Supporting Information

**Microfluidic Label-free Bioprocessing of Human Reticulocytes from Erythroid Culture**

Author(s), and Corresponding Author(s)*: Kerwin K Zeming¹,², Yuko Sato²,³, Lu Yin³,², Nai-Jia Huang⁴, Lan Hiong Wong⁵, Hooi Linn Loo⁵, Ying Bena Lim⁶, Chwee Teck Lim⁶,⁷, Jianzhu Chen⁸,⁹, Peter R Preiser⁵,¹⁰, and Jongyoon Han¹,¹¹,¹²,*

Figure S1. Synchronous differentiation of the *in vitro* erythroid culture from adult CD34⁺ HSPCs.
Figure S2. Expelled nuclei interfere with Plasmodium invasion.
Figure S3. Syto 16 and Sytox Red staining to distinguish reticulocytes, nuclei, erythroblasts and dead cells.
Figure S4. DFF microfluidic method results in substantial enrichment of reticulocytes.
Figure S5. Evaluation on the performance of DFF-based purification of reticulocytes in continuous perfusion culture system.
Figure S6. DLD device specifications and design for both circle and inverse-L pillars.
Figure S7. Reticulocyte and nuclei size and shape and Comsol simulated fluid streamlines for inverse-L DLD structure.
Figure S8. The FACS plots of the various cells from the four DLD outlets of inverse-L pillar.
Figure S9. Schematics and design of DLD device used for sorting of HSC erythroid cultures.
Figure S10. Composition of cell types collected from each outlets of DLD device at a lower cell concentration.
Figure S11. Comparison of different stages of reticulocytes (I-V) of FACS sorted and DLD purified samples.
Figure S12. Super imposed frame grabs from high-speed video of Nuc-Ret tethered cell.
Figure S13. Verification of *P. falciparum* invasion by CellTrace labeling.

Movie S1. Video of DFF sorting of reticulocytes, nuclei and erythroblasts.

Movie S2. Three videos showing the sorting mechanism for reticulocytes, nuclei and erythroblasts for DLD inverse-L shape structure within the region of Zone 1.

Movie S3. A 60x objective magnification video of DLD Zone 1 sorting with 400 million cells per mL concentration.

Movie S4. Video of a reticulocyte tethered onto a nucleus as it flow within the inverse-L DLD pillar array.
Figure S1

Synchronous differentiation of the in vitro erythroid culture from adult CD34⁺ HSPCs. a) Schematic of the 6 phases in vitro erythroid culture. b) Average cell number of erythroid culture starting from 1 million CD34⁺ HSPCs (n=4; ± SD). c) Percentage of cell recovery after thawing the cryo-preserved Day 12 erythroid cells (n=11; ± SD). d) Average cell number of erythroid culture. (n=11; ± SD). e) The representative flow cytometry plots from the left panels showing the granularity (SSC) and cell size (FSC), followed by histograms of CD49d, CD36, CD71, CD235α and CD34 surface expressions. Day of the cell culture is indicated on the left side of the columns (Day 5 from the top rows to Day 23). f-k) Wright-Giemsa stain of in vitro erythroid culture. Scale bar, 20 μm. f) Day 5, g) Day 9, h) Day 12, i) Day 16, j) Day 20 and k) Day 23 of erythroid culture with asterisks indicating expelled nuclei.
Figure S2

Expelled nuclei interfere with *Plasmodium* infection. a) Time course of *P. falciparum* infection using RBCs (dotted grey line) or crude erythroid culture (red line), monitored by microscopic examination of Giemsa stained culture smear (n=3; ± SD). b-c) Giemsa stain of *P. falciparum* culture. Asterisks indicate expelled nuclei. Arrows indicate *P. falciparum* parasites. Scale bar, 10 μm. b) Day 1 p.i. with ring-stage parasites. c) Day 6 p.i. with early schizonts.
Figure S3

