Accepted Manuscript

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PII: S1050-4648(16)30576-9

DOI: 10.1016/j.fsi.2016.09.028

Reference: YFSIM 4191

To appear in: *Fish and Shellfish Immunology*

Received Date: 17 March 2016

Revised Date: 12 September 2016

Accepted Date: 13 September 2016

Please cite this article as: Masterman K-A, Barnes AC, A reliable method for enrichment of neutrophils from peripheral blood in barramundi (*Lates calcarifer*), *Fish and Shellfish Immunology* (2016), doi: 10.1016/j.fsi.2016.09.028.

A reliable method for enrichment of neutrophils from peripheral blood in barramundi (*Lates calcarifer*)

Short Communication

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Abstract

Neutrophils are a short-lived, terminally differentiated, innate immune cell, that are critical first responders during infection. Research into neutrophil-pathogen interactions in fish has primarily employed cells derived from the pro-nephros and nephros. Since these sites are also the location of neutrophil and other immune cell development, there may be some ambiguity in maturation and functional ability of these cells, and difficulty in differentiating the effects of neutrophils from those of macrophages and monocytes. In contrast, peripheral blood circulating neutrophils are mature and ready to respond, thus it may be more physiologically relevant to use these cells for immune studies when evaluating interactions with blood-borne pathogens. The enrichment of tropical, euryhaline fish blood cells cannot follow classic mammalian enrichment methods for several reasons: Fish have nucleated red blood cells (RBC's), a high number of reticulocytes, a very low number of granulocytic leukocytes and an osmotic tolerance, rendering techniques such as water lysis ineffective. Enrichment of neutrophils while minimizing RBC contamination is imperative for studies where luminescence or fluorescence signals may be confounded by background from an overabundance of RBC's. We have optimized a method for enriching neutrophils from peripheral blood with an initial settlement step employing 6% dextran (Mr 450,000-650,000) for 30-60 min at room temperature, followed by density separation on an 8-step Percoll density gradient. This method provides a cell suspension comprising 20-50% neutrophils, free of contamination from reticulocytes. These are then suitable for luminometric or fluorometric downstream analyses.

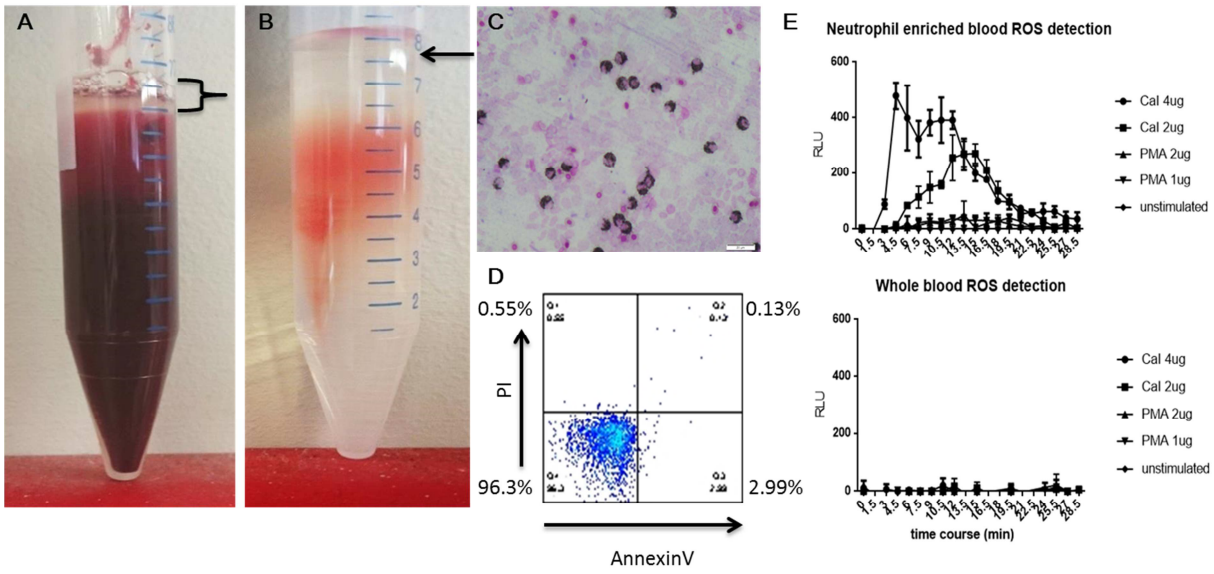
Neutrophils are a key early responder innate immune cell involved in detection and clearance of microbes. Isolation and/or enrichment of neutrophils can be problematic due to manipulation sensitivity and a short lifespan (Sepulcre *et al.*, 2011). In teleosts, the head kidney functions as the mammalian bone marrow equivalent, seeding granulocytic precursors which develop into neutrophils. These cells mature in the trunk kidney before moving into peripheral blood circulation for immune surveillance (Fijan, 2002). Upon microbial encounter, neutrophils phagocytose the foreign entity, secrete exogenous antimicrobial agents or cast neutrophil extracellular traps (NETs) (Brinkmann *et al.*, 2004,Finco-Kent *et al.*, 1987, Palic *et al.*, 2005). Assessment of blood neutrophils has proven difficult due to the low number of circulating cells (less than 5% in teleost) (Havixbeck *et al.*, 2016) and interference by red blood cells (RBC). Immature RBC, known as reticulocytes, may occur in a range of percentages in different teleost species (Catton, 1951), and have a range of densities that overlap with neutrophil density. This reduces the effectiveness of a routine Percoll density gradient separation, so alternative measures were sought to remove RBC, and particularly reticulocytes, from blood neutrophil preparations. Lysis of red blood cells using ammonium citrate buffer was ineffective since fish blood cells are nucleated. Fresh water osmotic lysis was also unsuccessful as barramundi are euryhaline and can tolerate a broad range of salinities. We have developed a method to enrich neutrophils from barramundi (*Lates calcarifer*) blood, utilising dextran settlement prior to Percoll density gradient separation to enrich neutrophils.

