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Fluorescence-activated cell sorting and quantitative real-time PCR to reveal VEGF expressing macrophage populations in the zebrafish larvae

Authors: Andrew Herman[1], Alexander Greenhough[2], David B. Gurevich[3]

1 –Faculty of Life Sciences, School of Cellular & Molecular Medicine, Biomedical Sciences Building, University of Bristol, UK, BS8 1TD.

2 – Centre for Research in Biosciences, Faculty of Health and Applied Sciences, University of the West of England, Coldharbour Lane, Bristol, UK, BS16 1QY.

3 - Department of Biology & Biochemistry, Centre for Therapeutic Innovation, Faculty of Science, University of Bath, Claverton Down, Bath, UK, BA2 7AY. Email: dbg29@bath.ac.uk

Abstract: The transparent, genetically tractable zebrafish is increasingly recognised as a useful model to both live image and uncover mechanistic insight into cell interactions governing tissue homeostasis, pathology and regeneration. Here, we describe a protocol for the isolation of macrophages from zebrafish wounds using fluorescence activated cell sorting (FACS), and the identification of specific pro-angiogenic macrophage populations that express high levels of vascular endothelial growth factor (vegf) using quantitative real time PCR (qPCR). The cell dissociation and FACS sorting techniques have been optimised for immune cells and successfully used to isolate other fluorescently marked populations within the wound such as neutrophils and endothelial cells. More broadly, this protocol can be easily adapted to other contexts where identification of pro-angiogenic immune cells is transformative for understanding, from development to pathologies such as infection, cancer and diabetes.

Key words: Zebrafish, wounding, VEGF, immune cells, macrophages, FACS, quantitative PCR

Running title: FACS sorting pro-inflammatory, VEGF expressing macrophages

1 – Introduction

Wound repair and regeneration is a complex and highly integrated process, dependent on interactions and collaborations between many cell types in order to restore damaged tissue [1]. Innate immune cells play significant roles throughout wound healing, and macrophages in

particular are vital for functions such as protecting tissue against infection, controlling the tissue inflammatory status, and co-ordinating the activity of other cell types such as endothelial cells to drive re-vascularisation [2, 3]. To facilitate this broad spectrum of functions, macrophages maintain plasticity that allows them to select and switch between differing phenotypic 'states' throughout tissue repair [4, 5]. Isolation of macrophages at specific timepoints during the wound healing process represents a powerful approach to identify the expression profile and changing functionality of these phenotypic states, to better understand how these cells interact with the surrounding tissue during repair.

The zebrafish is a tractable and highly versatile vertebrate model of tissue repair, possessing essentially all components of mammalian tissue but with the added advantage of being translucent and genetically tractable [6, 7]. This combination of strengths makes the zebrafish ideal for elucidating mechanistic insight by non-invasively observing tissue repair processes such as inflammation and angiogenesis. Recently developed transgenic reporter lines allow for the visualisation of macrophages [8-10] and their identification as pro- or anti-inflammatory cells [11-13]. These transgenic lines provide the key tools necessary for isolating macrophages based on fluorescence profile to unravel how their function changes between phenotypic states and throughout tissue repair, particularly with regards to the control over angiogenesis.

In this chapter, we provide a detailed description of how to induce wounds in zebrafish double transgenic Tg(*mpeg1*:mCherry); TgBAC(*tnfa*:GFP) larvae [9, 12], which mark macrophages in red and pro-inflammatory cells in green. Next, we describe how to dissociate larval tissue in a manner optimised for immune cells, and how to best FACS sort fluorescently labelled pro- and anti-inflammatory macrophage phenotypes from the resultant single cell suspension. We also explain how to use these cells for RNA extraction and qPCR, with the specific example of identifying which macrophage phenotype corresponds to the Vegf-expressing, pro-angiogenic population. The protocol described here can be utilised for numerous other contexts where identification of pro- and anti-angiogenic immune cell populations is vital, such as zebrafish models of infectious disease (reviewed [14]), cancer (reviewed [15]) and pathologies of impaired healing such as diabetes (reviewed [16]). With this protocol, we have also succeeded in isolating neutrophils and endothelial cells from cell sorting of dissected wounds taken from respectively Tg(*mpx*:GFP) [17] and Tg(*fli1*:GFP) [18] transgenic larval fish, which respectively label neutrophils or endothelial cells in green (data

not shown). We subsequently used the qPCR approaches described here on these cells, providing valuable mechanistic insight into how macrophages, neutrophils and endothelial cells interact with each other during tissue repair [19]: however, numerous other downstream applications can be performed on these purified cell populations e.g. RNAseq analyses [20] and proteomics [21].

