

(19) World Intellectual Property Organization  
International Bureau



(10) International Publication Number  
**WO 2009/135178 A2**

(43) International Publication Date  
5 November 2009 (05.11.2009)

- (51) **International Patent Classification:**  
A61K 49/00 (2006.01) A61K 9/50 (2006.01)
- (21) **International Application Number:**  
PCT/US2009/042606
- (22) **International Filing Date:**  
1 May 2009 (01.05.2009)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**  
61/126,344 2 May 2008 (02.05.2008) US
- (71) **Applicant and**
- (72) **Inventor: FLOWER, Robert, W.** [US/US]; 11 Dellwood Court, Hunt Valley, MD 21030 (US).
- (74) **Agents: BAUTISTA, Maria, T. et al;** Rissman Hendricks & Oliverio, LLP, 100 Cambridge Street, Suite 2101, Boston, MA 021 14 (US).
- (81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,

CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**  
— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) **Title:** METHODS FOR PRODUCTION AND USE OF SUBSTANCE-LOADED ERYTHROCYTES (S-LES) FOR OBSERVATION AND TREATMENT OF MICROVASCULAR HEMODYNAMICS

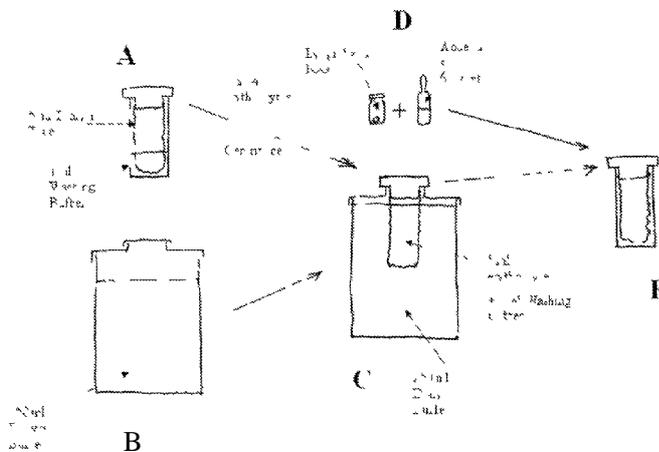


FIG. 11

(57) **Abstract:** Disclosed herein are methods, kits, and compositions for medical imaging relating to fluorescent dyes entrapped in erythrocytes. Also disclosed therein are methods, and compositions further comprising erythrocytes entrapping at least one therapeutically active agent.

WO 2009/135178 A2

**METHODS FOR PRODUCTION AND USE OF SUBSTANCE-LOADED  
ERYTHROCYTES (S-LES) FOR OBSERVATION AND TREATMENT OF  
MICROVASCULAR HEMODYNAMICS**

**RELATED APPLICATION**

5           **[01]** This application claims the benefit of priority under 35 U.S.C. § 119(e) to U.S. Prov. App. 61/1 26,344, filed May 2, 2008, the disclosure of which is incorporated herein by reference.

**FIELD OF THE INVENTION**

10           **[02]** Disclosed herein are kits, compositions, and methods for the clinical use of erythrocytes in the fields of medical angiography and therapy. The erythrocytes have been pre-loaded with substances for observation of blood flow under physiological conditions to detect circulation abnormalities. The erythrocytes may also be used for delivery of therapeutic substances to localized vascular areas. This technology can be applied to any vasculature that can be directly visualized by an optical means, such as  
15           ocular vasculatures.

**BACKGROUND**

20           **[03]** Medical angiographic imaging typically involves administration of a detectable substance to a subject (see U.S. Patent No. 6,91 5,154). In some instances the detectable substance may also be a therapeutic agent (see U.S. Patent Publication No. 2004/0206364). Most often the detectable substance is administered directly to a subject by intravascular injection, in which case the detectable substance mixes with and is carried through the vasculature by plasma, along with the blood cells. When using conventional angiography methodology, wherein the detectable substance is a  
25           liquid dye, blood flow physiology is treated too simplistically, especially at the microvascular level (i.e., the arterioles, capillaries, and venules).