Barramundi juveniles (40 – 100 mm fork length) were obtained from a local commercial hatchery and kept in aquaria with recirculated brackish water (15 ppt) at 28 +/- 2 °C. Water quality was checked daily for ammonia, nitrite, nitrate and pH, and water exchanges (~5-10% v/v) were done regularly as required. Fish were fed at a maintenance rate (approx. 5% body weight daily) twice per day, with a commercial diet for barramundi (Ridley Aqua Feed). Prior to bleeding, fish were anaesthetised with a lethal overdose of Aqui-S (Lower Hutt, New Zealand). Blood was collected via caudal venipuncture, with a heparinised 23G needle and immediately mixed with 10% final volume citrate-phosphate-dextrose anticoagulant. This mixture was added to an equivalent volume of 6% dextran (M_r 450,000-650,000 from *Leuconostoc spp*. Sigma-Aldrich, Castle Hill, Australia) in 0.9% NaCl, 0.22µM sterile filtered. The tube was inverted 3-5 times before being allowed to settle

undisturbed at room temperature (RT) for 30-60 min. Once settled, the tube was centrifuged at 10 x *g* (at RT) for a further 30 min with no brake and acceleration set to the minimum in an Eppendorf 5810R centrifuge. The upper opaque layer (Fig.1A) was removed via wide bore pipette and placed on top of a freshly prepared 8 step Percoll (GE Healthcare, Parramatta, Australia) gradient, of densities 1.092, 1.089, 1.086, 1.083, 1.08, 1.75, 1.07, 1.06 g/ml. The Percoll gradient was centrifuged at RT, 400 x *g* for 30 min, with no brake and minimum acceleration (Fig.1B). The uppermost buffy layer was collected and washed once with phenol-red-free RPMI-1640 (Sigma-Aldrich, Castle Hill, Australia). Cell counts and cytospins (Tharmac Cellspin, POCD, Artarmon, Australia) were prepared to assess yield and enrichment of neutrophils. Standard differential cell counts from blood smears from 4 fish were also performed by Brisbane Bird and Exotics Veterinary Service to enable comparison with cell counts post-neutrophil enrichment (Table 1).

Table 1. Differential cell counts derived from blood smears taken from healthy *Lates calcarifer* reared in brackish (15 ppt salt) recirculating water. Four fish were sampled. For comparison, ranges of white blood cell % (WBC) and post dextran-Percoll neutrophil enrichment are included, 100 field views were surveyed via light microscopy at 40 x magnification.

	Whole Blood	Post-Enrichment
packed RBC	30 - 43 %	n/a
reticulocytes	3.6 - 10 %	n/a
white cells	3.4 - 7.8 %	> 90%
lymphocytes (% WBC)	64 - 92 %	35 - 70%
monocytes/ macrophage (% WBC)	4 - 16 %	5 - 25%
neutrophils (% WBC)	6 - 36 %	25 - 50%



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79 **Fig.1**. Peripheral blood neutrophil enrichment from barramundi. A) Barramundi blood mixed 1:1 with 6%
80 dextran, 30 min gravity settlement followed by 30 min centrifuge at 10 x *g*, bracket shows opaque layer
81 collected for layering onto Percoll density gradient. B) Subsequent Percoll gradient, arrow indicates location of
82 buffy layer where neutrophils reside. C) Enriched neutrophils from blood stained with peroxidase (brown) and
83 hemacolor at 40x. Micrographs were taken using an Olympus BX41 epifluorescent microscope, images
84 captured with an Olympus DP26/U-CMAD3 camera. D) Flow cytometric assessment of neutrophil viability and
85 apoptosis post-enrichment. E) Reactive oxygen species (ROS) luminol assay demonstrating respiratory burst
86 activity of enriched neutrophil preparation (E top) and whole unprocessed blood (E bottom).