2 – Materials

2.1 Needle stab injury

1. Wild type larval fish (control), as well as larvae from the transgenic reporter line Tg(*mpeg1*:mCherry); TgBAC(*tnfa*:GFP), 4 days post fertilization (dpf) (see **Note 1**).
2. Incubator set to 28.5 °C.
3. Stereomicroscope.
4. Microscope slide.
5. 30-gauge needles
6. Plastic Pasteur pipettes.
7. 90 mm plastic petri dishes.
8. 0.4 % 3-amino benzoic acidethylester (tricaine) stock solution. Dissolve 400mg tricaine with 97.9 mL ddH_2O. Adjust to pH 7.2-7.4 with Tris-HCL (pH9). Store solution at 4 °C.
9. E3 buffer (embryo water). Prepare a 100 X stock in advance. 14.61 g NaCl, 0.63 g KCl, 2.43 g mM $CaCl_2$ and 1.99 g $MgSO_4$, mix well in 1 L of deionized water. Can be stored at room temperature. Prepare 1 X working solution prior to fish breeding and egg storage.

2.2 Cell Dissociation and FACS

1. Dissociation solution. Prepare a stock solution of 1 mg/mL of Collagenase type II, dissolve in ddH_2O and store at -20 °C. Immediately prior to use, thaw collagenase stock and prepare

(20 mg/mL) Collagenase solution in 0.05 % Trypsin-EDTA (also stored at -20 °C) (see **Note 2**).

2. Stop/resuspension solution. Prepare Hank's balanced Salt solution (HBSS) (Ca^{2+}, Mg^{2+} free), supplemented with 2.5 % fetal bovine serum (FBS). Store solution at 4 °C.
3. Scalpel blades.
4. Fine tipped forceps.
5. Microcentrifuge tubes, 1.5 mL.
6. Refrigerated tabletop microcentrifuge.
7. Water bath set to 32 °C.
8. Pipettes and filtered tips (P1000, P200, P10).
9. Ice.
10. Sterile disposable 40 μm cell strainers (adaptable to 50 mL Falcon tubes).
11. 50 mL Falcon tubes.
12. Becton Dickinson InFlux cell sorter.
13. Sterile Falcon 5 mL Polypropylene test tubes (Catalogue number 352063) for quality control of particles and for zebrafish samples.
14. Spherotech Calibration 8 peak beads (3-3.4 um) for laser alignment and QC (Catalogue number RCP-30-5A 3).
15. BD Biosciences Accudrop fluorescent beads 6 (μm diameter) for sort drop delay calculation (Catalogue number 345249)
16. Cell viability dye, e.g. Propidium Iodide (PI). Prepare 400 X stock of PI using Sigma Aldrich 1.0 mg/mL in H_2O, and use at 2.5 ug/mL final concentration (see **Note 3**).
17. Milty Zerostat anti-static remover Gun (see **Note 4**).

2.3 RNA/cDNA prep and qPCR

1. RNase-free workstation.
2. RNase-eliminating solution (such as RNaseZAP).
3. RNase-free filtered pipette tips and microcentrifuge tubes.
4. RNeasy Micro kit (Qiagen), with DNase I.

5. 100 % ethanol, ACS grade.
6. Nuclease- and DNA-free water.
7. Refrigerated microcentrifuge capable of at least 10,000 x g.
8. Ice.
9. Nanodrop ND-1000 spectrophotometer (Thermo Scientific) to assess RNA concentration and purity.
10. cDNA Synthesis Kit (e.g. Thermo Fisher Maxima First Strand cDNA Synthesis Kit).
11. PCR and qPCR machine (e.g. Agilent MX3005P QPCR cycler).
12. qPCR Primers with a Tm of 56 °C (see **Table 1**).
13. Optical 96-well reaction plates with transparent sealing.
14. Real-time PCR kit (e.g. Qiagen QuantiTect SYBR Green PCR kit).
15. 1 % agarose gel (stained with ethidium bromide at 0.5 μg/mL).

