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ORIGINAL PAPER

Fluorescent Function-Spacer-Lipid Construct Labelling Allows for Real-Time *in Vivo* Imaging of Cell Migration and Behaviour in Zebrafish (*Danio Rerio*)

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Abstract Real-time *in vivo* imaging of cell migration and behavior has advanced our understanding of physiological processes *in situ*, especially in the field of immunology. We carried out the transplantation of a mixed population of blood cells from adult zebrafish (*Danio rerio*) to 2 day old embryos. The blood cells were treated *ex vivo* with Function-Spacer-Lipid constructs (FSL) incorporating either fluorescein or Atto488 fluorophores (FSL-FLRO4-I or -II). Excellent labeling efficiency was demonstrated by epifluorescence microscopy and FACScan analysis. Real-time video imaging of the recipient fish showed that the functionality of these cells was retained and not affected by the labeling. The usefulness of FSL-FLRO4-I as a contrast agent in microangiography was explored. Overall, we found both FSL-FLRO4-I and-II promising labeling dyes for real-time *in vivo* imaging in zebrafish.

Keywords Kodecyte · Labeling · Fluorescence · Zebrafish

Introduction

Animal models have contributed significantly to medical science in order to understand better the pathogenesis of human diseases. Once developed and validated, they can be used as a pre-clinical therapeutic platform to assess drug toxicity as well as drug efficacy. Transgenic indicator strains are animals in which only certain type of cells, tissues, or organs express fluorescent protein(s), allowing the real-time imaging of disease pathologies. In rodent models, such real-time imaging often requires surgical intervention or post-mortem examination [1]. The zebrafish (*Danio rerio*) is a tractable model species as embryos and larvae offer the optical clarity to visualise developmental events, physiological processes, and disease progression. The chorion membrane encloses the embryos for the first 2 to 3 days, but this does not impair the visualisation of embryonic morphology, such as the heart. Embryos/larvae then become free-swimming at 48 to 72 h post fertilisation (hpf). The development of the brain, the cardiovascular system (heart and blood vessels), the digestive system (gut, liver, gall bladder), and the skeletal system (cartilage) can be visualised *in situ*.

Transgenic *fli*^{eGFP} fish express green fluorescent protein (GFP) in endothelial cells of all blood vessels, so angiogenesis can be studied over the course of development [2]. Alternatively, fluorescence microangiography in zebrafish can be achieved by microinjecting fluorescent latex microspheres and quantum dots into the blood circulation [3, 4]. This labelling is transient, and these agents are gradually cleared from the blood stream over several hours post-injection. Apart from latex microspheres and quantum dots there have not been reports investigating other fluorescent dyes for microangiography.

Historically, our understanding of the continuous traffic of leukocytes between blood and lymph has been gained from murine studies in which leukocytes are labelled with radioisotopes and introduced back to the recipient. There are several drawbacks to the procedure, including time-consuming autoradiography of histological sections, the hazardous nature of the radioisotopes, and the decay of high

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energy radio-isotopes [5]. Over the last 20 years, fluorescent dyes have replaced radioisotopes in cell migration studies as they can be monitored in real-time. Membrane-inserting dyes including C18 DiI, C18 DiO, PKH2, PKH3 and PKH26 have been used in tracking erythrocytes, leukocytes, and stem cells in murine models. Such studies have not been reported in the zebrafish [6].

KODE cell surface modification technology uses Function-Spacer-Lipid (FSL) constructs [7]. The lipid component allows the construct to spontaneously attach to phospholipid membranes. The functional moiety can comprise a peptide (either carboxyl or amino proximus linked), carbohydrate, polysaccharide, biotin or fluorophore. FSL constructs have been used in a wide range of applications [7]. For example, cells that have been coated with FSL-Biotin constructs can be recovered using avidin-paramagnetic beads [7–9]. Live cells, sperm, mouse embryos and competent virions have been fluorescently labeled using FSL fluorophores [7, 8, 10, 11]. FSL construct modified cells are known as kodecytes [7] and the process of modifying a membrane surface with FSL constructs is termed koding. The protocol for *in vitro* koding of live or fixed cells is simple, flexible and robust. The incubation time of cells with FSL constructs can vary between 30 and 120 min at temperatures ranging from 4 °C to 37 °C [7].

In this study, we investigated the possibility of using FSL molecules as a microangiography contrast dye. An optimized protocol was developed for *ex-vivo* labeling of a mixed population of blood cells from adult zebrafish. These FSL modified cells (kodecytes) were introduced into zebrafish and tracked in real-time *via* epifluorescence microscopy.

Materials and Methods

Microangiography

Microangiograms were performed as previously described [3]. 52 hpf embryos were manually dechorionated using fine syringe needles (1 mL insulin syringe). Anaesthetised embryos were placed in a trough of a 3% agarose tray flooded with E3 medium containing 0.016% tricaine. Two percent low melting point agarose supplemented with 0.016% tricaine was added to overlay the trunk of the embryo to prevent the embryos from moving. Borosilicate needles (1 mm O.D., Harvard Apparatus) used for injections were pulled using a Flaming Brown micropipette puller (Sutter Instrument Co.). The needles were back filled with a 1 mg/mL suspension using a microloader tip (Eppendorf). 10 nL of FSL-FLRO4-I solution was delivered to the *sinus venosus* over the course of 1 min (5×2 nL injections). Injected embryos were released from the agarose tray, recovered in E3 medium supplemented with 0.016% tricaine for 2 min, and then immobilised in 2% methyl cellulose for fluorescence imaging using an Axiovert 100 microscope (Carl Zeiss) equipped with an FITC/GFP filter.

