Supporting Information

Enrichment of Reticulocytes from Whole Blood using Aqueous Multiphase Systems of Polymers

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Additional Information on Materials and Methods

Formation and Analysis of AMPSs. The system C1 was a solution containing 12% (w/v) Ficoll (MW = 400 kDa), 12% (w/v) dextran (MW = 500 kDa), 5 mM disodium ethylenediaminetetraacetic acid (EDTA), 5 mM of sodium phosphate monobasic, and 5 mM sodium phosphate dibasic. A 100-mL solution of C1 was created using the following procedure: in a 100-mL volumetric flask, we added 1) 12 grams of Ficoll and 12 grams of dextran 2) 1 mL of a 0.5 M solution of EDTA, 3) 10 mL of a solution that contained 50 mM each of sodium phosphate monobasic and sodium phosophate dibasic, 4) 490 mg of solid NaCl, and 5) deionized water (MilliQ) sufficient to bring the final dissolved volume of the solution to 100 mL. To adjust the pH of the C1 solution to 7.40 ± 0.02, we mixed the solution well with a magnetic stirrer and measured the pH (Orion Star, Thermo Scientific). We adjusted the pH by titrating with concentrated (1-10 M) NaOH and HCl. After adjusting pH, we adjusted the osmolality. We removed a small aliquot (100 μL) of the mixture and centrifuged it for 10 minutes at 2000 *g* to separate phases. We then removed 10 μL of the top phase and measured the osmolality by vapor pressure osmometry (Vapro 5600, Wescor). To increase the osmolality, we added solid NaCl (1 M of NaCl ~ 2 Osm/kg) and mixed it in the solution until dissolved to reach the desired level (330 ± 10 mOsm/kg for C1). If the osmolality was modified, we measured and adjusted the pH again. After these steps, we transferred 4 mL of solution into 15-mL polycarbonate conical tubes, and centrifuged them for 20 minutes at 2000 *g*. We then separated the phases using a pipette to remove the top phase and a syringe to puncture the bottom of the tube and drain the bottom phase. We then characterized the pH, osmolality, and density of each phase. An oscillating U-tube densitometry (Anton Paar DM35N) measured the density of each phase.

For initial screening of different AMPS, we followed the steps above to prepare stock solutions of AMPS at concentrations higher (15% (w/v) of each polymer) and lower (5% (w/v) of each polymer) than those used in applications of aqueous multiphase systems (AMPSs). We added less salt in the initial solution to make solutions that were isotonic or hypotonic compared to blood. We mixed stock solutions in different ratios to attain a series of AMPS with different densities and osmolalities. To ensure thorough mixing, we vortexed the solutions for 30 seconds.

Phase separation in AMPSs due to gravity alone may occur inconveniently slowly (hours) because the difference in density between layers of an AMPS can be small ($\Delta\rho \approx 0.001–0.100$ g/cm^3). Centrifugation (2 – 30 minutes at 2000 g) increased the rate of separation of phases in AMPSs.

Separations of Blood with AMPS. We performed separation experiments within one week of the blood being drawn. Blood was stored at 4 °C and brought to room temperature before use. We introduced the blood by layering it gently above the top phase of the AMPS in all of our experiments. Samples were spun at 4000 *g* for one hour at a temperature of 32 °C.

Extraction of Fractions of Cells after Separation. For separations on whole blood performed in conical tubes with AMPS, blood enriched for reticulcoytes concentrated at the liquid/liquid interface. After blunting a pipette, we removed the clumps of packed red cells that could be seen by eye at this interface. Depending on the yield and the tube used, the total volume extracted ranged from 100 μL to 1 mL. 5 μL of packed cells from the pellet at the bottom of the tube were also collected for analysis.

DC-Percoll Separations. To compare our enrichment method to a standard technique, we used a standard density separation with a combination of differential centrifugation and layered Percoll. Centrifugation of blood in 50 mL tubes at 4000 *g* for one hour packed cells with

the least dense cells at the top of the packed volume (i.e. differential centrifugation). After removing the serum, the top 4 mL of packed blood was collected and resuspended in the previously collected serum at ~50% hematocrit. We layered 5 mL of this blood on top of 6 mL of 70% isotonic Percoll. Centrifugation for 15 minutes at 1200 *g* at 30 °C in a swinging bucket rotor (SX4750A, Beckman Coulter) left a band of erythrocytes below the serum and above the Percoll, and a pellet of erythrocytes at the bottom of the tube, below the Percoll. A pipette collected the band from above the Percoll.

Washing Cells to Remove Excess Polymers or Percoll. Cells enriched by either AMPS or DC-Percoll were washed before characterization or use for invasion assays. For screening experiments, washing extracted cells in roughly a five-fold volume of isotonic PBS a total of three times removed excess polymers or Percoll for analysis (i.e., microscopy on thin smears or flow cytometry). During each wash, we suspended the cells gently with a pipette and then spun the cells to a pellet at 1,500 *g* for 6 minutes. After the supernatant was removed, the cells were suspended again until all washes were completed. For invasion experiments, increasing the volume of PBS to be 20-fold the volume of the sample provided a more thorough washing to remove excess polymers or Percoll.

