



Genetic tools for multicolor imaging in zebrafish larvae



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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.

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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.

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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 nuclear 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 – photo-switchable 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).

Table 2

Cell and tissue specific fluorescent reporter lines. A selection of published transgenic lines that have been obtained by different methods that express fluorophores in defined cell types and tissues (see text in Section 4.). The gene from which regulatory elements were used to drive such expression is shown (in the case of BAC-transgenics, the nearest gene where the trap vector has inserted randomly is shown).

Gene	Cell type	Method	Fluorophore	References
<i>huc</i>	Neurons, (pan-neuronal)	2.8 kb upstream, (promoter element, 4.1)	GFP	[74]
<i>pu1</i>	Subsets of hematopoietic cells	9 kb upstream (promoter element, 4.1)	GFP	[43]
<i>olig2</i>	Oligodendrocytes, eurydendroid cells	(BAC-transgenic, 4.2)	GFP, RFP	[92]
<i>sp7</i>	Otic placode and vesicle, skeletal structures	(BAC-transgenic, 4.2)	GFP	[21]
Multiple insertions	Midbrain	(gene trap, 4.3)	GFP	SAGm17A [51]
1.5 kb down-stream of <i>egr2b</i>	Rhombomeres 3/5	(gene trap, 4.3)	mCherry	Mü4497_18 [24]

solutions are available, including free or commercial software to exploit imaging data, e.g. ImageJ (<http://rsbweb.nih.gov/ij/download.html>). For many applications commonly used standard laser scanning confocal microscopes with multiple channels are sufficient [56]. Some experiments like the study of subcellular organelle dynamics at high speed require special microscopes or signal detection at significant depth and speed, e.g. [36]. Spinning disk confocal microscopy, resonant-scanning confocal microscopy, two-photon microscopy or photoacoustic imaging have been used to study living zebrafish, reviewed for example in [96,79,25].

Light sheet fluorescence microscopy (LSFM), also known as single plane illumination microscopy (SPIM), is an up-and-coming imaging technology for light-efficient *in vivo* imaging of zebrafish embryos [84]. While confocal fluorescence microscopes (CFM) illuminate the whole tissue of the embryo and generate confocality by means of a pinhole that blocks light out of focus, LSFM-systems illuminate only the focal region of interest, using a thin light sheet from the side, thereby causing significantly less photobleaching and phototoxicity [47]. The whole emitting fluorescence from within the confocal plane is immediately imaged on a CCD camera, allowing for extremely fast data acquisition. Optical sectioning can be achieved by moving the sample through the light sheet, while the sample could be freely rotated, depending on the setup. Sample embedding, data-acquisition by multichannel optical sectioning of tissues up to several 100 micrometers into depth, and the release of an embryo back into the medium can be accomplished in less than 10 min, as we have experienced with recent commercially available LSFM-systems. The resolution of LSFM-images is comparable to that of point laser confocal microscopes and depends only on the system optics used. Notably, in a single multichannel experiment, huge amounts of data – in the range of gigabytes – can be produced very quickly posing a challenge on computing power, which is also true for subsequent data processing and 3D rendering. In summary, LSFM-based systems are especially well suited for gentle live-imaging experiments with zebrafish embryos, including time lapse-imaging, and are cost-effective alternatives to two-photon microscopes, however at the cost of tissue penetration. The use of LSFM is preferable when fast acquisition speed and minimum photobleaching are crucial, while LSCM provides a better spatial resolution and more possibilities for simultaneous multispectral imaging.

To plan and perform multicolor imaging-experiments, support software programs are freely available online. Such spectra viewers help to determine the right combination of fluorescent proteins and detection filters, thereby avoiding overlapping excitation or emission of fluorophores and the false interpretation of experimental data.

Protocols for the preparation of zebrafish larvae for live imaging have been published [60,23,56]; see also the zebrafish Model Organism Database (ZFIN, <http://zfin.org/>).

Together, the technical improvements and developments acknowledged so far provide a solid basis for efficient multicolor fluorescent imaging today [64].

4. Cell and tissue-specific *in vivo* labeling

Using genetic expression systems, fluorescent proteins can be specifically targeted to defined tissues or cells, given that a suitable promoter/enhancer combination for a cell type of interest is available. Precise spatial control over fluorescent protein expression can be achieved by choosing promoters of genes that are exclusively expressed in cells of interest. Today, a considerable number of reporter lines are available that express various fluorophores under the control of cell and tissue-specific promoters or enhancers. These lines can be viewed as a swimming toolbox for multicolor live imaging. To create such cell- and tissue-specific reporter lines, several molecular techniques are commonly used, including BAC-transgenesis and enhancer trap approaches (Section 4.1–4.3). As exemplified in Table 2, either strategy can give rise to specific reporter lines that show expression in whole tissues (SAGm17A, *sp7*), in certain types or subtypes of cells (*huc*, *olig2*, *pu1*), or transiently in specific developmental structures (Mü4497_18).

Owing to the well established molecular techniques and the need for tissue and cell-specific enhancers, further expansion of this “swimming toolbox” can be expected in the future, hopefully comprising reporter lines for most tissues and cell types of the zebrafish. Ideally, the characteristics of these line would be accessible from a single database.

4.1. Cloning of enhancer elements

A straightforward approach to isolate regulatory elements that determine gene-specific expression is to subclone approximately 10 kb of the genomic DNA upstream from the ATG-codon of a candidate gene with the desired expression pattern into an expression vector containing a fluorescent protein nearby, flanked by Tol2-sites [3]. This can be done by BAC-recombineering or by using long-distance PCR [18,62]. The next steps are to inject the vector containing this reporter construct into the one-cell stage of zebrafish eggs and to test whether it suffices to induce the expected fluorescence expression *in vivo* by screening injected individuals (F0), e.g. [43]. The coinjection of Tol2-RNA will significantly increase the rate of genomic integration of such constructs when analyzed in the next generation (F1). If no carriers can be identified in F0 that show the desired fluorescence expression, it is possible that the construct did not contain all the regulatory elements required to drive the gene-specific expression. In this case other strategies can be employed (Section 4.2). Due to their moderate sizes, such isolated regulatory units can be subcloned into other vectors, thereby representing versatile genetic tools to drive tissue specific expression of fluorescent proteins (Fig. 1). For further reading we recommend a recent review article [3]. Software that predicts core promoter elements and putative transcription factor binding sites within the genomic sequence are available online and should be used for planning such experiments (e.g. Genomatix suite). However the “promoter bashing” approach proposed here is mostly empirical and the elements required for gene-specific