Isolation of Whole Kidney Marrow Cells from Adult Fish

The isolation of the whole kidney marrow (WKM) cells was performed as previously described [12]. One to 2 year old adult zebrafish were euthanased in icy water for 1 min, then placed in a sterile petri-dish. Kidney tissue was scraped from the body cavity and immediately immersed in 10 mL of “PBS+” containing 0.5X PBS with 5% (for optimisation experiments) or 2.5% (routinely used concentration) FBS (Fetal Bovine Serum; Sigma Aldrich), 100U/mL penicillin and 100 μg/mL streptomycin. Three kidneys were pooled into 10 mL PBS+. For each transplantation procedure, two pools of three kidneys were combined. The kidney tissues were first disrupted by gently pipetting up and down with a 1 mL pipette tip, and further disrupted through an 18.5 gauge needle (attached to a 5 mL syringe). The tissue suspension was centrifuged at 400*g* at 20 °C for 8 min. After centrifugation, the pellet was resuspended in fresh 20 mL PBS+. The suspension was filtered through a 40 μm cell strainer and then centrifuged at 400*g* for 8 min at 20 °C. After centrifugation, the supernatant was removed and the pellet was resuspended in approximately 100 μL PBS+. The cell suspension from each tube was pooled at this point. Trypan blue exclusion assays were carried out to determine cell viability and concentration.

FSL-FLRO4 Constructs

FSL-FLRO4-I (FSL-FLRO4(fluorescein)-SA2-L1 Cat # 721472) was obtained from KODE Biotech Materials (Auckland, New Zealand; kodebiotech.com) and is also available from Sigma-Aldrich (Cat # F1058; St. Louis, MO, USA). FSL-FLRO4-II was custom synthesized by conjugation of Atto488 to a conjugate of bis(N-hydroxysuccinimidyl) adipate and 1,2-O-dioleoyl-sn-glycero-3-phosphatidylethanolamine.

FSL Labeling (Koding)

Aliquots of FSL stock solution were pre-equilibrated to 28 °C in the dark. In the case of FSL-FLRO4-I (fluorescein) experiments, 25 μL of 2 mg/mL FSL-FLRO4-I was mixed with 75 μL of cell suspension. Another 75 μL of cell suspension was incubated with 25 μL of PBS+

as an untreated control. For slide preparation of FSL-FLRO4-II (Atto488) experiments, 10 μL of 1 mg/mL FSL-FLRO4-II (or PBS+ for control slides) was mixed with 40 μL cell suspension. For transplantation experiments, due to the limited availability of FSL-FLRO4-II, the cells were treated with 0.125 mg/mL FSL-FLRO4-II (final concentration), rather than 0.5 mg/mL as was the case for FSL-FLRO4-I. In murine red blood cell experiments [8], 0.1 mg/mL was used, so the concentration 0.125 mg/mL was considered reasonable for the koding experiments described here. In the case of transplantation experiments, 25 μL of 0.5 mg/mL FSL-FLRO4-II was mixed with 75 μL of cell suspension. Another 75 μL of cell suspension was treated with 25 μL of PBS+ as a control. All kodecytes were prepared by incubation at 28 °C in the dark for 2 h. Each kodecyte suspension was then centrifuged at 420*g* for 8 min at room temperature, and the supernatant was removed. 1 mL of PBS+ was applied to resuspend the pellet, and the centrifugation was repeated. This procedure was repeated three times. In the final step of washing, the pellet was resuspended in 10 μL of injection solution containing 0.5X PBS, 3U heparin, 1U DNase and 2.5% FBS. Heparin (Sigma) and DNase (Amplification grade, Invitrogen) were added to reduce cell aggregation.

Transplantation

50–52 hpf embryos were manually dechorionated using fine syringe needles (1 mL insulin syringe). Anaesthetised embryos were placed in a trough of 3% agarose tray flooded with E3 medium containing 0.016% tricaine. Ten nanolitres of cell suspension (either untreated controls or kodecytes) were injected into the *sinus venosus* of embryos using borosilicate glass capillary needles (1 mm outside diameter, no filament; Harvard Apparatus) that were made using a Flaming/Brown micropipette puller (Sutter Instruments). Cell suspensions were back-loaded into each needle and injected into the zebrafish circulatory system using a Femtojet injection unit (Eppendorf). The injected embryos were placed in fresh E3 medium containing no tricaine in order to recover for 15 min, and then anaesthetised with 0.016% tricaine for subsequent imaging. Embryos were placed in a glass bottom dish, and fluorescence (with FITC/GFP filter) and bright field imaging were performed using an Axiovert 100 microscope (Carl Zeiss). As the software for the Axiovert 100 microscope did not have a video-recording function, the free-streaming video software CamStudio (http://camstudio.org/) was used to capture live streaming *via* the 'live' window. The video clips were usually 20 s long.