Syto 16 and Sytox Red staining to distinguish reticulocytes, nuclei, erythroblasts and dead cells. a and b) Doublet exclusion gating to examine the single cells. c) Debris was excluded from the analysis by gating on cell populations. d) Reticulocytes are distinguished from erythroblasts and free nuclei by Syto 16 staining. Dead cell population is identified by Sytox Red staining. 3) The erythroblasts and free nuclei are further distinguished by forward (FSC) and side (SSC) scatter.
Figure S4
DFF microfluidic method results in substantial enrichment of reticulocytes. Top panels are representative FACS plots for Syto 16 and Sytox Red staining to verify the reticulocyte purity of pre-sort, after FACS sorting, and cells collected from collection and waste outlets by DFF sorting. Bottom panels are FACS plots of nuclei and erythroblasts gating. The reticulocyte purity (percentage) for each category is shown below in maroon. The total percentages of nuclei (blue) and erythroblasts (black) are indicated.
Figure S5
Evaluation on the performance of DFF-based purification of reticulocytes in continuous perfusion culture system. a) Percentage of reticulocytes (red), nuclei (blue) and erythroblasts (green) from the pre-sort, purified and waste populations. Results represent mean values (± SD) from three independent experiments (n=3). b) Absolute number of reticulocytes and recovery percentages from outer (purified) and inner (waste) outlets are counted after 2 hours of DFF microfluidic sorting (n=3; bars represent mean, and error bars represent SD).
Figure S6
DLD device specifications and design for both circle and inverse-L pillars. a) CAD drawing of the DLD device with 3 inlets and 4 outlets. The red boxes indicate b) the inlet region, c) the outlet region and d) DLD zones. d) Design specifications of DLD zones where each zone comprises of 6 DLD segments. Each segment consist of N rows of pillars for which complete deflection of particles will have a net lateral displacement of $\lambda$. Each Zone will determine the cut-off size to be deflected within DLD. The three sorting zones namely zone 1, 2 and 3 have a $D_c$ of 2.4, 3.8 and 5.2 µm sorting specifications. With three DLD zones, 4 DLD sorting outlets can be achieved for particles <2.4 µm, between 2.4 and 3.8 µm, between 3.8 and 5.2 µm and particles larger than 5.2 µm.
Figure S7. Reticulocyte and nuclei size and shape and Comsol simulated fluid streamlines for inverse-L DLD structure. Particle capture at the protruded end of the inverse-L DLD structure shown for (a) and (b) with vertical and horizontal cell dimensions. (c) shows the corresponding simulated fluid streamlines.
Figure S8
The FACS plot of the various cells from the four DLD outlets of inverse-L pillar. DLD microfluidic method results in 97.4% reticulocyte purity. Top panels are representative FACS plots for Syto 16 and Sytox Red staining of cells collected from outlet 1 (red, termed collection) and waste outlets 2-4. Bottom panels show nuclei and erythroblasts populations. The reticulocyte purity (percentage) for each outlet is shown below in maroon. The total percentages of nuclei (blue) and erythroblasts (black) are indicated.
**Figure S9**

**Schematics and design of DLD devices used for comparison sorting of erythroid cultures.**
a) The input sample is sandwiched between two buffer inlet sheath flow as it enters the DLD sorting zones. Microscopic images of b) Device 1 with conventional circle pillars and c) Device 2 with novel inverse-L shape pillars. Scale bars, 15 µm. d) The output region consists of a distribution of sorted particles in a range of 24 sub-channels. Every 6 sub-channels correspond to a sorting output. Scale bar, 100 µm. a) and d) are image overlay of particles from a high-speed video capture with false color added.
Figure S10
Composition of cell types collected from each outlets of DLD device at a lower cell concentration. a) Representative FACS plots for the cells collected from outlets 1-4 using DLD inverse-L pillar structure. Sample concentration of 50 million cells per mL was sorted. Samples are plotted against Syto 16 and Sytox Red. b) Distribution of Nuc and Eb from the FSC and SSC plots. c) Percentage of cell types obtained by the FACS analysis of Syto 16 and Sytox Red stained samples. d) Hoechst staining cells collected from outlets 1-4. Unstained cells are mainly reticulocytes, while stained cells (blue) are Nuc and Eb. Scale bar, 100 µm.
Figure S11
Comparison of different stages of reticulocytes (I-V) of FACS sorted and DLD purified samples. Using new methylene blue stain, different stages of reticulocytes were counted for FACS sorted and DLD sorted samples.
Figure S12
Super imposed frame grabs from high-speed video of Nuc-Ret tethered cell. The screen grabs show a cell in the process of enucleation where the nucleus is still tethered to the reticulocyte. Due the fluid flow, the nucleus and reticulocyte oscillate and flips between Inverse-L pillars. Scale bar is 30 µm.
**Figure S13**

**Verification of *P. falciparum* invasion by CellTrace labeling.** Representative FACS plots to verify the labeling of FACS purified reticulocytes (RET) and RBCs by a) 2.5 μM CellTrace CFSE (top left) or b) RBCs by 0.5 μM CellTrace Far Red (bottom left). FACS plots on the right are unstained control of RET and RBCs to check the background/autofluorescence. c) Immunofluorescence micrographs of infected RET (top rows), infected RBCs (middle rows) and residual parasite from purified schizonts inoculation (bottom rows). RBCs was pre-labeled with 0.5 mM of CellTrace Far Red and RET with 2.5 mM of CellTrace CFSE. 1.0%-purified schizonts were inoculated to pre-labeled host cells. Samples were stained against Hoechst and PfEXP2.1 antibodies (with Alexa 568 secondary antibodies). All the fluorescence images were merged with DIC images (right). Scale bars, 5 μm.
**Movie S1 (additional file).** Video of DFF sorting of reticulocytes, nucleus and erythroblast.

**Movie S2 (additional file).** Three videos showing the sorting mechanism for reticulocytes, nuclei and erythroblasts for DLD inverse-L shape structure within the region of Zone 1.

**Movie S3 (additional file).** A 60x objective magnification video of DLD Zone 1 sorting with 400 million cells per mL concentration.

**Movie S4 (additional file).** Video of a reticulocyte tethered onto a nucleus as it flow within the inverse-L DLD pillar array.