3 – Methods

3.1 Needle stab injury

1. Store eggs and larvae in 1 X working solution of embryo water. At 4dpf, remove larvae from the incubator and anaesthetise by adding tricaine solution (approx. 1 mL per 30 mL embryo water, see **Note 5**).
2. Transfer individual larvae in a drop of liquid onto a microscope slide under a stereomicroscope using a plastic Pasteur pipette (see **Note 6**).
3. Manoeuvre larvae into appropriate lateral lying position and remove excess liquid using the plastic pipette (see **Note 7**).
4. Perform a needle stick injury into the dorsal somites opposite the cloaca using the 30-gauge needle held at a 75° angle (see **Note 8** and **Figure 1**).
5. Immediately transfer the injured larvae into a petri dish containing clean embryo water. Once injured larvae are

observed to be moving, place back into incubator to fully recover (see **Note 9**).

3.2 Cell Dissociation

1. Remove appropriately staged injured larvae from the incubator and anaesthetise by adding tricaine solution (approx. 1 mL per 15 mL embryo water).
2. Transfer anaesthetised larvae in a drop of the tricaine containing solution onto a microscope slide under a stereomicroscope using a plastic Pasteur pipette. Load up to 10 larvae in this manner.
3. Make two incisions on the trunk of each larval using a scalpel blade, dissecting the wound away from the head and tail (see **Note 10**).
4. Transfer all wounded tissue of interest into a 1.5 mL microcentrifuge tube containing 300 µL of chilled dissociation solution using fine tipped forceps. Samples should be kept chilled on ice until tissue collection is completed (normally 50 wounds per sample).
5. Incubate in a water bath at 32 °C for approx. 20 minutes, gently pipetting up and down every 3-5 minutes using a P200 pipette to promote tissue disruption and dissociation.
6. While incubation is in progress, chill resuspension solution on ice. Add 4 mL of resuspension solution to a 50 mL Falcon tube.
7. Once cells are fully dissociated, stop the reaction by adding dissociated cells to the 50 mL Falcon tube containing 4 mL of resuspension solution. Rinse the 1.5 mL microcentrifuge tube with another 1 mL of chilled resuspension solution and reunite with the remainder of cells.
8. Gently pipette the homogenised material 15-20 times against the bottom of the 50 mL Falcon tube to minimise cell clumping.

9. Centrifuge the dissociated cells at 300 x g for 10 minutes at 4 °C.
10. Discard the supernatant and resuspend the pelleted cells in 4 mL resuspension solution, on ice.
11. Pass the 4 mL of resuspended cells through a 40μm cell strainer placed into a clean 50 mL Falcon tube. Rinse the first 50 mL Falcon tube with another 1 mL of chilled resuspension solution and add this to the strainer.
12. Centrifuge the dissociated cells at 300 x g for 10 minutes at 4 °C.
13. Discard the majority of the supernatant, leaving approx. 500μl of resuspension solution.
14. Resuspend cells in the remaining 500μl of resuspension solution, resulting in a maximum concentration of 5-10 million cells/mL.

3.3 FACS sorting

1. Prepare cell sorter as per manufacturers' guidance (see **Note 11**).
2. Once the instrument has been calibrated, proceed to cell sorting, set up to FACS sort samples at 4 °C using a 100 μm nozzle at 21psi.
3. Add viability dye (e.g. Propidium Iodide in a 1:400 dilution) to the cell suspension to identify the live cell population.
4. Determine optimal excitation voltages and gating strategy using non-fluorescent ("no stain") (see **Note 12)** and single fluorophore controls (see **Note 13)**.
5. Use the above gating strategy to define the double positive population (see **Figure 2)**.

~~4.~~6. Collect single positive and double positive populations directly into 500 μL lysis buffer (RLT buffer from RNeasy Micro kit) in 1.5 mL eppendorf tubes.