Fluorescence-Activated Cell Sorting (FACS)

200 μL of FLRO4-I kodecytes were mixed with 300 μL of PBS+ to bring the volume to 500 μL for FACS. PBS+ treated cells were included to determine the level of autofluorescence detection. Cell suspensions were analyzed based on GFP fluorescence using a FACS Aria II platform (BD Biosciences).

Slide Preparation

20 μL of kodecytes were placed within 2 cm diameter circles that were drawn on poly-L-lysine coated slides using a PAP pen (DAKO). The slides were placed in a humidity chamber and incubated at 28 °C for 30 min. Following the incubation, coverslips were applied and the cells were imaged using an Axiovert 100 microscope (Carl Zeiss) equipped with a FITC/GFP filter.

Results and Discussion

Transient Decoration of Vasculature by FSL-FLRO4-I

Zebrafish microangiography involves injecting 0.02 μm (diameter) fluorescent latex beads into the *sinus venosus*. The vasculature can be visualised by epifluorescence and confocal imaging within a few minutes of injection. Microangiography was therefore carried out by the direct injection of FSL-FLRO4-I following the same method as if using fluorescent microspheres. Within 5 min, fluorescence could be detected in most blood vessels (Fig. 1a). The gap between the dorsal aorta (DA) and posterior caudal vein (PCA) could be resolved. Blood flows from the dorsal aorta to the caudal aorta (CA) makes a 180° turn when it reaches the most caudal region then enters the caudal vein, which is formed by a plexus of vessels [3]. Some vessels in the head vasculature, such as the metencephalic artery (MtV) and Prosencephalic artery were located (Fig. 1a). At 2 h post injection there appeared to be an accumulation of high fluorescence signal in the pericardial cavity, suggesting pericardial edema in FSL-FLRO4-I-injected fish (Fig. 1b). While the latex beads injected into the zebrafish still displayed sharp labeling of intersegmental vessels (Fig. 2a), the resolution was lost in the FSL-FLRO4-I injected fish (Fig. 2b), possibly due to the preferential location of FSL-FLRO4-I to the pericardial cavity. Whether this is a FSL-FLRO4-I-mediated cell aggregation/fluid accumulation or a toxic phenotype directly associated with FSL-FLRO4-I remains to be investigated. The latter scenario is less likely as such toxicity has not been observed in mice [9].

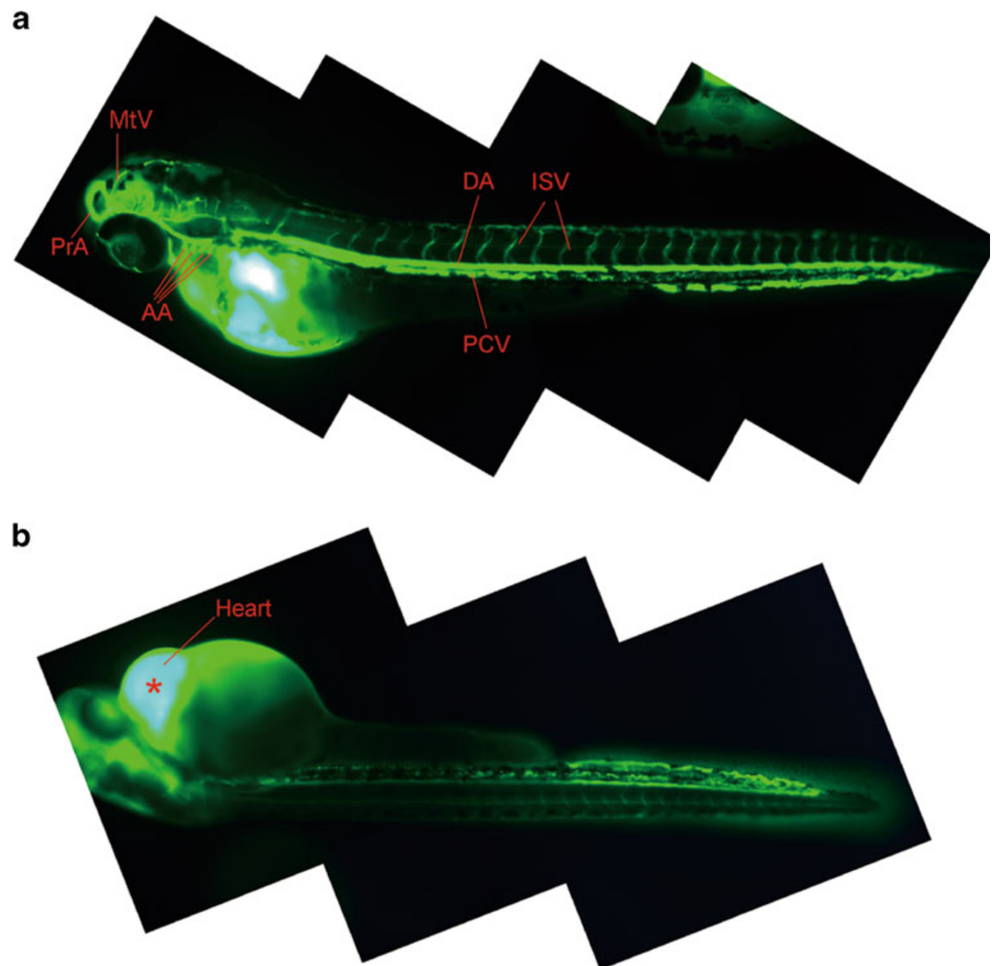

Fig. 1 Epifluorescence microangiograms of a 2 dpf embryo injected with 10 nl of 1 mg/mL FSL-FLRO4-I solution. Panel a: An epifluorescence image was captured 5 min post injection. Panel b: The image was taken 2 h after the injection. Note that fluorescence accumulation in the heart cavity is marked with an asterisk. Abbreviations: Dorsal Aorta (DA), Posterior Caudal Vein (PCV), Metencephalic Vein (MtV), Prosencephalic Artery (PrA), Aortic Arch (AA), and Intersegmental Vessels (ISV)