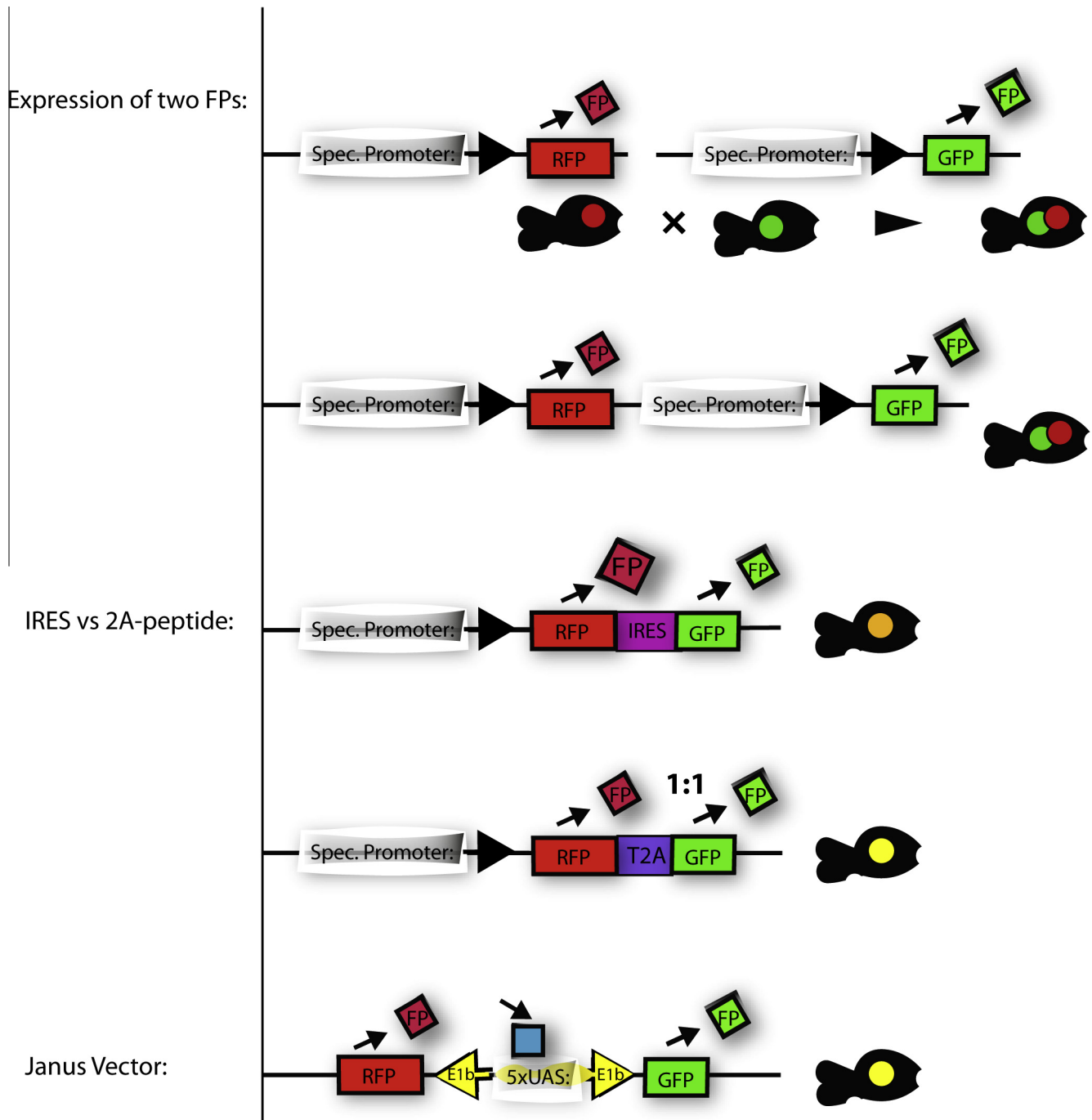


Fig. 1. Expression of two FPs in zebrafish using different vectors. From top to bottom: (1) crossing two transgenic lines expressing either RFP or GFP from individual expression vectors under the control of tissue specific promoters. (2) Cloning two promoter/FP constructs into a single vector. (3) Placing an IRES sequence between the two fluorescence proteins. Note that the upstream gene (RFP) will be expressed at higher levels than GFP. (4) Linking the two fluorescence proteins by a short 2A-peptide. (5) Expressing RFP and GFP on opposite strands from a Janus vector. Note that the transcription factor Gal4 (blue) is required to drive expression from the bidirectional promoter (Fig. 2). (1–2) The level of overlapping expression is determined by the individual promoters. (3–5) RFP and GFP will be expressed in the same cells. (4–5) Equimolar expression of RFP and GFP.

expression are hard to predict *in silico*. Moreover, positional effects at the site of insertion can influence the expression from the transgenic promoter/reporter construct leading to weak, unspecific or to mosaic expression. Flanking the construct by insulator sequences might be helpful to shield such effects [9].

4.2. BAC-transgenesis

If the direct isolation of gene-specific enhancer elements is not possible, BAC-transgenesis can help to obtain the desired

transgenic zebrafish reporter line. Bacterial artificial chromosomes (BACs) contain pieces of genomic DNA in special vectors that are usually larger than 100 kb of size. Their sequences are accessible from genome browsers and the vectors can be obtained too (e.g. ZFIN, <http://zfin.org/>). For many genes of interest a BAC can be identified which contains the complete introns and exons of the respective gene, flanked by long stretches of genomic DNA, likely comprising the complete gene with all the gene-specific regulatory elements. This BAC can be genetically modified by homologous recombination so that a fluorescent protein is replacing the gene

encoded by the BAC or will be coexpressed, e.g. by using an IRES element (see below). The modified BAC-vector is used for injection as described in the previous section. In transgenic fish, expression of the fluorescent protein will be subject to the regulatory elements contained in the BAC, and such lines usually express fluorescence in a gene-dependent manner and without positional effects [92,108,21].

4.3. Enhancer traps and gene traps

These approaches allow for the generation of large numbers of cell- and tissue-specific reporter lines. Enhancer traps are based on the random integration of a reporter cassette (Fig. 3(1)) into regulatory regions of endogenous genes, which is usually mediated by viruses, e.g. [58,28] or by transposons e.g. [6,51,73,20,4,24] to increase the efficiency. The genomic locus where a trap vector has integrated can be determined afterwards by PCR, and the fluorescence expression pattern of the respective trap line can be compared with that of the endogenous gene closest to the insertion site, to evaluate whether the trap line truly mimics its expression pattern. The usefulness of enhancer trap lines can be significantly increased when, together with a fluorescence reporter, a transcriptional activator for combinatorial genetics is introduced into the trapping construct (Fig. 3(1)).

Gene trap vectors also integrate randomly into the genome and they contain a splice acceptor site followed by a reporter. Vector integration becomes detectable by fluorescence when it occurs closely to a splice donor site of an actively transcribed, “trapped” gene, thereby proper splicing of the native transcript is disrupted. Recently, a special gene trap has been performed using a FlipTrap vector that initially does not interrupt the function of the trapped gene but subsequently the insertion can be rendered mutagenic (Section 6.2, [98]). The tissue and cell-specific reporter lines generated in large screens are usually accessible from online-databases that are maintained by the laboratories involved in the respective screens, e.g. FlipTrap, zTrap, ZETRAP2 [52,55].

5. Multicolor options

Multicolor *in vivo* imaging requires the simultaneous expression of several fluorescent proteins that emit light at discernable wavelengths, with each fluorophore illuminating different organelles or cellular structures. As outlined, tissue specific promoters regulate the spatial and temporal cellular expression patterns of fluorescent proteins. The latter determine the emission wavelengths to be detected and separated by fluorescence microscopy, and – when fused to certain tags – also their subcellular expression patterns. These components are implemented in vectors that combine various genetic and combinatorial systems. Certainly, these molecular tools do not only allow for the staining of tissues but also for the combined expression of fluorescent proteins and various other proteins, to analyze the consequences of protein overexpression directly *in vivo*. This aspect makes multicolor imaging especially attractive.