Analysis of the Fractions of Blood. We counted reticulocytes by flow cytometry (MACS Quant). Reticulocytes were stained with acridine orange (Retic ONE) following the manufacturer's protocol. Using known volumes of sample, we counted cells and also quantified the fraction of all cells that were reticulocytes. Comparing samples before and after enrichment allowed us to estimate the total number of reticulocytes that were added to each AMPS, and the total number of reticulocytes recovered. The fraction of these two numbers provided a measure of the yield of reticulocytes.

We also made thin smears stained with New Methylene Blue (Retic Stain) and quantified reticulocytemia by microscopy. To analyze other cell parameters, we used a hematology analyzer (Advia 2120, Siemens).

Experimental Details

Selection of AMPSs. The dextran–Ficoll AMPS exhibited a small difference in density between the top and bottom phases. Without additives, dextran–Ficoll AMPSs prepared in distilled, deionized water that are in the density range of blood cells are acidic and hypotonic. We titrated the pH to 7.40 with NaOH and HCl. We added NaCl to the solutions to reach a final osmolality of 295 ± 15 mOsm/kg (i.e., isotonic).

The small step in density in the dextran–Ficoll AMPS allowed us to create a bottom phase with a low enough density to allow mature erythrocytes to pass through it, and a top phase dense enough to prevent the blood from mixing instantaneously after being layered over the AMPS. We explored two other systems—a poly(ethylene glycol)–Ficoll AMPS and a poly(ethylene glycol)–dextran AMPS—as alternative systems.

The poly(ethylene glycol)–Ficoll AMPS exhibited differences in density between phases that were greater than 0.030 g/cm^3. We could not produce a top phase with a density significantly greater than that of plasma (ρ = 1.026 g/cm^3) while keeping the osmolality of the phases isotonic; this AMPS was, thus, not suitable for blood separations.

The poly(ethylene glycol)–dextran AMPS had similar characteristics to the poly(ethylene glycol)–Ficoll AMPS. The poly(vinyl alcohol)–poly(ethylene glycol) AMPS could not produce a bottom phase that was dense enough to separate most reticulocytes from mature erythrocytes in the range of osmolality that is required for the separation of cells.

Centrifugation Parameters. Our separations used swinging-bucket rotors for centrifugation to avoid smearing cells along the walls of centrifuge tubes during sedimentation. Centrifugation at a relative centrifugal force (RCF) of 4000 *g* for one hour provided a clear separation between blood cells at the liquid/liquid interface of the two-phase AMPS and cells below the bottom phase for a sedimentation distance of 40 mm (e.g., 4 mL of AMPS in a 15 mL conical tube) (**Figure 3.1**). Experiments with a greater distance for sedimentation (e.g., 60 mm for 25 mL blood over 25 mL AMPS in a 50 mL conical tube) required additional centrifugation time. For larger volumes that had up to a 50% increase in the sedimentation distance (i.e., 25 mL of AMPS in a 50 mL conical tube) the centrifugation time was increased to 90 minutes. The limitation of a long period of centrifugation can be overcome by using a centrifuge that operates at higher relative centrifugal forces.

Detailed Results from Enrichment of Reticulocytes from Whole Blood. Table S1 details the parameters of the different AMPS that were screened as well as the results from enrichments of reticulocytes. We performed additional enrichments using the hypertonic systems (C1-C6) with different loading volumes (**Figure S1**). From this we found multiple systems that were capable of attaining enrichments to reticulocytemias over 50%. **Table S2** summarizes the enrichments from different donors with hemochromatosis. **Table S3** details the enrichments of all donors with four different AMPS.

The volume ratio of blood to AMPSs affects both yield and purity of enrichments. We expected that the performance of hypertonic and hypotonic AMPSs would be dependent on the volume ratio of blood and AMPSs. In an isotonic system, we anticipated that this dependence would be negligible since water exchange between polymers and blood cells would be minimal. We found, however, that the volume ratio of blood and AMPSs did affect performance in an

Table S1. The enrichment of reticulocytes from blood with hemochromatosis at the interface of dextran–Ficoll AMPSs with varying density and osmolality.

	Concentration (% w/v)		Tonicity	Density (g/cm^3)		Initial	AMPS Interface	
ID	**Ficoll**	**dextran**	**(mOsm/kg)**	**Top**	**Bottom**	**Retic[a]**	**Retic[a]**	**S.D.[b]**
Isotonic Systems (295 ± 15 mOsm)								
A1	12.0	12.0	299	1.089	1.092	1.1	1.1	0.4
A2	11.4	11.4	295	1.084	1.087	1.1	2.1	0.7
A3	11.1	11.1	306	1.082	1.085	1.8	21	5.2
A4	10.8	10.8	301	1.080	1.083	1.8	24	4.1
A5	10.3	10.3	289	1.076	1.080	1.8	28	10
Hypotonic Systems (260 ± 10 mOsm)								
B1	10.5	10.5	250	1.079	1.083	1.1	2.0	1.0
B2	10.0	10.0	252	1.074	1.078	1.1	4.7	1.5
B3	9.5	9.5	252	1.071	1.075	1.1	19	1.6
B4	**9.3**	**9.3**	**269**	**1.068**	**1.072**	**1.8**	**55**	**8.4**
B5	9.0	9.0	264	1.067	1.071	1.8	43	5.1
Hypertonic Systems (330 ± 10 mOsm)								
C1	12.0	12.0	327	1.089	1.092	2.4	12	0.7
C2	11.6	11.6	340	1.086	1.089	2.4	27	3.1
C3	11.4	11.4	336	1.084	1.088	2.4	43	4.7
C4	11.0	11.0	332	1.081	1.085	2.4	50	1.5
C5	10.6	10.6	328	1.078	1.082	2.4	31	2.8
C6	10.1	10.1	329	1.075	1.079	2.4	21	2.4