Exploration of Ex Vivo Cell Labeling Using FSL Fluorescent Derivatives, and its Uses in Real-Time Imaging of Blood Cell Migration

Optimisation of Transplantation Protocol Using FLRO4-I Kodecytes

FSL-FLRO4-I can label mouse embryos and RL95 endometrial cells [11], and so we examined if zebrafish cells could be similarly labeled. The transplantation of zebrafish adult kidney marrow cells (blood cells) into 48 hpf embryos has been described by Traver et al. [13]. The cell population of interest was whole kidney marrow cells as they contain erythrocytes (~41%), myeloid cells (~24%), lymphocytes (~19%), progenitor cells (~6%) and other cells (~10%) [13]. Whole kidney marrows (WKM) were dissected from euthanised fish, gently teased, and filtered through a 40 μm cell strainer. The dissociated cells were washed several times and then resuspended in fresh PBS supplemented with FBS. Typically ~4×10^6 cells WKM cells could be isolated from six whole kidneys.

FSL-FLRO4-I was used for transplantation optimisation experiments. WKM kodecytes were prepared with 0.2 mg/mL FSL-FLRO4-I in 0.5X PBS with either 2.5% or 5% FBS. The latter concentration was considered critical as lipids in FBS can interfere with FSL labelling efficiency, and the insertion process [8]. Labelling appeared to be equally efficient at both FBS concentrations; however, at 2.5% FBS, stronger signals were observed (Fig. 3a). A more quantitative method using FACS

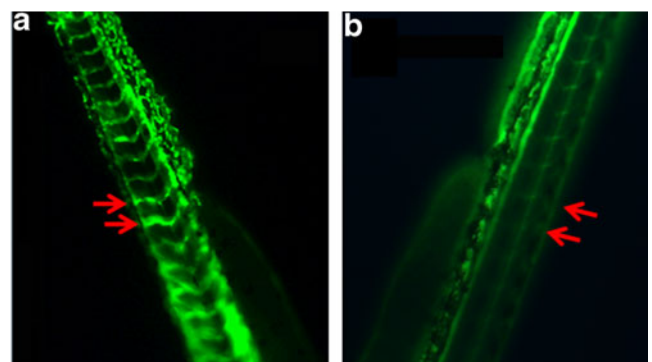

Fig. 2 Comparison of 2 h post injection microangiograms of 54 hpf embryos. Embryos were injected with 10 nl of either a microsphere suspension (MS) (panel a) or FSL-FLRO4-I (1 mg/mL) (panel b) or. No strong fluorescence could be detected in the intersegmental vessels (arrows) in FSL-FLRO4-I injected fish