5.1. Co-expression of various fluorophores and proteins

5.1.1. Fluorescence tagged fusion proteins

One way to visualize the expression of a protein of interest by fluorescence imaging is the direct genetic fusion to a fluorescent protein, either to the N- or C-terminus of the coding cDNA in an expression vector. Expression from this vector results in a single recombinant protein that can be observed by *in vivo* imaging techniques. The advantage of such a fluorescent fusion protein is that one has precise spatial control over its expression. Important

examples are fusions with targeting proteins, as emphasized in Section 2.2. For example fusion to histone 2B (H2B) is commonly used as a “tag” for nuclear targeting of fluorescent proteins in zebrafish. An H2B-tagged fluorescent protein can give instructive information about the cell cycle state of cells *in vivo*, – allowing for the discrimination between interphase, prophase and mitotic nuclei – similar to post-fixation staining with DAPI [49,19]. Basically, all cellular compartments and structures for which a specific targeting protein exists can be highlighted by using tags, including the cytoskeleton and organelles, which greatly enhances the multicolor options by adding subcellular spatial dimension to imaging experiments e.g. [87,25].

5.1.1.1. Applications as epitope tags. A special type of tagged fusion proteins are epitope tagged proteins which are invaluable when antibodies against a protein of interest are not available. Such tags are short (e.g. Myc-, Flag-, Strep- or Poly-Histidin tag/6xHis tag), can be fused to proteins of interest, and later recognized by high quality commercially available antibodies or purified using nickel resins for biochemical experiments. Fluorescence tagged fusion proteins can be used in the same way, although fluorescent tags are larger (GFP tag 238 amino acids), since for the most common fluorescent proteins good commercial antibodies are available. Moreover, the option to dissociate transgenic embryos and to isolate and enrich fusion protein expressing cells by fluorescence assisted cell sorting is convenient (FACS), e.g. [43,14,42].

5.1.1.2. Förster resonance energy transfer (FRET). FRET analysis is a powerful imaging application that requires fluorescent fusion proteins to reveal direct, physical interactions between two proteins. Therefore, one protein is fused to a fluorescent protein, which serves as FRET donor (e.g. CFP), the other protein is fused to a fluorescent protein that serves as FRET acceptor (e.g. YFP). Notably, while the excitation wavelengths of the two fluorescent proteins of a FRET-pair do not overlap, the excitation wavelength of the acceptor does overlap with the emission wavelength of the donor. When the donor is excited FRET can occur, thereby inducing fluorescence emission from the acceptor, which is only efficient over very short distances (less than 10 nm). Therefore, a high FRET-efficiency indicates that the proteins that had been fused to the FRET-partners are localized in very close proximity and are therefore likely to interact with each other. Such FRET-analyses can also be performed in living zebrafish, thereby supplementing biochemical methods by *in vivo* analyses. FRET-based biosensors, including Ca^{2+} -indicators and pH-sensors, are powerful novel tools to study functional processes in living zebrafish that involve distance-dependent molecular interactions within the FRET-radius [41,63,50].

5.1.2. Expression of two genes from two different promoters

The simplest way to express two individual fluorescent proteins is to cross two different transgenic zebrafish reporter lines and analyze individuals in the offspring that express both types of fluorescence (Fig. 1(1)). However, when suitable transgenic strains are not available it is often necessary to express two individual proteins simultaneously from a single plasmid. In this case the easiest way is to clone two transgenes each driven by its own promoter behind each other (Fig. 1(2)). Possible complications with this design are interferences between the two promoters leading to altered gene expression or strong expression of only one of the two transgenes, a problem that can sometimes be circumvented by choosing a head to tail orientation of the two genes (Fig. 3(3)), tagRFP vs. mTFP1) or by using insulators [90].

5.1.3. Janus-vectors

To create further options for the expression of two proteins from the same plasmid, “Janus vectors” have been developed

(Fig. 1(5)). The name of the vector alludes to the roman divinity Janus, who was depicted with two faces looking in opposite directions. Bidirectional promoters occur naturally in the mammalian genome, comprising about 10% of the predicted promoters in humans [99]. They regulate the expression of two genes encoded on opposite strands (head to head), or induce transcription in both directions, regardless whether the transcripts encode functional proteins [104,102]. These properties have been exploited for the design of vectors containing bidirectional promoters to coexpress two genes in transgenic zebrafish, possibly one functional gene variant from one strand and a fluorescent reporter gene from the opposite strand [5,72]. Notably, this design will result in the expression of two single proteins with similar expression levels but independent localization. Fig. 1 shows a bidirectional promoter that is off in the absence of Gal4, but can be turned on in any tissue following Gal4 expression. To achieve expression, Gal4 can be expressed from a tissue specific promoter in *trans* (Section 5.2.1).

5.1.4. IRES-vectors

An alternative to Janus vectors represent bicistronic expression vectors containing IRES sequences (internal ribosomal entry site). More than 500 IRES sequences from viral but also from cellular RNAs have been described, with an average length of 474 bp [67]. An IRES sequence located between the coding regions for two proteins oriented in the same direction recruits a second ribosome to the IRES on the mRNA, which results in the translation of two independent proteins (Fig. 1(3)). This is mediated by the flexibility for the ribosome not only to bind the capped 5'-prime end of the mRNA but also to secondary structures of the IRES in front of the second cistron. Therefore and due to its success in mouse genetics, the IRES system has been established also for the use in zebrafish [103]. Any isolated tissue-specific promoter can be cloned in front of transgenes linked by an IRES sequence to drive their expression (Section 4.1). Notably, the activity of IRES sequences is strictly context dependent and the downstream gene will usually be expressed at lower levels [106], see also [33]. The recent identification of an IRES from a zebrafish-gene [100], and the likely discovery of more such sequences might lead to the development of expression vectors for live multicolor imaging that mediate also high levels of gene expression from the 3'-prime cistron. Currently the use of the IRES from the encephalomyocarditis virus (ECMV) is a good choice to express two fluorescent proteins in zebrafish [59]. For *in vivo* imaging it can be useful to place the fluorescent protein 5'-prime to the IRES in the expression construct to achieve maximal fluorescence intensity, and another protein – preferably one where a lower expression level is desired to better reflect physiological conditions – downstream of the IRES.

5.1.5. 2A-peptide vectors

“Self-cleaving peptides” of the 2A-family are derived from viruses and consist of 18–22 amino acids [53]. Thus 2A-peptide sequences are much shorter than IRES-sequences. When placed between the coding regions of two proteins oriented in the same direction within a single open reading frame, 2A-linkers mediate the generation of two individual proteins (Fig. 1(4)). In contrast to IRES, the molecular mechanism involves the ribosome starting translation at the 5'-prime Cap structure of the mRNA, followed by a ribosome skipping event when the ribosome reads the 2A sequence [27]. This results in the break of the peptide backbone therefore producing equimolar amounts of these two proteins that afterwards can locate to different sites within the cell. Because of equimolar expression, 2A-driven fluorescent protein expression can be used to quantify transgene expression derived from the second cistron, e.g. [10]. Recently the relative cleavage efficiencies of four commonly used 2A peptides have been compared, showing cleavage efficiencies between fifty to close to hundred percent in

various cell culture systems, and an efficiency of almost hundred percent in zebrafish larvae for the P2A- and T2A-peptides [53]. In our hands, the T2A sequence has been found to work very reliably and both Western blot analysis and fluorescence microscopy showing virtually 100% cleavage efficiency *in vivo*.

It has to be taken into account, that the amino acids of the 2A-peptides will be part of the C- and N-termini of the ensuing proteins, where the downstream protein will begin with a proline residue and the upstream protein will contain a stretch of 17–21 amino acids at its C-terminus. Therefore, before generating such 2A vectors, one has to think carefully about the placement of the two cistrons to avoid interferences with protein localization or function. For example, a secreted protein has to be used as the first cistron since the proline-residue at the N-terminus of the second cistron will affect the functionality of the signal peptide. For the same reasons, N-terminally tagged proteins should be placed as first cistron. Alternatively, endomembrane and cytoplasmic membrane targeting of a fluorescent protein is also possible at the C-terminus by the addition of a CAAX-motif [13].