[a] Mean reticulocyte count per 100 erythrocytes (n = 3 technical replicates) by RNA stain
[b] Standard deviation of the replicates
b.f. System with the highest level of enrichment

Figure S1. The enrichment of reticulocytes from blood with hemochromatosis in hypertonic systems changes with different volume ratios (vol. blood (mL):vol. AMPS (mL)) of blood to AMPS. Both the reticulocytemia and yield show changes. A 4:4 ratio provides the best yield. Several systems provide enrichment to reticulocytemias over 50%.

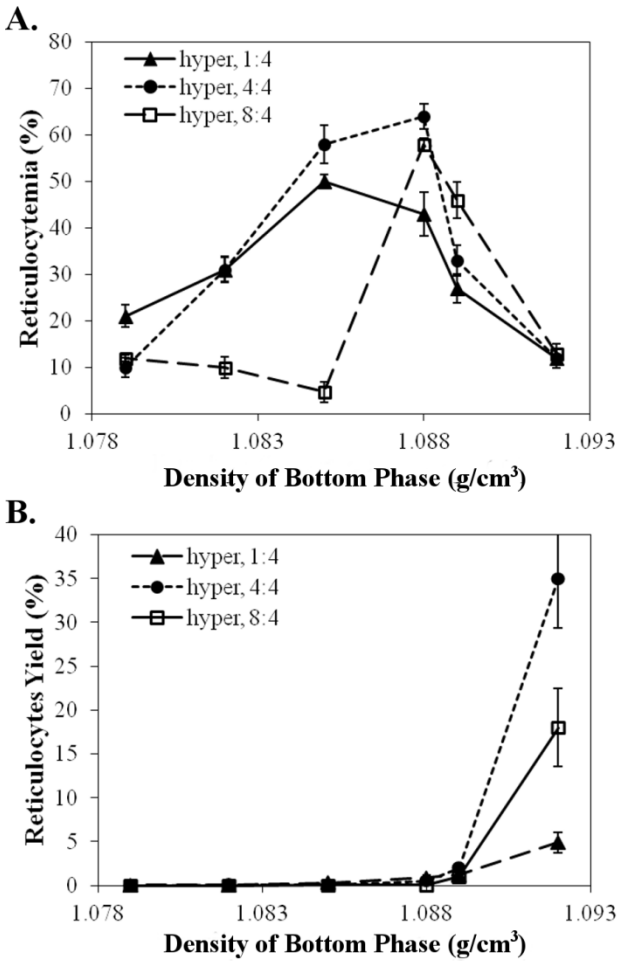


Table S2. Performance of AMPS-enrichment of reticulocytes with multiple (*n = 4*) donors compared to a combination of differential centrifugation and layered Percoll.

	Density (g/cm^3)		Osmolality	Reticulocytemia (%)		Yield of Reticulocytes[a] (%)	
ID[b]	ρ_{top}	ρ_{bot}	(mOsm/kg)	Median	[Min, Max]	Median	[Min, Max]
C1	1.089	1.092	330	19	[15, 21]	1.6	[0.24, 6]
C2	1.086	1.089	330	29	[15, 32]	0.90	[0.095, 1.7]
A5	1.076	1.080	295	36	[18, 49]	0.012	[0.0065, 1.1]
B3	1.071	1.075	260	40	[15, 45]	0.0061	[0.00079, 0.012]
DC-P[c]	--	--	--	38	[6.0, 86]	0.61	[0.12, 9.7]

[a] The percentage of the total reticulocytes from blood loaded on a system that are found in the enriched fraction.
[b] IDs refer to specific densities and tonicities of the dextran–Ficoll AMPS specified in Table S1.
[c] Enrichments done by differential centrifugation followed by centrifugation over layered Percoll (DC-P).

Table S3. Performance of AMPS over different individuals.