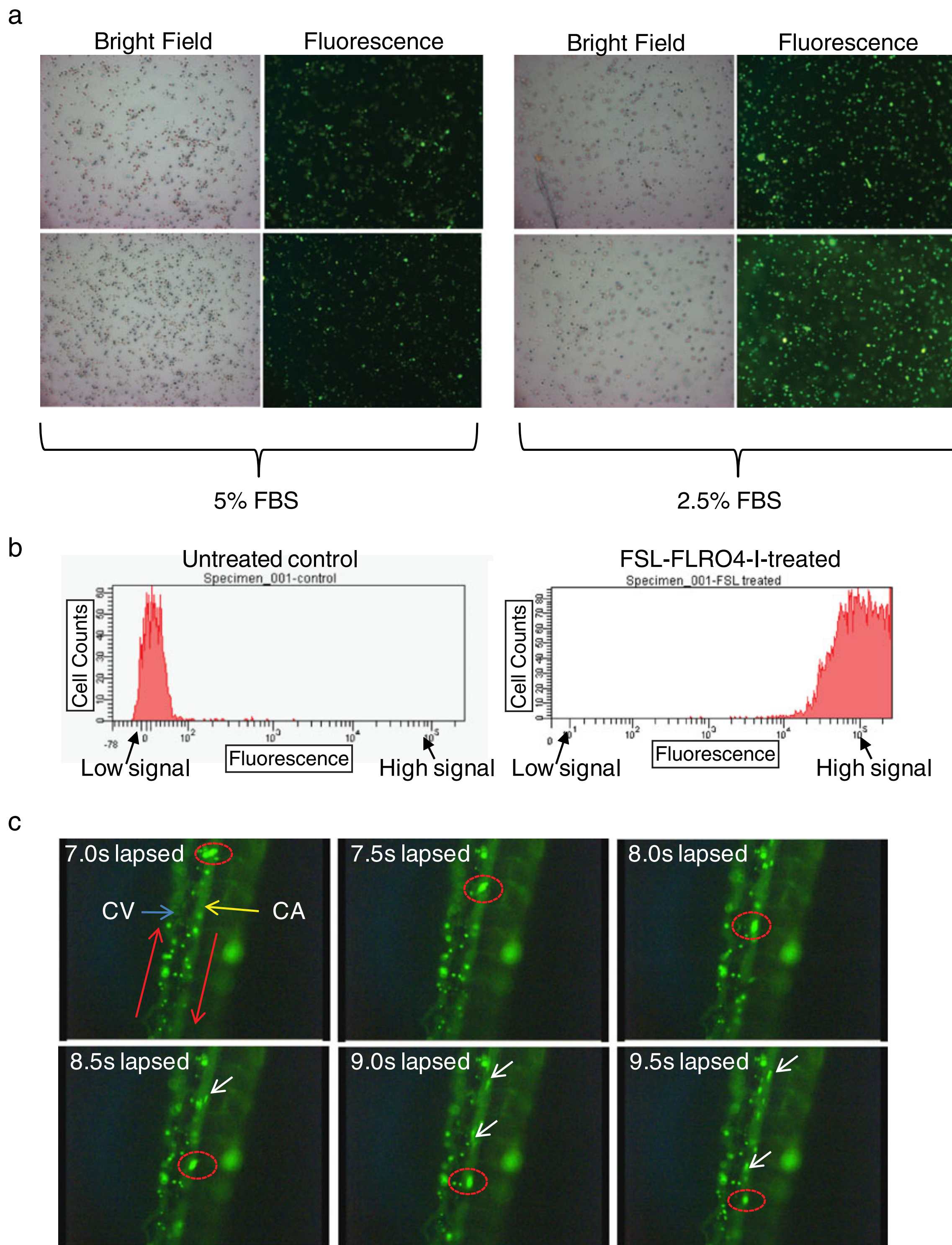

Fig. 3 Effect of different concentrations of FBS on labelling efficiency Panel a: FACS analysis of untreated and FSL-FLRO4-I-treated. Higher labelling efficiency was observed in cells suspended in 0.5X PBS with 2.5% FBS. Panel b: Lower background of green fluorescence in the untreated control and high efficiency of labelling in treated cells were confirmed. Two hours post injection imaging of the caudal vein plexus area of 52 hpf recipient zebrafish receiving 0.2 mg/ml FSL-FLRO4-I koded WKM cells. Panel c: Time-lapsed frames taken from video 1 [15], and each frame is separated by 500 ms. A large slow-moving cell (red dashed circle) tumbles along the endothelial surface. Elongated oval shaped erythrocytes move at a fast speed (while arrow). Red arrow indicates the direction of blood flow. CA: caudal artery. CV: caudal vein

was applied to confirm greater labelling efficiency at 2.5% FBS. FACS analysis revealed that nearly all the FLRO4-I-treated cells were labelled (becoming kodecytes) and emitted very strong fluorescence (Fig. 3b). By comparing the histograms between untreated and treated cells (Fig. 3b), there was no overlap of the signals (red area), indicating very low background fluorescence emitted by unlabeled cells.

The WKM cells were then transplanted into 2 dpf embryos. No graft rejection was expected at this time point as the onset of lymphopoiesis does not occur until 3–5 days after fertilisation [14]. Kodecytes were resuspended in injection solution containing 0.5X PBS, 3U heparin, 1U DNase and 2.5% FBS. No significant aggregation of kodecytes was observed. Approximately 10 nL of the cell suspension was injected into the *sinus venosus* of anaesthetised zebrafish embryos. Embryos were allowed to recover in E3 medium containing no tricaine, then anaesthetised again for imaging. A good injection