Due to their small sizes, 2A-peptide linkers are the method of choice when cloning capacity is limited, and also when it is important that two genes are expressed at equimolar levels in a tissue specific manner from a single plasmid.

5.1.6. Triple and quadruple Medusa vectors

Medusa vectors are multicistronic vectors that contain a Janus element plus additional UAS/reporter elements, thereby allowing for the expression of three or more fluorescent colors in zebrafish (Fig. 3(2)). In combination with a binary transcriptional system such as the Gal4-system, expression of the fluorescent proteins can be controlled in a tissue-specific manner [26]. Each of these fluorescent proteins can be tagged in a way that fluorescence will be detectable in different subcellular compartments.

The combination of two Janus elements, gives rise to a quadruple Medusa vector, designed to express four proteins simultaneously (Fig. 3(3)). In our example, three different – non overlapping – fluorescent proteins in the visible range highlight four different subcellular structures. The cell membrane and the nucleus – two compartments that do not overlap spatially – share the same color. Because the quadruple Medusa vector was generated by Gateway cloning, a combinatorial cloning system, this multicolor imaging tool can be quickly adapted to ones specific needs by exchanging fluorescent proteins or adding proteins of interest *in vitro* [59].

5.1.7. Heat shock vectors and inducible gene expression

Besides spatial control of gene expression, temporal control by means of inducible expression is often required. Heat shock vectors are still the method of choice to generate inducible gene expression in larval and adult zebrafish [37,93]. Heat shock proteins constitute components of the cellular stress response in fish, with the Hsp70 family being involved in repair and degradation of altered or denatured proteins. Stress factors – including heat – induce the promoters of heat shock genes, leading to the production of new proteins within minutes to hours. After the isolation of the regulatory units of genes encoding heat shock proteins, e.g. hsp70, heat shock promoters and heat shock elements have been implemented in vectors for the inducible expression of proteins in transgenic zebrafish [37,5]. To achieve efficient induction of a heat shock promoter, zebrafish are transferred from their natural environment of 28 °C to temperatures between 38 °C and 41 °C, thereby generating an environmental temperature difference of about 10 K (Fig. 2). This induces the expression of genes cloned downstream to the heat shock promoter. Localized activation of the promoter, e.g. in individual tissues, can be achieved using focused laser excitation, soldering irons or heat emitting optical fibers as “heat sources”,

making this system inducible in a defined area [37,39,78]. However even in stably transfected fish, the complete induction of all the cells expressing the heat shock vector might not be achieved [37]. In addition, induced gene expression is transient and not long lasting, although repeated activation of the promoter is possible. Recently, optical inducible systems have been developed that allow for improved spatiotemporal control of gene induction [94].

5.2. Combinatorial expression systems

In the previous sections we laid out how tissue specific promoters and genes encoding fluorescent and other proteins can be arranged in special vectors for efficient multicolor imaging. In this section we will introduce combinatorial genetic expression systems, which further enhance possibilities for multicolor imaging.

Such systems include binary transcriptional systems (Sections 5.2.1–5.2.5) and recombination systems (Sections 5.2.4–5.2.5).

A binary transcriptional system is based on a transactivator and an effector. The transactivator drives effector gene expression, and can be under the control of a tissue specific promoter, while effector genes are regulated by the transactivator via unique binding sites. These separate entities can be expressed from the same or two independent constructs as well as by crossing two independent transgenic zebrafish lines, so called activator- and effector lines, which allows a combinatorial use and results in stronger gene expression due to transcriptional amplification when strong activators are used.

Recombination systems can be used *in vitro* and *in vivo* for the manipulation of genes after introducing specific recombination sites.