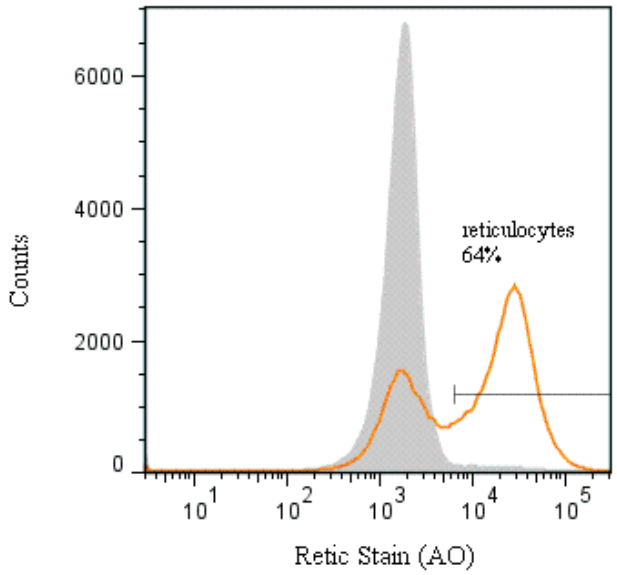
	Init.	C1 (%)		C2 (%)		B3 (%)		A5 (%)		DC-P	
ID	Retic	Retic	Yield	Retic	Yield	Retic	Yield	Retic	Yield	Retic	Yield
Blood from Donors with Hemocrhomatosis											
1	0.80	19	0.24	28	0.11	35	0.013	35	0.0086	--	--
2	0.85	21	0.75	30	0.095	49	0.053	45	0.013	--	--
C.HC	1.3	18	2.4	32	1.7	38	0.011	45	0.00079	--	--
	0.83	15	6.0	15	1.7	18	0.0065	15	0.0036	--	--
E	0.71	--	--	--	--	--	--	--	--	6.0	9.7
F	0.70	--	--	--	--	--	--	--	--	28	0.84
G	1.0	--	--	--	--	--	--	--	--	48	0.38
H	0.92	--	--	--	--	--	--	--	--	87	0.12
Med.	0.84	19	1.6	29	0.89	36	0.012	40	0.0061	38	0.61
Min.	0.80	15	0.24	15	0.095	18	0.0065	15	0.00079	6.0	0.12
Max.	1.3	21	6.0	32	1.7	49	0.053	45	0.013	87	9.7
Blood from Normal, Healthy Donors											
A	--	14	11	27	8.5	--	--	--	--	3.6	2.2
B	--	8.9	92	19	2.3	13	--	15	--	2.1	34
C	2.2	22	0.45	30	0.19	--	--	--	--	39	0.047
D	2.3	5.5	96	6.1	49	20	1.3	17	1.6	26	9.0
Med	2.3	7.2	52	23	5.4	17	--	16	--	15	5.6
Min	2.2	5.5	0.45	6.1	0.19	13	--	15	--	2.1	0.047
Max	2.3	22	96	30	49	20	--	17	--	39	34

isotonic system. A range of volumes of blood (1 mL, 4 mL, and 8 mL) were loaded onto 4 mL of the hypertonic AMPS (C1-C6) (**Figure S1**). From these systems we found a maximum enrichment of 64% ± 3% (**Figure S2**).

Dispersal of Plasma Proteins into AMPS. We hypothesized the dependence of enrichments on the volume ratio of blood to AMPS might be due to a slight amount of mixing of plasma which would dilute the phases and would depend on the volume ratio of blood to polymer. We checked the density of the phases after a separation to see if there was a change in the phases. When 2 mL of blood was layered on top of 4 mL of A5, the density of both phases decreased ($\Delta\rho_{top}$ = -0.013 g/cm^3, $\Delta\rho_{bottom}$ = -0.016 g/cm^3); we used isotonic A5 rather than hypertonic AMPS to avoid changes in volume that would result from the cells shrinking or swelling.

If a boundary layer of plasma were to penetrate the top phase with the cells and then mixes with the phase, we would expect to see plasma proteins dispersed in the top phase. To visualize the dispersal of plasma proteins into an AMPS during an experiment—layering blood, introduction into a centrifuge, fractionation by centrifugation, and removal from a centrifuge—we added a fluorescent protein into whole blood as a marker. We prepared fluorescein-labeled bovine serum albumin (FITC-BSA) by following the Thermo Scientific protocol for coupling reactions using fluorescein isothiocyanate. After dialysis against isotonic PBS, the final concentration of FITC-BSA was ~ 7 mg mL^{-1}. We mixed this solution with whole blood at a ratio of 1:9 to create an experimental sample. A control sample was made by mixing PBS with blood at a ratio of 1:9. The blood samples were each layered over dextran–Ficoll AMPS (1 mL blood over 3 mL AMPS). The samples were sedimented by centrifugation at an RCF of 2000 *g* for 100 minutes at 25 °C in an Allegra-6R swinging bucket centrifuge.

Figure S2. A 1:1 volume ratio of hemochromatosis blood to a hypertonic AMPS provided the maximum enrichment of reticulocytes. Using system C3, we attained a reticulocytemia of 64 ± 3% as measured by flow cytometry. The gray, filled curve shows the blood before enrichment, which had a reticulocytemia of 2.2%. After centrifugation through AMPS, the fraction of cells at the interface is dominated by reticulocytes (orange curve). Acridine orange (AO) preferentially stains the RNA in the reticulocytes, and causes the shift to the right.



We imaged the systems after fractionation using a combination of brightfield and fluorescent techniques (**Figure S3**). For the fluorescence images, the tubes were kept in a dark box, illuminated with longwave UV light (λ = 365 nm), and imaged using a bandpass filter (500 – 600 nm) in front of the camera. Although the fluorescence intensity is highest in the plasma layer, we observe that some FITC-BSA is present in the top phase of the AMPS. The diffusion length of serum albumin in whole blood over the course of a 100-minute experiment is approximately 5 mm ($D_f \sim 2.1 \times 10^{-10}$ m^2 s^{-1}).[1] The distance between the plasma/top phase boundary and the AMPS interface is 14 mm. Diffusion alone, therefore, cannot account for the presence of FITC-BSA in the top phase of the AMPS. Dilution of the top phase from the mixing of the boundary layer of cells would change the equilibrium of the AMPS, shift the water and polymer contents of the bottom phase, and reduce the bottom phase density.