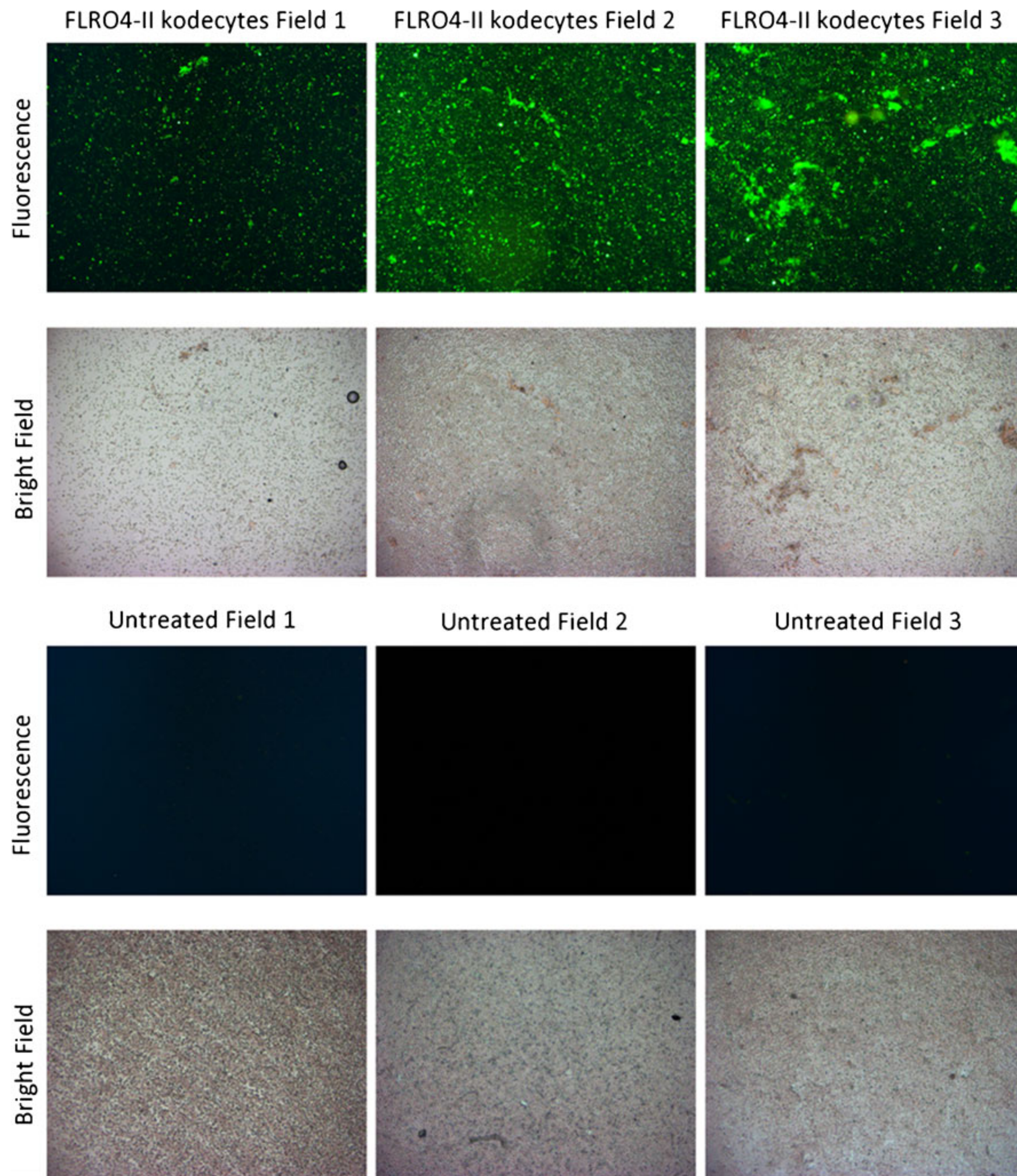

Fig. 4 Efficient labelling of WKM cells by FSL-FLRO4-II. Three fields were randomly imaged from the same slide containing either 0.2 mg/mL FSL-FLRO4-II-treated (FLRO4-II kodecytes) or PBS control treated cells. Very low background fluorescence was seen in PBS control cells. Some degree of aggregation of cells was observed in both fluorescent and bright field images of FLRO4-II kodecytes