[04] Blood is a shear-thinning, non-Newtonian fluid. However, in diagnostic applications, blood often is treated as if it were water-like (i.e., a Newtonian fluid), and not an homogeneous mixture of two distinctly different non-Newtonian fluids: (1) liquid (plasma) and (2) particles (blood cells, especially erythrocytes). Limitations inherent in conventional angiography contribute to ignoring that the dynamics of plasma movement do not necessarily reflect the dynamics of erythrocyte movement, especially at the microvascular level where their movement is far more important to the circulation's metabolic efficiency than that of plasma. For example, in conventional sodium fluorescein and indocyanine green angiography (SFA and ICGA) of the ocular vasculatures, observed fluorescence arises from dye molecules associated primarily with blood plasma, not erythrocytes. Even in capillaries, where they deform in order to pass through one-at-a-time in boxcar fashion, erythrocytes cannot be seen in conventional angiogram images, so metabolically significant phenomena such as vasomotion, which results in periodic suspension of erythrocyte movement through individual capillaries, cannot be directly visualized. Yet, it has been postulated that all the clinical findings concerning edema in diabetic maculopathy can be explained as a result of disturbances in retinal vasomotion (Bek 1999, *Acta Ophthalmol Scand* 77:376). Moreover, in conventional angiography, dye molecules leave the plasma and become associated with vessel walls, so those blood vessels rapidly exhibit steady-state fluorescence, thereby obscuring further visualization of blood movement. Consequently, conventional fluorescent dye angiography is limited as a diagnostic tool, since what hemodynamic information it conveys is misleading with respect to metabolic efficiency and capacity of microvascular blood flow. An example of this would be relying on observation of conventional angiograms to assay the metabolic capacity of blood flow through a choroidal neovascular (CNV) membrane. Due to the well-known phenomenon of plasma skimming (likely to occur where the CNV feeder vessel arises at an acute angle from the choroidal arterial vessel feeding it), only erythrocyte-deficient plasma would profuse the CNV, but this deficiency would not be reflected in the

angiogram images since the fluorescence arises only from dye in the plasma, not the erythrocytes.

[05] Alternatively, a detectable substance (e.g., sodium fluorescein dye) has been administered in a particle carrier, heat sensitive liposomes (Kiryu et al. 1994, *Invest Ophthalmol Vis Sci* 35:3724). However, such artificial particles are rigid and are small to assure that they can pass unobstructed through the smallest capillary vessels. They may not serve as faithful models of erythrocyte dynamics.

#### SUMMARY OF THE INVENTION

[06] Disclosed herein are kits, compositions, and methods that take advantage of the ability of erythrocytes to be preloaded with various substances, such as fluorescent dyes that facilitate medical imaging. Human erythrocytes, despite their large diameters and volumes, readily conform to the small capillary diameters, and they have been demonstrated to possess properties that make them useful as carriers of molecules other than haemoglobin. Erythrocytes are capable of reversible deformation, such as occurs when they are in hypotonic solution; their volumes increase, causing 200-500 Å pores to open transiently in the cells' membranes (Seeman, 1967, *J Cell Biology* 32:55), allowing two-way trans-membrane exchange between their normal content (haemoglobin) and high-molecular-weight substances placed in their externally vicinity. Then, by returning the solution to normotonicity, the pores close, and the cells return to normal size, trapping the added substances inside; remaining non-entrapped substance can be washed away, leaving substance-loaded osmotically competent erythrocytes; these cells contain reduced amounts of hemoglobin, rendering them incompetent—or at least inefficient—with regard to oxygen delivery. Nevertheless, substance-loaded erythrocytes (S-IEs) appear to have a normal life span of up to 120 days, and they have been used for studying membrane morphology, physiology and biochemistry (see, e.g., Wu et al., 2005, *Biochem Pharmacol* 69(8): 1257; Rodnenkov et al., 2005, *Pathophysiology* 11(4):209). S-IEs have been used in the field of medical

imaging (see, e.g., Thelwall et al., 2002, *Magnetic Resonance in Medicine* 48:649; Kleszcynska et al., *J. Fluoresc.* 15(2):137).

**[07]** One embodiment relates to the discovery that the amount of ICG dye inserted into each erythrocyte can produce detectable fluorescence without exceeding safe levels of retinal illumination. Another embodiment relates to the discovery that the amount needed to optimally induce fluorescence and the much larger amount needed to absorb sufficient energy to enhance photocoagulation are mutually exclusive. Yet another embodiment relates to the re-sealing of the cells of the substance-loaded erythrocytes.