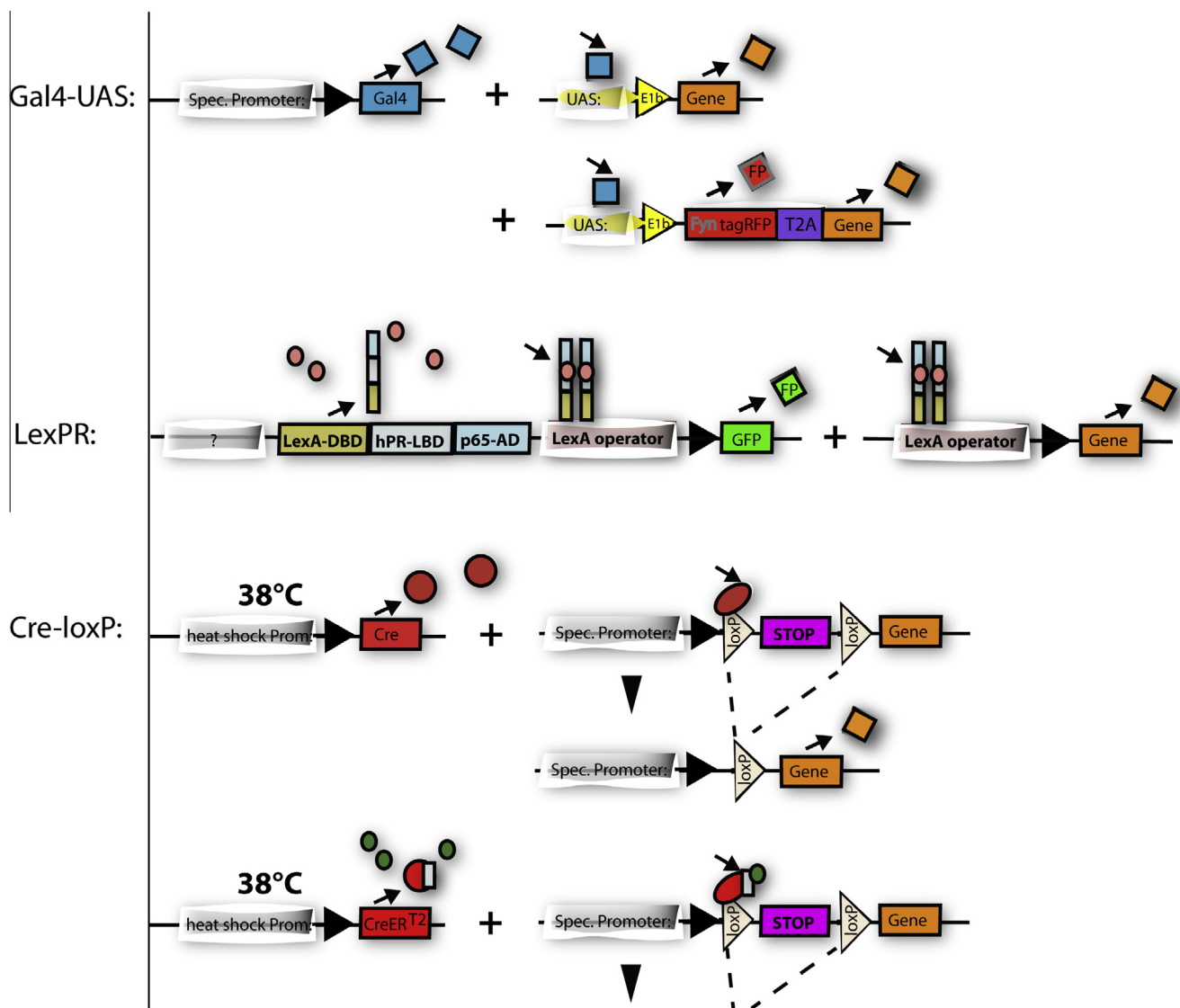


Fig. 2. Combinatorial genetics to express fluorescent proteins and genes of interest. The Gal4/UAS-system and combinatoric. Transgenic UAS-effector line containing a silent expression cassette for a gene of interest (gene). Conditional expression of the gene requires the transactivator Gal4 (blue), usually expressed from a tissue specific activator line that has to be crossed in. Various effector lines can be crossed with the Gal-activator line that for example express fluorescence proteins together with a gene of interest using a 2A-peptide linker (Fig. 1(4)). Note that tagRFP contains an H2B-tag resulting in nuclear expression, while the gene of interest is not targeted. The LexPR-system and inducible expression. A hybrid protein containing a DNA-binding domain, a ligand-binding domain and a transactivation domain is expressed from a gene trap promoter. In the presence of the ligand mifepristone (dots) this fusion protein can induce gene specific expression of a reporter (GFP) by binding to the LexA operator located on the same construct. In crosses with LexA-effector lines other genes can be expressed in an inducible manner. The Cre-loxP-system and heat shock-inducible expression. Upper: Cre recombinase is expressed from a heat shock promoter at a permissive temperature. Cre recombinase recombines two loxP-sites located in *trans*. Such activity releases a STOP cassette thereby enabling the specific expression of a gene of interest. Lower: fusion of Cre recombinase to the estrogen receptor ligand binding domain (CreER^{T2}) allows for heat shock-inducible gene expression only in the presence of the ligand tamoxifen, thereby eliminating potential leakiness of the heat shock promoter.

The development of combinatorial systems and their implementation for the use in zebrafish allow for a broad variety of live imaging possibilities in zebrafish, especially when used in combination with other genetic systems.

5.2.1. The Gal4/UAS system and combinatoric

This system has been reviewed in great detail by other groups and us, and significant improvements have been made to achieve sufficient levels of expression for the imaging of multiple fluorophores, especially in zebrafish [24,70]. It is based on the yeast transcription factor Gal4, which recognizes specific DNA-binding sites that are not present in the genome of vertebrates and insects. Gal4 has been fused to repeats of the minimal transactivation domain of the transcriptional activator VP16 to display efficient activity in zebrafish [7]. The activator lines express Gal4 under the control of a tissue specific promoter (Fig. 2). The effector lines instead contain an expression cassette comprising a Gal4-specific binding site named upstream activating sequence (UAS) and a minimal promoter, followed by a downstream gene of interest. This UAS-sequence can be considered as silent ubiquitous promoter, which is only activated upon binding of the transcription factor Gal4, thereby inducing the expression of a downstream gene. Expression is achieved by crossing carriers of activator and effector strains, giving rise to offspring carrying both alleles. Gal4 can act from a gene expressed in *trans* or from a single transgene cassette construct in *cis* (Fig. 2 vs Fig. 3(1)). Today this combinatorial system is probably the most popular to express genes in zebrafish, providing the convenience that many tissue specific activator lines are already available allowing for numerous combinatorial possibilities. Some of these lines are listed on the ZFIN database <http://zfin.org/>. In binary systems a single activator line can be crossed with many effector lines and vice versa and such combinatoric enables the use of transgenic lines for multiple experiments. Moreover, due to the separation between the enhancer and the

effector gene, pathogenic transgenes can be expressed for genetic modeling of human diseases while the effector line can be maintained without complications. The freely diffusible activator Gal4 can activate more than one transgene, as indicated in the quadruple Medusa vector (Fig. 3(3)). It is also possible within a certain range to modulate the expression level of the UAS-driven effector gene by varying the number of UAS-sites preceding it [24]. However, too many repeats of identical UAS-sites can provoke DNA-methylation which results in silencing of transgenes and their mosaic expression [34]. While in our hands 5xUAS tandems work well when effector strains have been carefully screened for a good expressing carrier, the use of non repetitive UAS sites can further prevent such methylation [2]. To gain some temporal control over Gal4 induced protein expression a natural inhibitor of Gal4 (Gal80) was used to delay the activity of the original yeast Gal4 activator, however the stronger activating Gal4-VP16 cannot be repressed [31]. Another way is by expressing Gal4 under the control of a heat shock promoter, which results in the loss of tissue specificity. Recently, an efficient tissue specific inducible system has been developed which is a modification of the tamoxifen-inducible CreERT²-system (see Section 5.2.4). Instead of Cre, Gal4 was fused to ER^{T2} (ERT²Gal4), now allowing for temporally controlled Gal4 expression [32].

Importantly, many enhancer trap lines described in Section 4.3 are designed to express Gal4 together with a fluorescent protein, thus reporting tissue-specific expression of Gal4. These lines are both, useful reporter lines and Gal4-activator lines (Fig. 3(1)).

5.2.2. The LexA system and inducible gene expression

Like the Gal4-system, the LexA-system is a binary transcriptional system. It has been extensively used in yeast two hybrid assays. The activator consists of a chimeric protein, where the DNA-binding domain from the bacterial repressor LexA is coupled to a transactivation domain. The activation domain can be derived