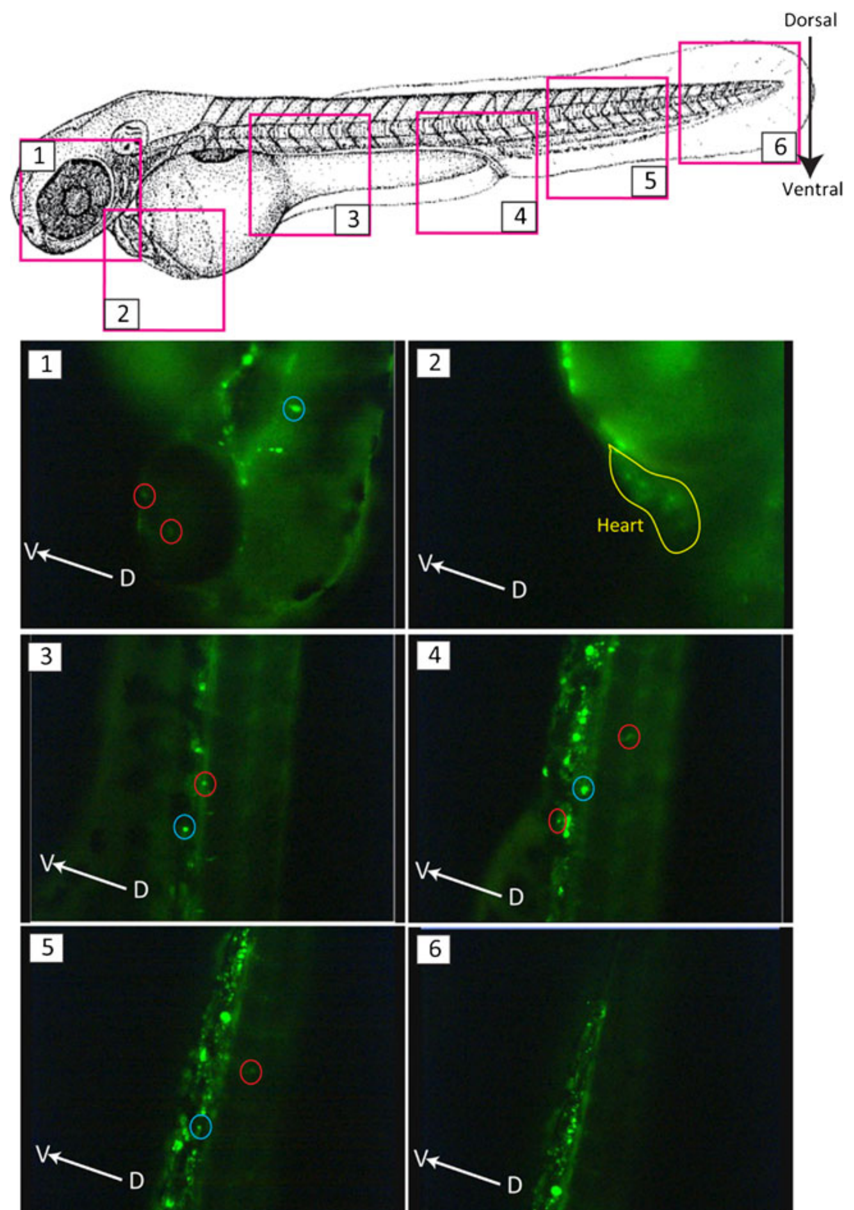

Fig. 5 Visualisation of in vivo FLRO4-II kodecytes in different regions of the recipient fish. Videos 2–7 can be found in [15], and representative captured images are shown in panels 1–6, respectively. Moving cells in each video are labelled red and static cells are labelled blue. D: dorsal. V: ventral

outcome consisted of the presence of heart beats, good circulation (erythrocytes moving fast), few slow moving/ static cells, and a low fluorescence background (indicating efficient washing and low levels of uninserted FSL-FLRO4-I in the cell-suspension). Figure 3c shows time-lapsed snapshots taken from video 1 [15]. In each frame, kodecytes travelled at speed making it difficult to visualise them as single cells. There were also cells moving at a much slower speed, and travelling in the opposite direction to that of the circulation, or travelling in between intersegmental vessels; cell size varied. A large sized cell shown in Fig. 3c (represented by a red dashed circle), illustrates a 'slow moving' cell with pseudopodia, which could be a myelomonocytic cell [13].

Real-Time Tracking of Transplanted FLRO4-II Kodecytes

FSL-FLRO4-I demonstrated excellent *in vitro* labeling efficiency and allowed the kodecytes to be visualised *in vivo*, but they quenched upon repeat exposures to UV light. This quenching hampered the potential of FSL-FLRO4-I to be used in tracking cells over time. Such photo instability has also been seen in other model systems (DB, unpublished results). During the development of the transplantation protocol, a new more photostable FSL fluorophore, using Atto488 (FSL-FLRO4-II), was developed. As with FSL-FLRO4-I, very good labeling efficiency of WKM cells was achieved using FSL-FLRO4-II (Fig. 4), although there was some degree of cell clumping that was not observed using FSL-FLRO4-I.