**[08]** Use of erythrocytes as a drug delivery system has been investigated (see, e.g., Rossi et al. 2001, *Biotechnol Appl Biochem* 33:85; Magnani et al., 1998, *Biotechnol Appl Biochem* 28:1). In the method disclosed, erythrocytes that had been loaded with various therapeutic substances were autologously re-injected into a subject and were subsequently distributed throughout the body; there they continuously released the encapsulated substance as the erythrocyte population gradually underwent normal cell death over a span of about 120 days. Although useful for delivery of certain drugs where maintenance of some level of substance throughout the circulation is desirable, this method does not readily facilitate targeted release of therapeutic substances in high concentration. Therefore, its use for delivery of substances to targeted vascular areas requiring therapeutic concentrations too high to be tolerated throughout the body may be prohibited. Another embodiment relates to the discovery that in situations where the targeted vascular area is optically accessible (e.g., the ocular vasculatures or the vasculatures of hollow organs such as the bladder), erythrocytes loaded with appropriate substances can be lysed by means of optical delivery of appropriate radiation, thereby delivering their entrapped contents to precisely localized areas.

**[09]** Also disclosed herein are kits and methods for the relatively facile preparation of substance-loaded erythrocytes (S-IEs) for use in clinical application for autologous re-injection. Alternatively, pre-loaded erythrocytes suitable for homologous re-injection in an off-the-shelf form can be prepared. To that end, disclosed herein are

methodologies for producing and stabilizing substance-loaded cells, for both diagnostic and therapeutic applications in human subjects, which can obviate need for end-user access to extensive laboratory facilities in order to obtain and process cells under blood-banking sterile conditions.

5 [10] Another embodiment relates to the discovery that S-IEs having increased fluorescence (beyond that which can be achieved by optimal loading of dye alone into each erythrocyte) for cell detection can be achieved by incorporation of metallic silver colloids.

[11] Another embodiment provides novel approaches to detection and  
10 controlled release of therapeutic substances encapsulated in S-IEs that result in delivery of high substance concentrations with respect to targeted vascular areas, but which at the same time amount to micro-dose concentrations with respect to the circulation as a whole; this makes possible use of substances that would otherwise be rejected due to the significant systemic toxicity they demonstrate when delivered by conventional  
15 intravenous injection. Various substances and combinations thereof can be pre-loaded into erythrocytes in ways that take into account a spectrum of desired biological, chemical, and physical properties of those elements in ways without which those combinations would not produce useful results.

[12] One embodiment relates to the use of pre-loading substances into  
20 erythrocytes and use of S-IEs in ophthalmic diagnostic angiography and drug delivery. Alternatively, this technology can be applied to other vasculatures that can be directly visualized, as well as to other substances that facilitate similar ends in those other vasculatures.

25

#### BRIEF DESCRIPTION OF THE DRAWINGS

[13] Various embodiments of the invention will be understood from the following description, the appended claims and the accompanying drawings, in which:

[14] FIGs. 1A-1 D are near-infrared fluorescence micrographs at 40x magnification of erythrocytes, comparing the brightness of cells "stained" with ICG (A - stained in 1 mM ICG solution for 30 min. and B - stained in 2mM ICG solution for 60 min. to cells into which ICG has been loaded through pores in their membranes; C -  
5 stained plasma resulting from exposure for 20 min. to the stained erythrocytes from A; D - dialysed in 1 mM ICG solution for 30 min.);

[15] FIGs. 2A-C are near-infrared fluorescence micrographs at 40x magnification of erythrocytes into which ICG has been loaded, demonstrating the effect of introducing different dye concentrations into the dialyzed cell suspension;

10 [16] FIGs. 3A-3D are near-infrared fluorescence micrographs at 40x magnification comparing brightness of ICG-"stained" (A = initial brightness, B = brightness at 20 s) and ICG-loaded erythrocytes (C= initial brightness, D = brightness at 20 s) after continuous exposure to the same illumination, due to bleaching;

[17] FIG. 4A is a near-infrared fluorescence micrograph at 40x magnification of  
15 ICG-loaded human erythrocytes;

[18] FIG. 4B is a photograph of a vial containing freeze-dried ICG-loaded human erythrocytes;

[19] FIG. 4C is a near-infrared fluorescence micrograph at 40x magnification of freeze-dried ICG-loaded human erythrocytes reconstituted by addition of 1 ml\_ of H<sub>2</sub>O;

20 [20] FIG. 5A is a sodium fluorescein angiogram image (left) and a conventional ICG angiogram image (right) for orientation of a patient's normal right eye;

[21] FIG. 5B is an angiogram image of ICG-loaded erythrocytes of the patient's normal right eye; individual erythrocytes appear as white dots;

[22] FIG. 5C is a sodium fluorescein angiogram image (left) and a conventional  
25 ICG angiogram image (right) for orientation of the patient's diseased left eye;