Additional studies could further elucidate the connection between sample volumes and enrichment, but were beyond the scope of this work.

Reticulocyte enrichments scale to 25 mL of blood without a loss in purity. The purity and yield of reticulocytes remain fairly constant as volumes are increased provided that the volume ratio of AMPS to blood remains constant (**Figure S4**). Using the same isotonic dextran–Ficoll AMPS as used in the volume ratio experiments, we performed a series of enrichments on blood from a single donor with volumes of blood ranging from 2 mL to 25 mL. In all cases, a volume ratio of 1:1 between blood and AMPS was used; this ratio provided the best combination of yield and enrichment (**Figures S1 & S4**). Both 15 mL and 50 mL conical tubes were used. Interestingly, the yield initially decreased as volume increased. At 6 mL, the results were similar for both the types of tubes used. Above this volume, however, yield and reticulocytemia remained relatively constant.

Figure S3. Dispersion of protein from plasma into AMPS. Brightfield images of whole blood after fractionation by AMPS without (A) and with (B) the addition of 700 µg/mL fluorescein-labeled BSA. A fluorescence image (C) of the tube in (B) after illumination with longwave UV light.

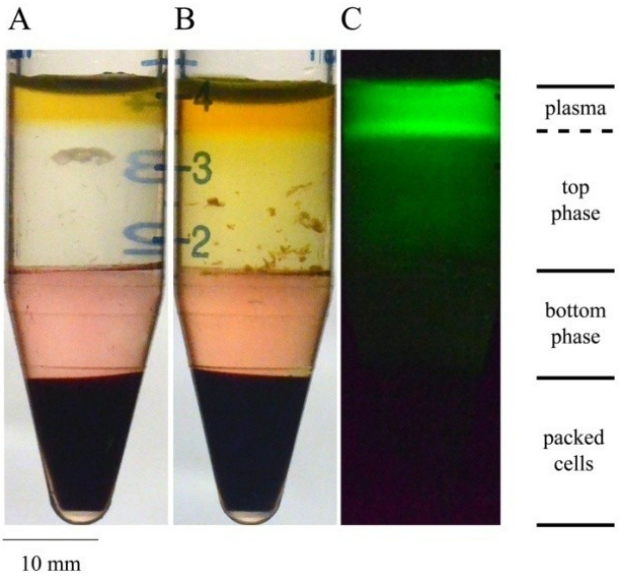
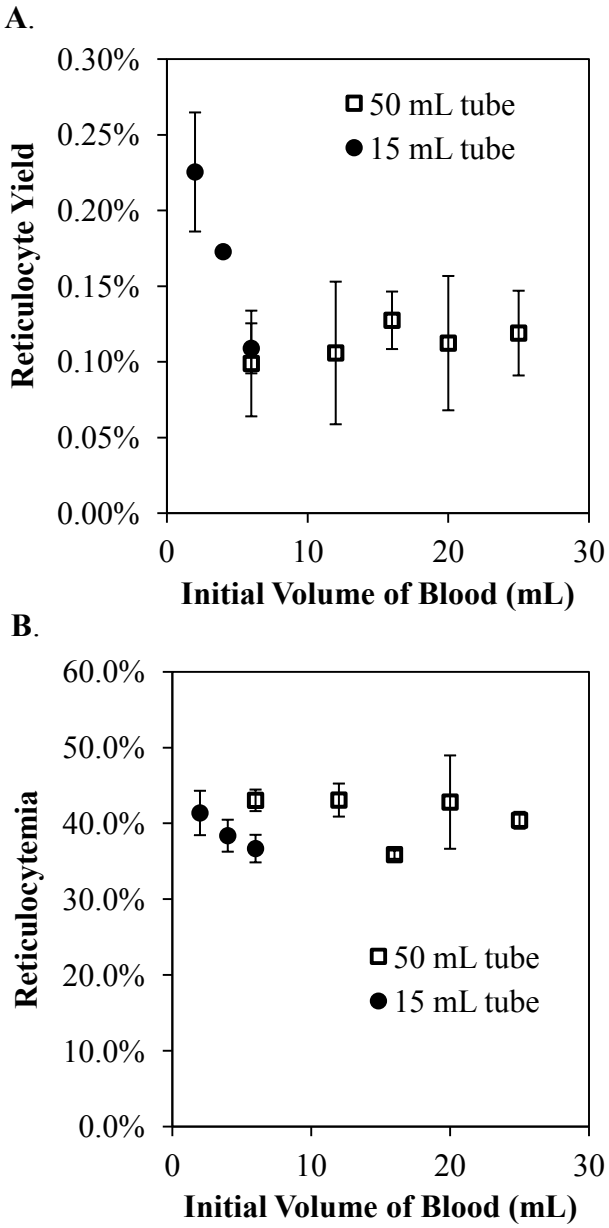


Figure S4. Reticulocyte enrichment over AMPS C2 was comparable over multiple scales of volume. For both reticulocyte yield (A.) and reticulocytemia (B.), only one datum fell outside one standard deviation of the mean over all volumes, and all data were within two standard deviations of the mean.