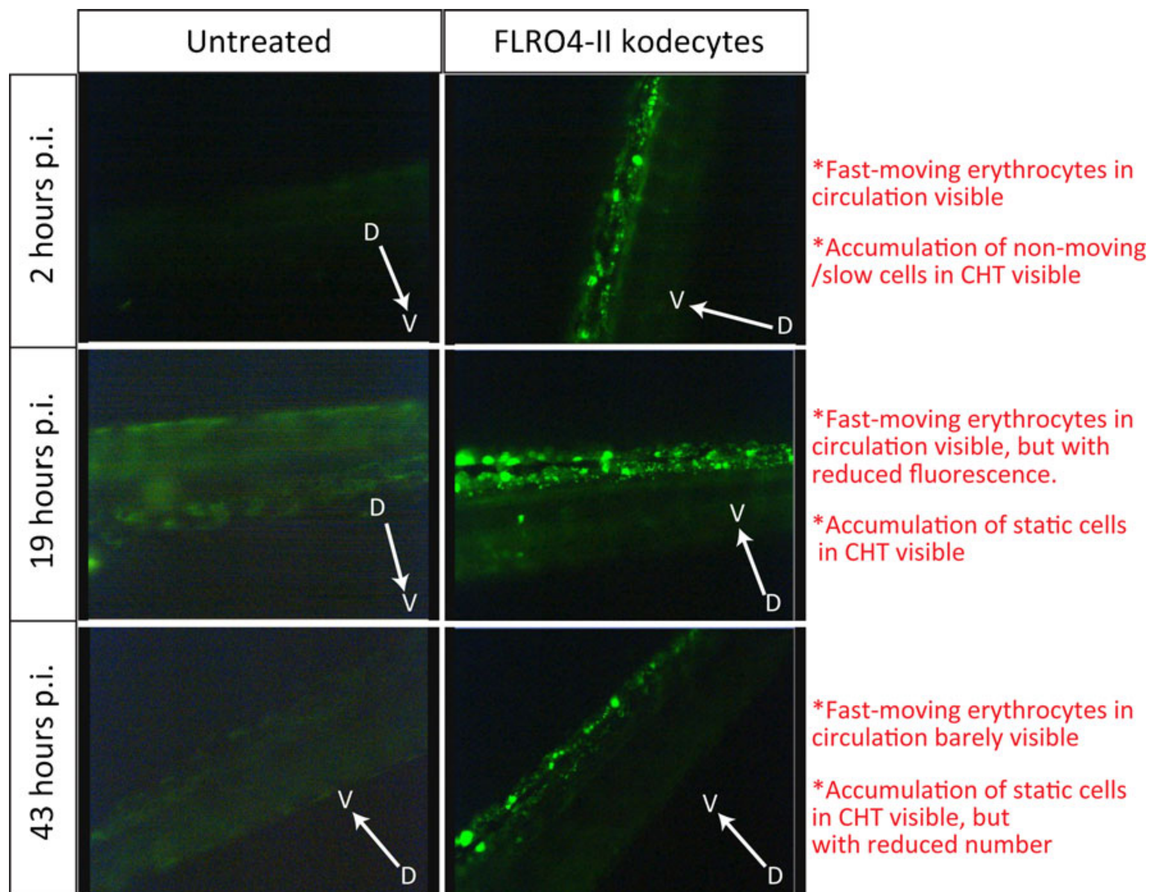

Fig. 6 Temporal assessment of FLRO4-II kodecytes in the lower trunk area of fish 11, and a control treated recipient. Three time points were assessed: 2 h (video 8:control; video 9:kodecytes), 19 h (video 10:control; video 11:kodecytes), and 43 h (video 12: control; video 13:kodecytes) post injection (p.i.).. D: dorsal. V: ventral. Videos 8-13 can be found in [15]. The above panels are captured images from these videos

Using the optimised protocol established with FSL-FLRO4-I, transplantation was performed using embryos aged between 50 and 52 hpf. The survival rate following transplantation was 100% (n=12). The focus for imaging lay in the caudal vein plexus area, which contains caudal haematopoietic tissue (CHT). This area allows imaging of the circulation in the caudal aorta and the caudal vein, and homing behaviour of progenitor or mature leukocytes to be undertaken. 20-second videos were taken 2 h post injection and manually inspected. Single cell resolution could be achieved, which indicates sufficient labeling and adequate washing. The accumulation of static kodecytes in the caudal vein plexus area was observed in all fish. Apart from rapid-moving erythrocytes in the circulation, other types of cells including small, round cells that rolled steadily along the vessels (lymphocytes; rarely observed) and large, amoeboid cells with visible pseudopodia that moved along vessels (more commonly observed) were found in recipient fish. These cells have also been described by Traver et al [13] where WKM cells from the adult kidneys of doubly transgenic zebrafish (*gata1*^{dsRed} and *bactin*^{eGFP} in which erythrocytes and leukocytes express DsRed and eGFP, respectively) were transplanted to 2 dpf recipient zebrafish embryos.

Overall, recipient fish displayed good transplantation outcomes: good circulation, high intensity, CHT cell accumulation, and single cell resolution (both moving and static). 20-second video clips were taken of six different regions (Fig. 5). In the videos 2–7 [15] both rapid-moving and static labeled cells could be seen. For example in the head region (frame 1), circulating erythrocytes were evident in the inner optic circle vessel around the eye. The temporal investigation of FLRO4-II kodecytes was also carried out (Fig. 6 and videos 8–13; see ref. 15). Circulating erythrocyte kodecytes exhibited the strongest fluorescence 2 h post injection (p.i.), but signal strength decreased at 19 h p.i., and was barely visible at 43 h p.i. Cell accumulation peaked at 19 h p.i. in the caudal vein plexus area, but declined by 43 h p.i.

Again, special attention was paid to the caudal vein plexus where CHT are located. Even at 43 h p.i. (recipient age: 4 dpf), fluorescently labeled cells could still be observed. The accumulation of transplanted leukocytes in this region has also been reported by Traver et al [13], while transplanted GFP-labeled thymic T cells. *CD41*^{eGFP} cells (marking definitive haematopoietic precursor cells) also appear to colonise the CHT [16, 17]. It appears that both

mature and precursor blood cells home to CHT, which is a transient haematopoietic site between approximately 35 h to 14 days post fertilisation [18]. Apart from the precursors, mature macrophages and neutrophils are also found at this site. Leukocytes at CHT are capable of migrating to wounds and phagocytosis [19, 20]. The wound healing assay to study leukocyte migration and phagocytosis has, to date, used transgenic fish such as the *mpo*^{eGFP} or *lysC*^{eGFP} lines [21, 22]. A wound is usually introduced in the small area of the ventral fin, and the migration of leukocytes is tracked in real-time. The earliest time point for carrying out a wound assay is at 3 dpf [23]. Critically, the experiments described here show that FLRO4-II kodecytes are still present in the CHT area at 3 dpf. Therefore, transplanted FLRO4-II WKM kodecytes could be used in wound healing studies in the zebrafish. In addition, *in situ* hybridisation could be undertaken to identify transcripts of macrophage/neutrophil marker genes such as *myeloperoxidase*, *lysozyme C* and *L-plastin* to confirm the identity of cells that migrate to the wound.

Conclusion

In this study, we evaluated FSL-FLRO4-I fluorescein construct solution as a microangiography contrast agent. The results indicate a good distribution of FSL-FLRO4-I within the blood vessels in zebrafish embryos, but the accumulation of the molecules in the pericardial cavity 2 h post injection was unexpected and requires further investigation. It should be noted these constructs are expected to associate with lipids and their distribution and fate will be linked with that of circulatory lipids. We also show efficient *in vitro* labeling (forming kodecytes) of erythrocytes, leukocytes and other support cells in whole kidney marrow cells with FSL-FLRO4-I and FSL-FLRO4-II constructs. Transplantation studies indicated that the functionality of kodecytes did not appear to be hampered, which is consistent with other studies [7, 10]. With the availability of transparent juvenile and adult zebrafish [24], the simple and efficient FSL labeling protocol described here may be useful for labeling tumors or cells in transplantation experiments.

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