[23] FIG. 5D is an angiogram of ICG-loaded erythrocytes of the patient's diseased left eye; individual erythrocytes appear as white dots;

[24] FIGs. 6A and 6B are angiogram images from the same rabbit eye comparing ICG fluorescence in the choroidal vasculature following conventional

intravenous injection of a bolus of aqueous ICG dye (6A) and following injection of an identical bolus containing silver colloid (6B);

[25] FIG. 7 is an infrared fluorescence image comparing the fluorescence enhancement effect of silver colloid in 0.05 mg/mL ICG solutions; samples in the left-  
5 hand side contain no colloid, while those in the right-hand side contain colloid; samples at the top are in 1-cm thick tubes, while those at the bottom are 100 microns thick;

[26] FIG. 8 is an infrared fluorescence image of the samples of FIG. 7 showing reflection of the 805-nm wavelength light used to stimulate fluorescence; no barrier filter was placed in front of the CCD camera in this case;

10 [27] FIG. 9 is a schematic diagram of an embodiment of a kit containing sterile containers used in substance loading of erythrocytes (see Example 1);

[28] FIG. 10A is a schematic representation of the steps involved in removing a plasma- and leukocyte-free volume of erythrocytes from the bottom of the blood sample collection vacutainer after centrifugation, using a long needle;

15 [29] FIG. 10B is a schematic representation of steps involved in removing a volume of erythrocytes from the bottom of the two 15-ml washing solution tubes after centrifugation, using a long needle; and

[30] FIG. 11 is a schematic representation of the sequence ordered steps (A-E) detailing the use of those particular containers from the kit depicted in FIG. 9 to  
20 accomplish dialysis of the erythrocytes, including addition of ICG dye to the solution once dialysis is complete and pores in the cell membranes are open.

#### DETAILED DESCRIPTION

##### Preparation of Substance-loaded Erythrocytes for Re-injection

25 [31] One embodiment relates to encapsulation of fluorescent dyes in erythrocytes for diagnostic observation. Optionally, the erythrocytes can be used for therapeutic substance delivery. In the former application, re-injection of S-IEs can

improve performance of angiography by imaging movement of fluorescent erythrocytes, rather than dye-tagged plasma. In the latter application, the impetus for substance encapsulation in the cells is that many desirable substances do not bind well to the outer cell membrane (as is true for ICG dye). However, encapsulation of those  
5 substances in erythrocytes and localized release by laser-induced lysis facilitates delivery of high substance concentrations at the targeted areas, which amount only to micro-dose concentrations with respect to the circulation as a whole. This makes possible use of substances that otherwise may have had to be rejected. In the former application of diagnostic observation, the association of fluorescent dyes with  
10 erythrocytes may depend on one or more of three circumstances, each of which relates to why dye insertion into the cell rather than simply "staining" the outside of the cell is useful:

(1) Although many fluorescent dyes, such as ICG dye, do bind to the erythrocyte outer cell membrane, binding is weak and non-covalent, and  
15 regardless of the dye concentration and conjugation time, the maximum fluorescence brightness that can be achieved is considerably less than optimum (see FIG. 1). The erythrocytes in Frame B were "stained" by conjugation with ICG dye in a solution at twice the dye concentration and for twice as long as the cells in Frame A; yet the fluorescence brightness  
20 of both is the same since there only are a finite number of available cell-surface binding sites, regardless of the abundance of dye molecules in a particular solution. Frame C demonstrates that once "stained" cells come into contact with plasma proteins, ICG molecules are more attracted to plasma proteins than to the cell membrane, so "stained" cells become  
25 even less bright during circulation; this does not occur with dye-loaded cells.

(2) Exposure to fluorescence excitation energy for diagnostic purposes must not exceed the safe maximum permissible exposure level for the sensory retina. Since ICG dye is a fairly weak emitter of fluorescence, care must