Pre-enrichment increases the purity of the final enrichment. When using differential centrifugation, naturally pre-enriched blood has a higher final enrichment than normal blood [2,3]. Similarly, we expected that blood pre-enriched for reticulocytes by density would have a higher final enrichment after centrifugation through an AMPS.

Using blood from a single donor with an initial reticulocytemia of 2.2%, we pre-enriched reticulocytes from 100 mL of blood with two different methods: a) differential centrifugation, and b) centrifugation through AMPS C1. The enriched fractions collected had a reticulocytemia of 4.7% and 14%, respectively. The total amount of cells recovered differed as well. We recovered approximately 8 mL of packed cells from differential centrifugation and resuspended them in a volume of 16 mL using homologous plasma recovered from the centrifugation. We only recovered ca. 100 μL of packed cells from AMPS C1. After washing these cells three times in PBS, we resuspended them in a final volume of 3 mL. For the final enrichment, we split each suspension into thirds and layered them over 4 mL of AMPS C1. After centrifugation, we recovered cells from the AMPS interface and washed them three times in PBS. Using flow cytometry, we measured the reticulocytemia of the final enrichments. The results were similar for the fraction pre-enriched by differential centrifugation and that pre-enriched by centrifugation through AMPS. Final reticulocytemia was 20% and 21%, respectively.

Osmotic effects on reticulocyte enrichment by density. As discussed in the main text, systems with different osmolalities achieved different yields when final reticulocytemias were similar. Also, different osmolalities had maximum enrichments for different densities of AMPS (**Figure 2**). The effect of osmolality on the density of the cell populations of interest—reticulocytes and mature erythrocytes—may explain the difference in yields.

A cell at osmotic equilibrium with a system has a concentration of solutes, c, and a volume, V. Both the cell and the surrounding environment have a osmolarity: $\phi = \frac{c}{V}$. If the cell is now placed into a hypertonic environment with osmolarity, $\phi' = \phi + \delta\phi$, where $\delta\phi > 0$, then the volume of the cell will change to compensate by losing water. We assume that the concentration of solutes remains fairly constant due to the presence of ion pumps to maintain internal ion concentrations. Although there still may be some solute exchange between the cell and environment, we assume that at least, $\frac{\delta c}{c} \ll \frac{\delta V}{V}$. With this assumption then, the cell volume changes to V', given by Equation 1:

$$V' = \frac{V}{1 + \frac{\delta\phi}{\phi}} \qquad \text{(Equation S1)}$$

This change in volume shifts the density of a cell ($\rho_1 = \frac{m_1}{V_1}$) by changing both the mass and volume. The mass can be split into both dry mass and water mass, $m_1 = m_{1d} + m_{1w}$. The density then shifts as follows:

$$\rho_1^{'} = \frac{m_1^{'}}{V_1^{'}} = \frac{m_1 + \left(V_1^{'} - V_1\right)\rho_w}{\frac{V_1}{1 + \frac{\delta\phi}{\phi}}} \qquad \text{(Equation S2)}$$

$$\rho_1^{'} = \rho_1\left(1 + \frac{\delta\phi}{\phi}\right) - \left(\frac{\delta\phi}{\phi}\right)\rho_W \qquad \text{(Equation S3)}$$

$$\rho_1^{'} - \rho_w = \left(1 + \frac{\delta\phi}{\phi}\right)(\rho_1 - \rho_w) \qquad \text{(Equation S4)}$$

Two different cells (e.g., a reticulocyte and a mature erythrocyte) with a density different of $\Delta\rho = \rho_2 - \rho_1$, will have a new density difference of $\Delta\rho' = \Delta\rho\left(1 + \frac{\delta\phi}{\phi}\right)$. On a population

scale, this means that the distance between the two peaks of the density distribution of cells will scale with the osmolality.

Morphology of Cells after Centrifugation through AMPSs. Washing cells was an important step to restore morphology and remove excess polymer. Dextran adsorbs to the surface of cells [4–6]. Repeated washing removes some, but not all of the dextran [5]. Washing cells in an isotonic buffer such as PBS appeared to return them to their physiological volumes; that is, unwashed cells from hypotonic systems appeared swollen when observed by microscopy. After extracting and washing cells from the interface of the AMPSs, we found that both mature erythrocytes and reticulocyte had a similar morphology to fresh blood on thin blood smears (**Figure S5**).

We compared the mean corpuscular volume (MCV) and the mean corpuscular hemoglobin (MCH) of RBCs isolated from five random donors before and after separations by AMPS (**Table S4**). These quantitative results support the hypothesis that sedimentation through the AMPS does not drastically affect the morphology or the contents of cells. Evaluation of the percentage of cells that were hypochromic, hyperchromic, microcytic, and macrocytic revealed very little change between the original blood and the cells from the bottom fraction of the AMPS for the latter three indices. There was a slight increase in the percent of hypochromic cells and decrease in the percentage of hyperchromic cells that indicates minor swelling, but the effect is small and could be further reduced by increasing the salt content of the AMPS. Donors A and E had the lowest mean cellular hemoglobin concentration (MCHC) and, hence, had potentially more cells near the threshold of being hypochromic; the effect of a slight swelling would be more pronounced in these two samples.

Figure S5. Morphology of cells after separation using an AMPS. Representative micrographs of mature erythrocytes and reticulocytes before and after centrifugation in an AMPS demonstrate no significant morphological change as a result density-based separation. The blue stained clumps of RNA identify the reticulocytes in the right hand micrographs.