3.4 RNA and cDNA preparation

1. For RNA extraction, use RNeasy Micro kit and proceed according to manufacturer's protocol (see **Note 14**).
2. During the procedure, perform on-column treatment with DNase I provided in the RNeasy Micro kit to remove DNA contaminants.
3. At the end of the procedure, elute RNA extracted from sorted fluorescent cells in 10 µL of RNase-free water. Transfer to a fresh Eppendorf tube and store at -80 °C.
4. Prior to cDNA synthesis, measure RNA quality and quantity using a Nanodrop spectrophotometer. Normalise all samples to the same concentration by diluting with RNase-free water.
5. Using equal concentrations of each RNA sample, synthesise cDNA using the Maxima First Strand cDNA Synthesis Kit according to manufacturer's protocol (see **Notes 15 and 16**).
6. Store synthesised cDNA (final volume of 20 µL) at -80 °C.

3.5 qPCR

1. Dilute each cDNA sample 1:10 in nuclease-free water to obtain the final concentration used in the qRT-PCR reaction (see **Note 17**).
2. Prepare the SYBR Green mix for each condition (target and control samples at each of the different time points). For each condition, prepare three replicates for each cDNA sample, together with data normalisation using expression of a reference gene (see **Note 18**). Calculate 7.5 µL SYBR Green master mix (2x), 1.5 µL primer mix (100 nM final concentration for forward and reverse primers) and 4µl nuclease-free water per well (see **Note 19**). Prepare the SYBR Green master mix in an Eppendorf tube for the number of

wells required, plus an excess (two extra volumes) to allow for pipetting error.

3. Put 13µL of SYBR Green mix in each well and then add 2 µL of cDNA.
4. Perform real-time PCR using the following qPCR program:
   (a) 95 °C for 15 minutes (initial denaturation).
   (b) 94 °C for 15 seconds; 56 °C for 30 seconds; 72 °C for 30 seconds → 40 cycles.
   (c) Perform dissociation (melting curves) analysis using a final step of 95 °C for 30 seconds; 56 °C for 30 seconds; followed by gradual temperature increases to 95 °C (approximately 10 minutes)
   (d) 4 °C, hold.
5. Analyze the qPCR results (see **Notes 20 and 21**).
6. Run qPCR products on a 1 % agarose gel (stained with ethidium bromide) to verify quality and specificity of the qPCR reactions (see **Figure 2I)**.

4 - Notes

1. There are numerous published zebrafish transgenic reporter lines that mark macrophages, such as *mpeg1* [9], *mfap4* [8] and *cfms* [10]. Furthermore, numerous transgenic reporter lines exist that mark pro-inflammatory cells, such as *il1β* [11] and *tnfα* [12]. In this protocol, we have chosen the *mpeg1* marker to identify wound macrophages, and overlaid the *tnfα* marker to help separate pro-inflammatory macrophages (which express both markers) from anti-inflammatory macrophages (which express *mpeg1* only). Other markers could be used to segregate macrophage phenotypes, including markers of anti-inflammatory macrophages such as *spp1* [13].
2. The composition of the dissociation solution is critical for maximising the efficiency of cell dissociation process and cell survival. Through testing numerous combinations of reagents

from different suppliers we have identified that Collagenase from Sigma (Catalogue number C8176) and Trypsin-EDTA from Thermo Fisher (Catalogue number MT25051CI) are the optimum reagents for wound tissue dissociation, especially for immune cells.

3. PI was used as a viability marker in these studies and dead cells were detected using the violet (405 nm) laser in the 610/20 BP detector (via the 600 nm LP filter). It is critical to use a viability dye to exclude dead or dying cells from the sorted populations and there are many choices, depending on the FACS configuration and the fluorescent markers used in the experiment. For FACS instruments without a violet laser, one could use DRAQ7 (Biostatus) as an alternative (detected with the red laser (e.g. 640 nm) through the 750 nm LP filter.
4. Using this static gun on collection tubes prior to sorting improves yields during sorting, as it minimises drops (containing cells) striking the tube wall instead of the reservoir of buffer.
5. This ratio of tricaine to embryo water has been optimised to anaesthetise larvae at 4dpf. Early larvae are less sensitive and may require more tricaine (approximately 1:20), while older larvae are more sensitive and require less (approximately 1:50). Assessment of heartbeat and blood flow is critical throughout the wounding procedure – if either of these processes stop, fish must be immediately recovered in fresh embryo water until heartbeat and blood flow is restored.
6. Have all equipment necessary for wounding ready and easily accessible by this step, to allow for wounding experiments to be performed as rapidly as possible while maintaining reproducibility of injuries.
7. Minimising the amount of water surrounding the larvae also minimises the 'bolus' within which the larvae may move or float in, making the stab procedure easier and more reproducible. However, it is important to maintain a thin layer

of liquid on the fish, as allowing the larvae to dry out will result in much higher mortality – if the larvae appears to be drying out, immediately transfer to fresh embryo water.