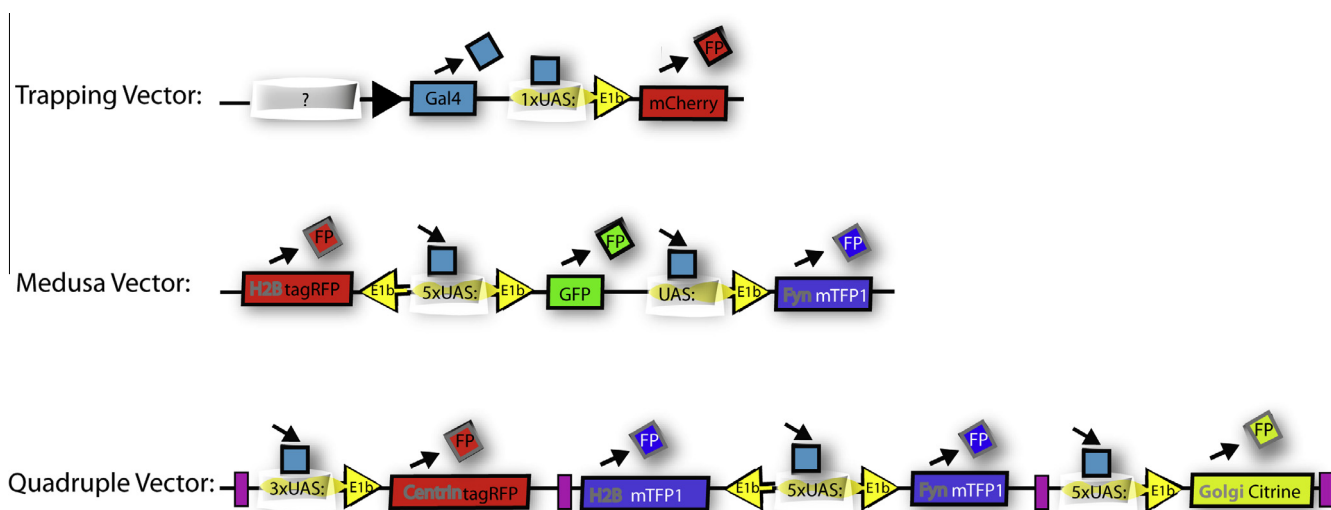


Fig. 3. Multicolor options. From top to bottom: (1) an enhancer trapping vector containing a minimal promoter and a Gal4/UAS/reporter cassette has randomly inserted into the genome, close to an active enhancer or promoter (?). Such promoter activity drives expression of the transcription factor Gal4, which binds to the 1xUAS site and induces the expression of the red fluorescent protein mCherry – thereby “reporting” the trapping event. This includes lines expressing the Medusa, Quadruple Medusa or Janus vectors shown in Figs. 3(2–3) and 1(5), where Gal4 induces multicolor fluorescence expression. (2) A Medusa vector containing a Janus cassette (Fig. 1(5)) and an additional UAS/reporter element. Gal4-activity induces the simultaneous expression of three non-overlapping FPs that can be observed in the same cell. Note that in this example tagRFP contains a H2B-tag resulting in nuclear expression, green fluorescence is cytoplasmic and expression of cyan fluorescence (mTFP1) is targeted to the cell membrane due to the Fyn-tag. (3) A Quadruple Medusa vector can be used to drive the expression of four fluorescence proteins: Centrosome-targeted tagRFP, Golgi-targeted Citrine and two versions of mTFP1, targeting cyan fluorescence either to the nucleus (H2B-tag) or to the membrane (Fyn-tag). Note that the fluorescence intensity can be modulated by varying the number of UAS-sites. Pink elements indicate att-sites, allowing for the exchange of UAS/reporter or UAS/gene elements using the Gateway system. Since enhancer trap lines expressing Gal4 in this way (1) can be crossed with any other UAS-effector line and since all the UAS-effector lines expressing e.g. the vector constructs shown here (Fig. 1(5), Fig. 2 upper, Fig. 3(2–3)) can be crossed with any cell and tissue-specific Gal4-activator line, the respective fluorescence expression patterns can be recapitulated in any cell context defined by the activator-line, further underlining the combinatoric character of the Gal4-system.

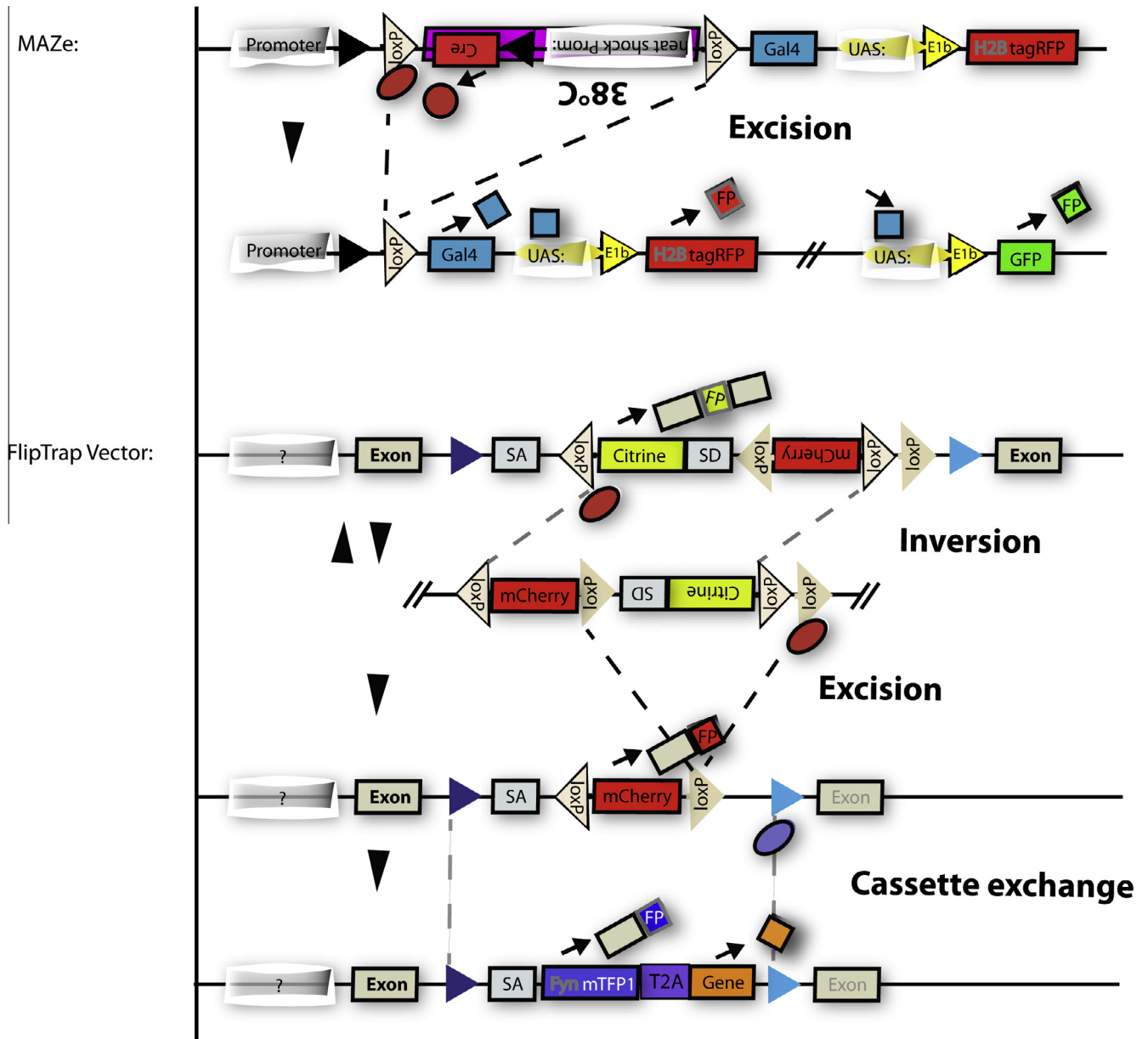


Fig. 4. Multicolor applications. From top to bottom: (1) mosaic analysis in zebrafish (MAZe). Cre recombinase is expressed under the control of a heat shock promoter, which is embedded in a cassette flanked by two loxP sites. Heat shock (38 °C) induces excision of the complete cassette, thereby interrupting further expression of Cre recombinase. Importantly, excision of this STOP cassette now allows for Gal4 expression from a nearby promoter, which induces the expression of a downstream fluorescence reporter cassette, or – as indicated by the interrupted DNA strand – other fluorescent proteins located in *trans*. Such expression will not be ubiquitous but result in a random mosaic, because the heat shock will not be efficient enough to remove the STOP cassette in every cell. Repeated heat shocks can modulate the pattern of mosaic expression, see text (Section 6.1). (2) A FlipTrap vector containing a Citrine reporter flanked by splice donor (SD) and acceptor (SA) sites has randomly inserted between two exons of a gene. Citrine can be expressed as fusion with the trapped gene product which will not be affected by such insertion. Part of the vector is a second reporter cassette in inverted orientation encoding mCherry-polyA which is silent. Inversion: Cre activity (ovals) recombines two pairs of heterotypic loxP sites causing the transient inversion of the reporter cassettes (only recombination from the first pair of loxP sites is indicated within the affected DNA region). Excision: Cre-mediated recombination leads to the excision of the citrine-SD reporter cassette between two loxP sites. A truncated trapped gene will be expressed as fusion with mCherry. Note that such conditional mutation is indicated by a color switch from yellow (Citrine) to red (mCherry). Exchange: Blue triangles are heterotypic Flp-sites (Frt and Frt-F3) used for recombination mediated cassette exchange of the Citrine or mCherry reporter cassettes in the presence of the recombinase Flippase and DNA flanked by compatible Flp-sites, see text (Section 6.2).

from different sources, e.g. p53 domain from the human NFκB protein or the transactivation domain VP16 from the herpes simplex virus. This transactivator binds to an operator-promoter sequence (LexA_{OP}) consisting of a synthetic LexA operator, analogous to the UAS-sites of the Gal4-system [97]. Recently it was reported that a modification of this system (LexPR) works well in zebrafish to allow for chemical-inducible gene expression [29]. In this report the

transactivation domain was based on a fusion protein of two human proteins, the truncated ligand-binding domain of the progesterone receptor, and the activation domain of the p53 protein, rendering it responsive to mifepristone, a steroid derivative (Fig. 2). Adding mifepristone to the rearing medium efficiently induced gene expression in embryos and also adult fish. The disadvantage of the LexA and the LexPR system is that not many

activator lines are available yet and that only few reports exist concerning their efficiency in zebrafish, in contrast to the well established Gal4-system.