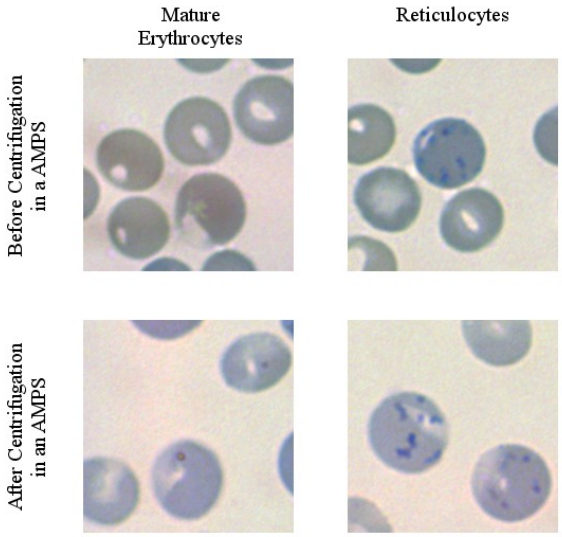


Table S4. Hematological indices of the size and contents of erythrocytes (RBCs) pre- and post-exposure to an AMPS.

	MCV[a] (fL)		MCH[b] (pg)		% Macro[c]		% Micro[d]		% Hypo[e]		% Hyper[f]	
Donor	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
A	82	84	27	26	0.0	0.1	1.2	0.8	4.8	12	0.2	0.1
B	92	93	31	30	0.7	0.7	0.2	0.1	0.3	0.4	0.5	0.0
C	90	91	30	30	0.4	0.4	0.3	0.2	1.0	1.5	0.3	0.0
D	94	95	31	31	1.3	1.5	0.3	0.3	0.4	0.7	0.4	0.4
E	85	86	27	26	0.1	0.1	0.9	0.7	7.6	15	0.1	0.0

[a] mean corpuscular volume
[b] mean corpuscular hemoglobin
[c] percentage of erythrocytes that are macrocytic
[d] percentage of erythrocytes that are microcytic
[e] percentage of erythrocytes that are hypochromic
[f] percentage of erythrocytes that are hyperchromic

Parasite Culture. Parasites were maintained *in vitro* in rhesus blood (purchased from the New England Primate Research Center, Southborough, MA) at 2% hematocrit, in RPMI-1640 supplemented with 25mM HEPES, sodium bicarbonate, 50 mg/L hypoxanthine, and 0.5% Albumax. Parasitemia of cultures are determined by microscopy. Reticulocytemias for each enriched sample are given in **Table S5**.

Invasion Assays. For invasion assays, late stage *P. knowlesi* H parasites were purified through magnet columns (MACS Miltenyi Biotec). They were plated at a final parasitemia of 0.5-1% in 150 µl cultures at 2% hematocrit in a 96 well plates. Normal human blood and rhesus blood were used as controls. Each red blood cell type was plated in triplicate. Parasites were incubated overnight to allow re-invasion. Parasitized erythrocyte multiplication rate (PEMR) was calculated by dividing the parasitemia after re-invasion to the initial parasitemia seeded.

Additional Background

Direct growth of reticulocytes and labeled methods to enrich reticulocytes are not practical for the routine cultivation of malaria. Reticulocytes may be obtained directly from the *in vitro* culture of hematopoietic stem cells (HSCs) [7]. This method has been used to culture *P. vivax* at a parasitemia below 0.0013%; access to HSCs remains expensive, and asynchronous erythropoiesis limits reticulocytemia [8]. Blood can be enriched highly for reticulocytes (>90% measured by microscopy) using antibodies that differentiate reticulocytes from mature erythrocytes based on characteristic surface proteins [9]. Recovering undamaged reticulocytes from affinity-based separations is difficult, however, and is expensive for routine use [10]. As a result, attempts to enrich reticulocytes for the cultivation of malaria parasites have focused on label-free methods using the physical properties of these cells (e.g., size and density) [2,3,11].

Table S5. Reticulocytemia of samples used for invasion assays.

	Matched Reticulocytemia (%)*	
Donor	AMPS[a]	DC-Percoll[b]
Hemochromatosis		
1	21	19
2	21	20
3	5.0	11
4	7.0	7.1
5	5.6	3.5
6	16	20
7	16	11
8	12	12
Healthy, Normal		
1	9.2	9.2
2	2.9	2.9
3	3.0	3.0
4	5.0	8.0
5	16	11
6	11	5.0

[a] blood enriched by aqueous multiphase systems (AMPSs)
[b] blood enriched by differential centrifugation followed by centrifugation over layered Percoll
***** reticulocytemia was matched by diluting the system with a greater reticulycemia with normal blood

Density provides a label-free parameter to enrich reticulocytes. Reticulocytes are generally larger in volume than mature erythrocytes and they contain ribosomal RNA; the average density of reticulocytes, thus, is slightly lower than that of mature erythrocytes ($\Delta\rho \approx 0.009$ g/cm^3) [12,13]. The reported values of the densities of these two populations differs with the study and the method, but most studies agree that the reticulocyte population is concentrated in the least dense quarter of the distribution of density of erythrocytes [13,14]. Differential centrifugation and centrifugation through a gradient in density are the most common methods to separate these types of cells by density [2,3,10].