8. It is critical to avoid injury to the notochord, as this will affect survival of injured larvae. To maintain a consistent 2-somite block of injury, hold the bevel facing the fish and perform the stab as a single wound.
9. When recovering the wounded larvae into clean embryo water, make sure these larvae 'sink' to the bottom of the dish by gently pipetting embryo water onto them. Fish that remain floating at the water surface may dry out sufficiently to cause mortality. It should be noted that other injury approaches, such as wounding using tungsten needles [22], tail fin amputation [23], or injuries using a laser microablation system [24, 25] can also be used in this pipeline.
10. In order to harvest similar amounts of macrophages for control unwounded fish, perform a single cut as per wounded fish at the anterior most point (near the cloaca) and collect the entire tail region. Perform all downstream steps as per wounds, with an increase in agitation to assist in tissue dissociation.
11. For fluidics & laser stabilisation, choose appropriate sheath pressure and drop drive frequency, optimise & align lasers and calculate sort drop delay. Use Spherotech Rainbow Calibration 8 peak beads for laser alignment and QC, BD Biosciences Accudrop fluorescent beads for drop delay calculation. Optimise sort stream deflection for 1.5 mL Eppendorf tubes. Clean sample line with 70 % IMS (5 minute wash at high flow rate). Set chiller to 4 °C (sample port and collection tube holder). "Sterilise" the cell sort chamber w/ UV light treatment for 30 minutes. Sort mode set to 1.0 drop purity for these studies. Based on drop drive frequency: ensured that event rate was <9400 per sec. e.g. to aim for no more than 1 event/4 drops.

12. Load an unstained or negative control sample from WT larvae (harvested from 50 WT wounds) and acquire at low flow rate. Adjust the forward scatter (FSC) and side scatter (SSC) detector voltages (linear scaling) to place the cell populations on scale, as well the relevant fluorescence detectors (logarithmic scaling) to ensure visualisation of the background signals. In these studies, the detector sensitivities for GFP (488 nm 530/30 nm BP), mCherry (552 nm 610/20nm BP) and PI (405 nm 610/20 nm BP) were adjusted.
13. Acquire single fluorophore control samples (e.g. GFP only or mCherry only expressing cells) to set the sort gates for single versus double expressing cells.
14. To minimise the risk of RNA degradation by RNases, ensure all equipment used for RNA extraction is thoroughly cleaned with RNaseZap (or autoclaved). If possible, keep separate workstation and equipment for RNA work.
15. We used 50 ng of total RNA per reaction for cDNA synthesis, extracted from approximately 10000 cells per sample (therefore 200 cells are needed per ng of RNA).
16. Always aim to use the same quantity of RNA in all samples, to permit direct comparison of gene expression between samples. Otherwise, internal reference genes are required to allow for a comparison of 'relative' levels between samples. We used internal reference genes as an additional control measure for our analysis.
17. cDNA will need to be diluted according to the amount of starting material used. Set up a 1:10 serial dilution from neat cDNA to a 1:10000 dilution in nuclease-free water. Perform qPCR and select a dilution that ideally yields Ct values between 15 and 30 cycles.
18. For our reference gene, we used Elongation Factor 1α (see Table 1). Other genes that may be used for this purpose include the ribosomal protein 28S, Calnexin and Cyclophilin A

[26]. In addition, evidence suggests beta actin 2 (bactin2) is a suitable zebrafish housekeeping gene due to its stable, high level expression across a wide range of tissues [27]. It is also worth noting a recent study identified several new reference genes for qPCR that can be used across different tissue types, development stages and chemical treatments [28].