87 Large polymer solutions, such as high molecular weight dextran (≥40 kDa), can be
88 used to bind RBC in a reversible process to make large linear or branched aggregates (Neu *et*
89 *al.*, 2008). These complexes can be allowed to settle via gravity or gentle centrifugation
90 leaving the leucocytes at the top. The leucocytes were further enriched via Percoll, a
91 colloidal silica solution, using gradients of differing concentrations of Percoll prepared in
92 Hank's Balanced Salt Solution (HBSS with calcium and magnesium, no phenol red, Sigma-
93 Aldrich, Castle Hill, Australia) and ultrapure water (Sigma-Aldrich, Castle Hill, Australia). The
94 resultant cellular enrichment gave a yield of 2-3 x 10^6 cell per ml of whole blood and the
95 neutrophil percentage of this cell preparation ranged from 25-50% as assessed via 3,3'-
96 diaminobenzidine peroxidase (DAB, Sigma-Aldrich, Castle Hill, Australia) and Hemacolor®
97 (HC) staining (Merck Millipore, Bayswater, Australia) of cytospin slides. Neutrophils were
98 identified via brown peroxidase staining, only expressed in neutrophils and monocytes, and
99 via HC staining in which neutrophils were identified with a multi-lobed horseshoe-shaped

nucleus (Fig. 1C and 1D). Optimisation experiments to reduce the number of layers in the multilayer Percoll gradient determined that enrichment was reduced, or contamination with other cell types increased, if any of the 8 density layers were omitted

Since *in vitro* manipulation of neutrophils can cause activation and apoptosis, neutrophil respiratory burst activity was assessed via luminol assay to confirm cell functionality, as adapted from a human blood luminol assay (Carulli *et al.*, 1995). In brief, enriched blood neutrophil cell preparations were resuspended to $5x10^6$ cells per ml in RPMI and seeded into a flat bottom black 96-well plate (Greiner, Germany) at 100 µl per well. Stimulatory agents were diluted and mixed with 10 mM luminol (Sigma-Aldrich, Castle Hill, Australia) in 20 M borate buffer, at pH9, and 100µL added to cell wells including negative and unstimulated control wells. The luminescent signal was immediately read every 30 s for 30 min using a BMG FLUOstar (BMG Labtech, Ortenberg, Germany). Units are expressed as relative luminescent unit and wells were set up in triplicate. Whole blood diluted 1:4 in RPMI assessed for luminol based ROS activity consistently gave little to no signal (Fig. 1E), luminol possibly being absorbed by RBC's rather than oxidised via ROS to produce chemiluminescence. Additionally, in order to determine whether the multi-step enrichment procedure was killing neutrophils, viability was assessed during each cell count via trypan blue exclusion and confirmed on several occasions via live/ dead cell kit based on uptake of annexinV and propidium iodide, which was detected using flow cytometry. In every case, viability was always greater than 90% (Fig. 1D).

In conclusion, these combined methods allow a reproducible enrichment of mature, circulating neutrophils from teleost blood. These may be used for further immunological assessment as evidenced by respiratory burst in response to positive controls. This enrichment process will substantially assist with assay design and development specifically for mature neutrophils, and progress understanding of the vital first responder role neutrophils play in pathogenic clearance. This should prove advantageous compared to evaluation of function of potentially immature cells derived from haematopoietic tissues such as head-kidney. Indeed, peripheral neutrophils in goldfish (*Carassius auratus*) seem to be deployed from the head-kidney reserve during inflammatory challenge (Havixbeck *et al.*, 2016). The rapid extravasation of neutrophils supports the theory that neutrophils play a

vital role in the early regulation of the innate response. In murine systems, myeloperoxidase secreted by stimulated neutrophils has been shown to enhance both phagocytosis and killing of microorganisms (Lincoln *et al.*, 1995) and trigger cytokine release, which in turn activate macrophages (Wei *et al.*, 1986). Studies of these regulatory functions by mature circulating neutrophils necessitate enrichment procedures to enhance numbers of quiescent cells for *in vitro* activation.

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Ms. Ref. No.: FSIM-D-16-00192

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Highlights:

- Dextran effectively separates reticulocytes and red blood cells from neutrophils in peripheral blood.
- Subsequent density separation on Percoll enables reproducible functional assays of peripheral blood neutrophils
- Functional assessment of respiratory burst via stimulated neutrophils requires removal of red blood cells