5.2.3. The Tet-system and reversible induction of gene expression

The TetON/OFF system is yet another binary transcriptional system that is well established *in vitro*, enabling inducible and reversible gene expression. It has found its way into routine mouse genetics and has recently been reported to also work in zebrafish [54]. A fusion of the Tetracycline (Tet) repressor and a VP16 activation domain generates a powerful transcriptional activator, similar to Gal4. Genes can be activated after binding of this activator to specific tetracycline response elements (TREs) as part of an inducible promoter. The difference between TetON and TetOFF is that this transcription factor either activates transcription in the presence or absence of doxycycline, a stable tetracycline derivative (Dox). Thereby Dox is rendering this system inducible, a drug that can be added to the rearing medium of zebrafish. As it can be washed out again this genetic system is reversible, however the time for wash-out is cell and tissue dependent and the reversal might be delayed. One caveat is that the induction with Dox is not 100% efficient leading to mosaic expression. However, since the targeting of subpopulations of defined cells can be useful in imaging experiments this limitation can also be considered an advantage. For example, TetOff was recently used to induce the expression of optogenetic tools in zebrafish neurons in a mosaic pattern [109]. It has to be mentioned though that due to its recent introduction to zebrafish not many reports exist currently to comment on the efficiency of the Tet-system in zebrafish.

5.2.4. The Cre/loxP system and recombination

This recombination system can be used in zebrafish to conditionally manipulate genetic elements or vector cassettes introduced by transgenesis [57]. It is based on the enzymatic activity of Cre-recombinase derived from the P1 bacteriophage, which specifically recombines 34 bp long, so called loxP sequences, that do not naturally occur in vertebrate genomes [68]. To apply this system to zebrafish, a transgenic strain – the activator line – is generated to express Cre recombinase under the control of a tissue specific promoter. Another transgenic strain – the effector line – is established that contains an expression cassette, which has been engineered to contain loxP-sites flanking a sequence that is to be manipulated (Fig. 2.). After crossing these two lines, Cre recombinase will excise the region within the cassette that is flanked by loxP-sites by site-specific recombination, provided that these sites have the same orientation. If two loxP sites have opposite orientations, Cre recombinase activity will cause an inversion of the DNA-region flanked by the two sites rather than an excision (Section 6.2 and Fig. 4(2)). After the recombination of two loxP-sites and the excision of the DNA between these sites as circular loop, one of the loxP-site will remain in place.

In our example in Fig. 2, Cre is used to excise a “stop” cassette, which is flanked by two loxP-sites, thereby initiating a downstream expression cascade. Recently a heat shock-inducible Cre-line Tg(*hsp-70*-Cre) was used in a zebrafish cancer model to over-express oncogenes in combination with fluorescent proteins [61]. Problems can arise when expressing the highly sensitive Cre recombinase from a heat shock promoter. Basal activity of the promoter at normal temperatures can lead to Cre activity. Therefore a heat shock inducible Cre line Tg(*hsp70*-CreER^{T2}) was generated which contains a fusion between Cre and the estrogen receptor ligand binding domain (CreER^{T2}), rendering Cre activity only inducible in the presence of the xenoestrogen tamoxifen. The inducer tamoxifen can be added to the rearing medium after the heat shock application thereby adding a second control element, which largely eliminates the problem of heat shock promoter leakiness [38].

5.2.5. The Flp/Frt system and recombination

An alternative or a supplement to the Cre/loxP system for site-specific recombination in zebrafish is the yeast-derived Flp/Frt-system [105]. Here the recombination enzyme is Flippase (Flp), which recognizes 34 bp long Frt sequences, analogous to the loxP sites. This system is very similar to the Cre/loxP system, but Flp is less efficient at higher temperatures than Cre, the latter being highly efficient at mammalian body temperature [11]. In mice the Cre/loxP system has been used very successfully, also in combination with the slightly less efficient Flp-system, for example to remove selection markers flanked by Frt sites from gene targeting cassettes – thereby preserving the more efficient loxP-system for experimental use, such as reporter gene activation or conditional gene knockout. Not many reports exist so far regarding the efficiency of the Flp-system in zebrafish, although Flp-recombinase seems to be predestined for the use in zebrafish working optimally at 28 °C, the temperature where zebrafish are maintained. Optimized variants for either system, FLPo in mice and FLPe for zebrafish have been reported [11,107]. In a recent gene trap approach though, both recombination systems – Flp/Frt and Cre/loxP – were successfully combined in zebrafish using a FlipTrap vector [98]. These data propose that Flp will obtain more attention in the future.

In mouse ES-cell clones which contained trapped genes, recombination mediated cassette exchange (RMCE) was demonstrated using the FLEX system, which utilizes a combination of different site specific recombination systems, including the gateway system [59], the Flp/Frt system and the Cre/loxP system [85,82]. RMCE allows for the integration of a DNA sequence into a trap-vector cassette that contains appropriate site-specific recombination sites (Fig. 4(2)). Therefore various genes of interest can be expressed from a single, well-characterized gene-trap line after successful RMCE. Such expression could even be rendered conditional by the incorporation of Cre-specific recombination sites [85]. Similar RMCE was recently demonstrated in the zebrafish system by the successful exchange of fluorescent proteins *in vivo*, using the phage PhiC31 integrase and the Flp recombinase, respectively [44,98]. In the latter study transgenic lines were generated that are compatible for RMCE, thus allowing for multi-purpose gene expression in the future.

6. Studying and manipulating zebrafish by multicolor imaging

So far we have emphasized the design of genetic expression systems for various fluorophores in live zebrafish using genetic vectors and diverse expression systems. In the remaining part we will show a few examples of how these concepts have been used to optimize imaging conditions (Section 6.1) and to improve the usefulness of transgenic reporter lines (Section 6.2). Finally we will show some examples of how multicolor imaging tools were used to obtain *in vivo* cell biological data using zebrafish (Section 6.3).

6.1. Mosaic expression and brainbow imaging in zebrafish

Some applications such as the analysis of cell migration by time lapse imaging or cell lineage tracing focus on the observation of single or a few individual cells. These applications benefit from embryos displaying mosaic expression of fluorescent proteins in defined cells rather than whole tissue expression, because contrast is optimal for fluorescent cells surrounded by dark neighbors. An elegant way to achieve mosaic expression in zebrafish using stable transgenics is MAZE, which stands for mosaic analysis in zebrafish [16]. This method combines different genetic systems and molecular tools to express multiple fluorescent proteins in a mosaic pattern, thereby providing an elegant solution for a common

imaging problem (Fig. 4(1)). MAZe is based on the heat-shock induced removal of a STOP cassette by Cre recombinase. This allows for the cell type specific expression of the transactivator Gal4 from a single vector construct. Gal4 can drive expression of a (multi-color) fluorescence cassette which can be part of the same plasmid or expressed from a different line that contains e.g. a Janus vector. As mentioned before, heat shock induction is not 100% efficient thereby inducing mosaic expression of fluorescence. This pattern can be further modulated by varying the duration of the heat shock or by performing repeated heat shocks, until a mosaic pattern has been achieved that fits experimental requirements, e.g. for lineage tracing.