In differential centrifugation, erythrocytes sediment and pack at the bottom of a container; the erythrocytes located at the top of the packed cells have a lower density than those below. As a result, the top quarter of the packed cells contain relatively more reticulocytes than the lower three quarters. Starting with whole blood from normal subjects, differential centrifugation results in an average enrichment of reticulocytes of 2.6% [3]. Using sources of blood with elevated reticulocyte counts (e.g., blood from umbilical cords [15] or from patients with hemochromatosis [2]) can increase the final enrichment obtained from differential centrifugation. Use of these sources is a barrier to the routine use of this method. Cord blood is expensive to purchase from vendors, and the total volume of blood that can be harvested from each cord is limited to an average of 75 mL [16]. Although arrangements can be made with facilities that store discarded samples, cord blood, by its nature, is generally less readily available than peripheral blood.

Gradients in density improve the enrichment of reticulocytes by separating reticulocyte-rich fractions and reticulocyte-poor fractions of erythrocytes into visible bands. Percoll—a suspension of colloidal silica stabilized by polyvinylpyrrolidone (PVP)—will form a time-

dependent gradient in a centrifuge with an angled rotor. Separations are highly dependent on the centrifugation parameters (e.g., rotor angle, applied relative centrifugal force, acceleration, and time). As a result, reproducibility suffers; initial reports of fractionation of reticulocytes over Percoll gradients achieved a maximum of 78% reticulocytemia in enriched fractions of blood, while average reticulocytemias % [3,17].

Layered gradients—manually assembled by carefully layering decreasing concentrations of aqueous solutions of a dense solute (e.g., sucrose or arabinogalactan) [10,18]—provide a means to tune the resolution of separations at multiple densities. The boundary between layers provides a location at which cells of specific densities will collect. Such gradients achieve 68% reticulocytemia in some subjects, but they are time-consuming to assemble and susceptible to mixing and destruction without careful handling [10].

References

1. Liu M, Nicholson JK, Parkinson JA, et al. Measurement of Biomolecular Diffusion Coefficients in Blood Plasma Using Total-Correlation NMR Spectroscopy. Anal Chem. 1997;69:1504–1509.

2. Golenda CF, Li J, Rosenberg R. Continuous in vitro propagation of the malaria parasite Plasmodium vivax. Proc Natl Acad Sci U S A. 1997;94:6786–6791.

3. Rushing D, Vengelen-Tyler V. Evaluation and comparison of four reticulocyte enrichment procedures. Transfusion. 1987;27:86–89.

4. Van Oss CJ, Arnold K, Coakley WT. Depletion flocculation and depletion stabilization of erythrocytes. Cell Biophys. 1990;17:1–10.

5. Brooks DE. The Effect of Neutral Polymers on the Electrokinetic Potential of Cells and Other Charged Particles. J Colloid Interface Sci. 1973;43:700–713.

6. Chien S, Simchon S, Abbott RE, et al. Surface Adsorption of Dextrans on Human Red Cell Membrane. J Colloid Interface Sci. 1977;62:461–470.

7. Giarratana M-C, Kobari L, Lapillonne H, et al. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. Nat Biotechnol. 2005;23:69–74.

8. Panichakul T, Sattabongkot J, Chotivanich K, et al. Production of erythropoietic cells in vitro for continuous culture of Plasmodium vivax. Int J Parasitol. 2007;37:1551–1557.

9. Brun A, Gaudernack G, Sandberg S. A new method for isolation of reticulocytes: positive selection of human reticulocytes by immunomagnetic separation. Blood. 1990;76:2397–2403.

10. Sorette MP, Shiffer K, Clark MR. Improved isolation of normal human reticulocytes via exploitation of chloride-dependent potassium transport. Blood. 1992;80:249–254.

11. Andreux A, Merino A, Renard M, et al. Separation of red blood cells by field flow fractionation. Exp Hematol. 1993;21:326–330.

12. Key J. Studies on erythrocytes, with special reference to reticulum, polychromatophilia and mitochondria. Arch Intern Med. 1921;1882:511–549.

13. Leif RC, Vinograd J. the Distribution of Buoyant Density of Human Erythrocytes in Bovine Albumin Solutions. Proc Natl Acad Sci U S A. 1964;51:520–528.

14. Haidmayer R, Kenner T, Hinghofer-Szalkay H. On the influence of Temperature on the Measurement of Density Distribution of Erythrocytes. Biomed Tech. 1980;25:258–260.

15. Russell B, Suwanarusk R, Borlon C, et al. A reliable ex vivo invasion assay of human reticulocytes by Plasmodium vivax. Blood. 2011;118:e74–81.

16. Hillyer CD, Strauss RG, Luban NLC. Handbook of Pediatric Transfusion Medicine. 2004. page 295–296.

17. Branch D, Hian A, Carlson F, et al. Erythrocyte age-fractionation using a Percoll-Renografin density gradient: application to autologous red cell antigen determinations in recently transfused patients. Am J Clin Pathol. 1983;80:453–458.

18. Martínez-Salas E, Martín JA, Vicente M. Relationship of Escherichia coli density to growth rate and cell age. J Bacteriol. 1981;147:97–100.