lower as in cell culture experiments, only a fraction of cells display green nuclear fluorescence, revealing intercalation of AO in dsDNA and disrupted chromatin integrity of these cells. Increased numbers of AO-positive cells can therefore serve as a first indicator for increased cell death *in vivo*, and staining experiments are usually combined with additional cell-death assays, the assessment of the nucelar morphology or with TUNEL-staining [75,72]. AO-stained zebrafish embryos are excited at 488 nm and measured using a bandpass filter (525 nm), allowing for the simultaneous detection of other fluorophores, e.g. in the red spectrum. Recently, dying neurons have been identified by AO-staining using *in vivo* time lapse imaging in a reporter line expressing the fluorescence protein dsRed in neurons [72]. Other vital dyes have been reported to faithfully label apoptotic cells in zebrafish embryos, such as AnnexinV-Cy5, that emits in the far red spectrum and can therefore be combined with fluorophores emitting in the visible spectrum in multicolor imaging experiments [75].

However, optimal penetration of vital dyes can be compromised in postembryonic larvae. Moreover, these synthetic compounds lack the advantage to be expressed genetically and – with the given exceptions – are more useful as counterstains. For example, secreted AnnexinV fused to fluorescence proteins and expressed in zebrafish under tissue-specific promoters can serve as genetic probes to detect apoptotic cells *in vivo* [101]. An updated list of available fluorescent dyes can be found online at http://pingu.salk.edu/flow/fluo.html, and in the database of Fluorescent Dyes, Properties and Applications (http://www.fluorophores.tugraz.at/).

2.2. Genetically encoded fluorophores

Cell-type-specific expression of fluorophores requires genetic systems. Since the breakthrough discovery of the green fluorescent protein (GFP), a protein that can be excited with blue light to emit green fluorescence, a large number of other fluorescent proteins have been discovered by systematic searches and constantly optimized over the last 50 years [91,86,87,76]. Today, they comprise a palette of proteins spanning the spectrum from ultraviolet to far red laying the basis for multicolor imaging [95]. Besides expanding the color palette, fluorophores have been improved to achieve increased brightness, photostability, faster folding, inducible or spontaneous photoconvertability, photoactivatability and clear cut excitation/emission properties by increases in the Stokes shift. The Stokes shift defines the spectral distance between the excitation and emission maximum of a fluorophore. Fluorescent proteins exist that – due to differences in their Stokes shifts – emit at non-overlapping wavelengths after the excitation with a single wavelength when coexpressed in a cell. Such long shifted fluorescent proteins are used for multicolor applications such as single laser dual FRET or flow cytometry [89]. Last not least, monomeric versions of fluorescent proteins have been developed, making them useful as epitope tags (Section 5.1.1.1). In super-resolution microscopy – an emerging application also in the zebrafish field – photoswitchable fluorescent proteins enable the observation of biological phenomena at increasingly higher resolutions and beyond the limits of Abbe's law, revolutionizing light microscopy [8,45].

Fluorescent proteins are genetically encoded, and can therefore be expressed in zebrafish within cells and tissues using transgenic methods. Importantly, they can be genetically fused to proteins with specific subcellular localization, thereby restricting fluorescence to certain organelles or structures (Section 5.1.1). In our hands, among the available fluorescent proteins, a few have established themselves as standard for live imaging, due to their brightness, tolerance in zebrafish cells at high concentrations, suitability for generating fusion proteins and ease of separation when being coexpressed (Table 1 and Fig. 3). For example, tagRFP-T was chosen over tagRFP due to its improved photostability (by ten times, Table 1). It was favored over tdTomatoe, an extremely bright red fluorescence protein (brightness 95, Table 1), because tagRFP-T (brightness 33) is a strict monomer. We used tagRFP-T instead of the photostable monomer mCherry (brightness 16), because tagRFP-T is just brighter (by 3.5 times). However, if bleaching is not an issue, we prefer the original tagRFP (brightness 40) as monomeric red fluorescence protein [88].

3. Technical aspects

The microscopic equipment, microscope configurations and settings for multicolor detection will not be discussed here in detail, but certainly need to be considered when planning a multicolor imaging project: For most experimental questions, microscopic

Table 1
Fluorescent proteins recommended by us for combined multicolor imaging of zebrafish larvae.

Name	Color	Excitation maximum (in nm)	Emission maximum (in nm)	Extinction coefficient	Quantum yield	Brightness*	Bleaching time**(in s)	References
mTFP1	Cyan	462	492	64×10^3	0.85	54	110	[1]
mCitrine	Yellow	516	529	77×10^3	0.76	59	49[a]	[86]
tagRFP_T	Red	555	584	81×10^3	0.41	33	337[a]	[88]
LSS-mkate1	Far red	463	624	31×10^3	0.08	2.5	60	[77]

* The product of the molar extinction coefficient and the quantum yield/1000.
** Bleaching time/photostability was measured in different labs, see references.
[a] (e.g. Shaner lab).

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