Brainbow constructs were first used in the mouse brain to stain groups of neurons in multicolor mosaics [65,66]. To generate color diversity, these constructs contain a promoter and three or four spectrally different fluorescence proteins that are recombined randomly for expression using the Cre/loxP system. The genomic integration of multiple cassettes at the same site can label cells in almost 100 different colors by combinatorial fluorescence expression. After removal of recombinase the fluorescence-pattern expressed in individual cells is genetically fixed and will be passed onto daughter cells, thereby allowing color-coded, fluorescent lineage tracing in developing tissues with the possibility to observe several clusters of cells at the same time. After confocal imaging of brainbow-labeled tissues in three to four channels, grey scale images are merged and converted into pseudo-color images. Therefore proper image processing is crucial to obtain maximal color diversity. For a detailed description of the technique, its use in zebrafish and an update on recent developments, we suggest to read the following manuscripts [71,12]. The principal of Brainbow imaging was adopted to study heart development in zebrafish in a unique way by transgenetically expressing a brainbow cassette in cardiomyocytes at different developmental time points by means of a tamoxifen-inducible system [35]. Inducible Cre (CreER^{T2}) was expressed from a cardiomyocyte specific promoter in one line and another line expressed the brainbow cassette under the control of an ubiquitous promoter (*-actin2*). This allowed the authors to induce mosaic fluorescent protein expression at different timepoints during heart development by the application of tamoxifen to the rearing medium of developing embryos that were obtained from crosses between the two lines. With this multicolor approach the authors were able to gain unprecedented insights into the morphogenetic processes that occur during heart development, identified the existence of three myocardial lineages in this process and proposed the clonal dominance model as a strong candidate to explain vertebrate organogenesis.

6.2. Visualizing gene function using Flip Trap vectors

Recent advancements are transgenic lines harboring specifically designed gene-trap vectors named FlipTrap [98]. A FlipTrap vector integrates several of the molecular tools and genetic concepts reviewed in this article and can therefore serve as an example how these concepts can be combined to create highly versatile transgenic lines for multicolor imaging. A FlipTrap vector contains a splice donor and an acceptor site flanking a multipurpose fluorescent reporter cassette equipped with sequences for recombination (Fig. 4(2)). When the vector integrates randomly within the intron of an actively expressed gene, the fluorescence reporter can be spliced into the native transcript so that it will be part of the protein, thereby preserving the function of the trapped gene. This is in contrast to traditional gene-trapping approaches where native gene function is usually disrupted by the trapping event when it occurs in introns near the 5'-prime end (Section 4.3). Thus when using a FlipTrap vector, a successful trapping event becomes

detectable by the gene-specific expression of a fluorescent fusion protein containing Citrine, which allows for the analysis of native protein expression and localization *in vivo*.

Another feature of FlipTrap lines is the possibility for recombination mediated cassette exchange *in vivo*, owing to the presence of heterotypic Frt-sites flanking the reporter cassette. Thus, in the absence of ES-cell based gene targeting technology in zebrafish, FlipTrap lines can be used for the site-specific integration of foreign DNA encoding other proteins. This was shown by site specific exchange of the Citrine reporter cassette with an mCherry reporter cassette, thereby inducing a color switch in heart and trunk muscle cells of a defined FlipTrap line. By exchanging the Citrine reporter with a photoconvertible fluorophore like Kaede, it will be possible to study the turnover of trapped proteins *in vivo*. Another option is the exchange with a cassette that contains a splice acceptor followed by a 2A sequence and a transcriptional activator, thereby generating well characterized gene specific activator variants for combinatorial gene expression (Section 5.2).

A third fascinating feature of a FlipTrap line is the “Flip and Excision (FLEX)” option, the possibility to induce a conditional mutation in the trapped allele. In the FlipTrap vector FLEX depends on Cre recombinase activity and on the positioning of two pairs of heterotypic loxP sites. Two loxP sites flank the Citrine reporter, the splice donor and a second reporter cassette encoding mCherry/polyA, which itself is flanked by two loxP sites, oriented in reverse and silent. Cre activity will induce recombination of the loxP sites, which causes a transient inversion of the regions placed between them in a first step. In the course of these reversible rearrangements Cre activity can excise the Citrine reporter and the splice donor which constitutes a second, irreversible step. Likewise, the silent mCherry/polyA cassette is inverted so that it can now be spliced into the native transcript. Such activity has two consequences: The first is a “color flip”, which means that expression of Citrine (yellow) is ceased and mCherry (red) is expressed instead. Secondly, since the cassette has lost its splice donor, the translation of the endogenous transcript will be terminated at the integration site of the FlipTrap vector, therefore exons located 3'-prime to the trapped locus will not be part of the final fusion protein. If such a truncated gene product is non-functional, the “color flip” from green to red is a fluorescent reporter for the acute loss of endogenous gene function. A phenotype could be directly observed in such mutants, however since many genes in zebrafish have two copies due to partial genome duplication, further genetic manipulations might be required to achieve a full “knockout” phenotype. Such an approach is now possible by using the TALEN-method, which can generate targeted mutations in the zebrafish genome after the design of gene-specific DNA-binding proteins fused to DNA-nuclease FokI [46,83]. Alternatively, genome editing could be performed by using a similar, most recently developed system that utilizes guide-RNAs to target specific genomic regions [48]. In FlipTrap lines, mutated alleles can be “genotyped” *in vivo*, thereby identifying heterozygous mutants by the coexpression of red and green fluorescence, and homozygous mutants by the continued exclusive expression of red fluorescence. This constitutes a tremendous advantage over traditional genotyping methods, which are invasive, laborious and time consuming.

Considering the versatility of lines generated by FlipTrap vectors, i.e. their usefulness as gene-specific reporter lines, as “conditional knockouts” and as vehicles for the expression of other gene products, it can be hoped that more such lines will be generated in the near future. FLEX-Trap has also been realized in other vectors to induce conditional alleles in zebrafish such as GBT and FT1 [15,69]. The work by Ni et al. demonstrates how embryonic lethality of a housekeeping gene can be overcome and rescued in a tissue specific way in living zebrafish.

6.3. Use of multicolor imaging tools in cell biology

Using Janus and Medusa vectors (Figs. 1(5) and 3(2)) in combination with Gal4-genetics we recently studied the behavior of the centrosome in migrating neurons of zebrafish larvae by *in vivo* time lapse imaging [26]. To this end relevant subcellular compartments were highlighted simultaneously by using different fluorophores with respective tags, including the nucleus, the centrosome, the plasma membrane and the actin cytoskeleton. One aim of this study was to clarify the position of the centrosome in migrating neurons, which had been postulated to be generally ahead of the nucleus and oriented towards the direction of cellular movement. This controversial concept was refined by our *in vivo* time lapse data, revealing a leading position of the centrosome only prior to the forward movement of the nucleus, which overturned the centrosome such that the centrosome iteratively has to surround the nucleus to prepare the following migratory step of the cell.

These few examples should illustrate how genetic, molecular and multicolor imaging tools can be applied to answer biological questions in living zebrafish embryos and larvae. Data obtained from *in vitro* studies are not always conform with *in vivo* data. We thus hope that we have convinced the readers, that the zebrafish model is a versatile and genetically mature system to study various aspects of vertebrate cell biology *in vivo*. As indicated in some of our figures, applications for multicolor imaging tools in zebrafish go beyond observation, allowing for *in vivo* physiological interrogation or manipulation of zebrafish for example in combination with electrophysiological studies, or by the overexpression of proteins and their variants with altered functions. Thus zebrafish awaits a bright and colorful future.

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