19. To maximise efficiency of the qPCR reaction, primers should be designed with a Tm of between 52 °C and 60 °C (we opted for 56 °C), and the PCR product should ideally be between 100 and 200 nucleotides in size.
20. Prior to quantifying gene expression levels, examine the dissociation curve analysis to ensure that there is only one PCR product peak per gene for all reactions. Several peaks indicate multiple PCR products and might suggest problems with sample purity, primer specificity or the formation of primer dimers.
21. qPCR data can be analysed by absolute or relative quantification strategies, as describe in detail elsewhere [29]. Briefly, we used the threshold cycle (Ct) values, to perform the comparative (ΔΔCt) method of qPCR analysis. Ct values from two different experimental samples are normalised to a housekeeping gene and then compared. For example, $\Delta\Delta Ct = (Ct_{,vegfaa} - Ct_{,ef1a})sorted - (Ct_{,vegfaa} - Ct_{,ef1a})unsorted$. This analysis results in the representation of data as fold change in gene of interest expression (*vegfaa*) in a target sample (sorted) relative to a reference sample (unsorted), normalised to a reference gene (e.g. *ef1a*).

Table 1

Gene name	**Forward primer 5'-3'**	**Reverse primer 5'-3'**
Elongation factor 1α (ef1α)	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC
Vascular endothelial growth factor aa (vegfaa)	AAAAGAGTGCGTGCAAGACC	AGCACCTCCATAGTGACGTT

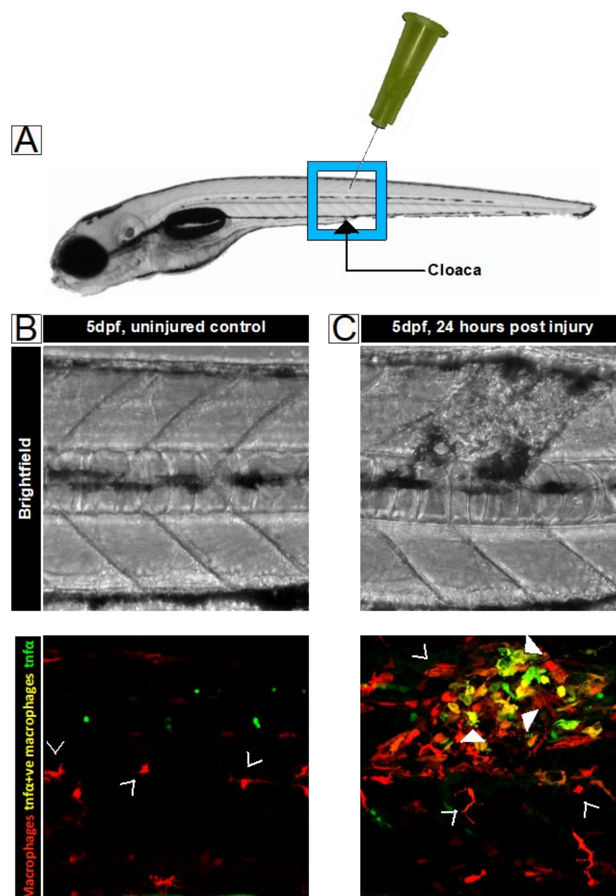

Figure 1: Macrophages expressing *mpeg1* (red), together with *tnfα*-expressing pro-inflammatory cells (green), accumulate at the sight of needle stab injury. (A) Schematic with boxed area showing location of needle stab injury at the dorsal somites above the cloaca. This area is subsequently excised to isolate the injury region for cell sorting. (B-C) Maximum intensity projection through a representative fluorescent Z-stack of a laterally mounted 5 dpf larvae, uninjured or at 1 day post injury, taken using a confocal microscope. An enrichment of macrophages is seen in the injury site compared to the ventral side of the fish, with some of these macrophages expressing the pro-inflammatory marker *tnfα* (yellow).

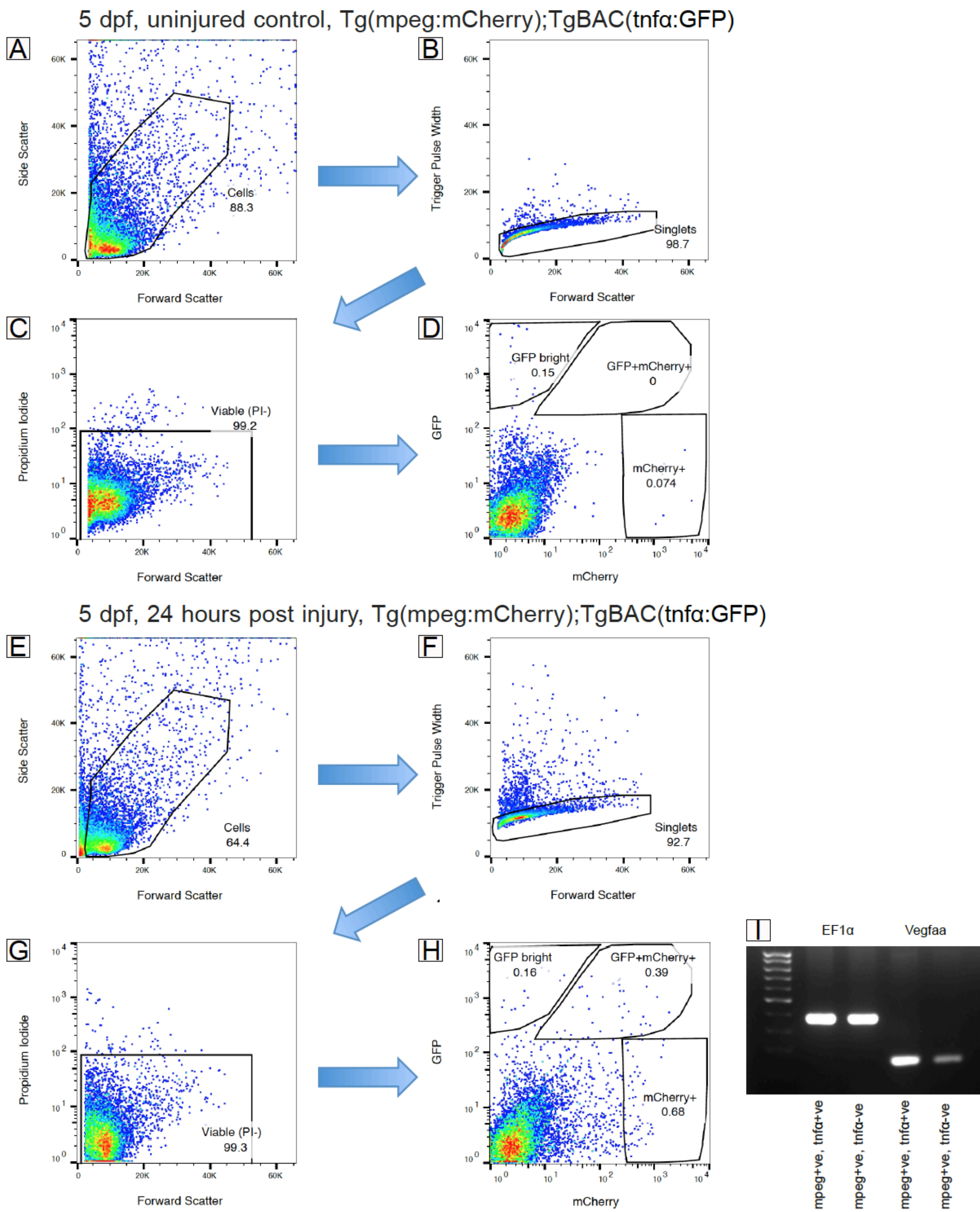

Figure 2: Fluorescence Activated Cell Sorting of tnfα+ve and tnfα-ve macrophages at 24 hours post needle stab injury. (A-D) Representative flow cytometry data showing gating strategy for sorting macrophages from unwounded control fish. (E-H) representative flow cytometry data showing gating strategy for sorting macrophages from wounded fish, with wounds harvest at

24 hours post injury. Cells are first gated for expected size (Forward and Side Scatter), singlets, and viability (Propridium Iodide). Live, single cells of expected size are subsequently gated on GFP and mCherry expression to identify *tnfα* and *mpeg* expression, respectively, to determine tnfα+ve and tnfα-ve macrophage populations. (I) Representative qRT-PCR showing equal levels of expression of housekeeping gene *ef1α* and differential levels of expression of *vegfaa* between tnfα+ve and tnfα-ve macrophage populations following 40 rounds of amplification.

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