be taken to ensure that the amount of ICG present post-loading is optimal, resulting in maximum fluorescence intensity possible at a given safe excitation energy level. This condition can be met by insertion of dye into the erythrocytes. FIG. 1, Frame D, demonstrates that ICG-loading of erythrocytes by dialysis in a dye solution having the same lower concentration used to stain the cells in Frame A. are considerably brighter; there are considerably more binding sites within a cell volume (including those associated with residual haemoglobin) than on its external surface. Moreover, since ICG is subject to concentration fluorescence quenching— even when inside erythrocytes—the amount of loaded dye must be controlled: too much or too little will not produce the maximum fluorescence. Equal brightness of cells in A and B demonstrates "staining" by conjugation in even high-concentration dye solution does not achieve the optimum brightness possible by dye insertion (D). Image C demonstrates that the affinity of ICG dye molecules is greater for plasma proteins than for the erythrocyte outer membrane; cells in A, B, and D are suspended in isotonic saline. Achieving optimal dye concentration is accomplished by controlling the amount of dye in the solution during dialysis once the pores in the cell membranes open; experimentation has determined the optimum amount to be approximately  $1.0 \mu\text{mol/ml}$  dialysed cell-solution (see FIG. 2). After equilibrium is established between the solutions internal and external to the cells, the pores are closed by returning the hypotonic solution containing the cells to a normotonic level, fixing the entrapped dye concentration. This also has the advantage of preventing plasma dye-staining and the associated reduction of contrast between cell fluorescence and background brightness once the cells are re-injected. FIGs. 2A, 2B, and 2C have concentrations of  $0.31 \mu\text{mol/mL}$  solution,  $0.89 \mu\text{mol/mL}$  solution, and  $6.14$

$\mu\text{mol}/\text{nnL}$  solution giving rise to an average 8-bit-gray-scale brightness of 159, > 255, and 165, respectively.

- (3) ICG dye is subject to bleaching, potentially making detection and imaging of ICG-loaded erythrocytes difficult, due to the resulting reduced levels of fluorescence for a given level of illumination. This can be a problem, especially when it is desirable to make observations for periods of many seconds, as would be required for observing the effects on erythrocyte movement of vasomotion, wherein individual cell motion may be temporarily halted for up to 30 sec. It has been experimentally determined that, whereas ICG dye-"stained" cells are susceptible to bleaching, ICG-loaded cells are not (see FIG. 3). Again, it is the limited number of dye molecules on an erythrocyte's outer membrane, compared to the number within the cell volume that makes this phenomenon significantly visible in the "stained" cells, but not in the loaded cells.

[32] In one embodiment, encapsulation of various substances in human erythrocytes is accomplished by a procedure of hypotonic dialysis, isotonic resealing and re-annealing. Placing washed erythrocytes in a hypotonic solution causes pores that can have a size ranging from 200-500 Å to open transiently in the cell membranes, thereby allowing substances (such as ICG dye) in the solution to cross the membranes. Making the hypotonic solution normotonic causes the pores close, and the cells (now with reduced hemoglobin content) return to an osmotically competent state, trapping the substances inside. Remaining un-entrapped substances can then be washed away, leaving only substance-loaded erythrocytes (S-IEs).

[33] In one embodiment, the dye entrapping (or encapsulation) is as follows. Fresh blood in acid-citrate-dextrose anticoagulant is obtained under sterile conditions and centrifuged to obtain erythrocytes. These are then washed in a buffer to remove surface proteins, leukocytes and platelets and then centrifuging. The erythrocytes are then suspended at about 70% hematocrit (Ht) in the washing buffer solution inside a

dialysis tube, where they are dialyzed against a dialysis buffer. ICG dye is then added to the dialyzed erythrocyte solution, and the mixture is incubated under gentle agitation. The erythrocytes (now with reduced hemoglobin content) are then resealed by returning the dialyzed erythrocyte solution to a normotonic state and incubating the cells. The  
5 resealed cells are washed several times and centrifuged each time. About 9 ml of whole blood yields 4 ml of packed (about 80% Ht) ICG-loaded erythrocytes. This same basic methodology can be elaborated to satisfy several approaches to providing S-IEs to be used in several different circumstances:

**1. Ad-hoc Preparation of Small Volumes of S-IEs for Autologous Re-injection:**

10 **[34]** One embodiment provides a method for an end-user who wants to use S-IEs for a specific subject, using the subject's own erythrocytes, or for introducing substances in a desired combination or concentration.

**[35]** One embodiment provides method comprising:

- 15 (a) providing erythrocytes in a blood anti-coagulant solution;
- (b) dialyzing the erythrocyte-containing solution in (a) against a dialysis buffer having an osmolality ranging from 50-70 mOsm/kg to cause pores in the erythrocyte to open;
- (c) combining the dialyzed solution in (b) with at least one fluorescent dye; and
- 20 (d) combining the dye-containing solution in (c) with a resealing solution having an osmolality of at least 1000 mOsm/kg to reduce the osmolality, thereby causing the erythrocyte pores to close and entrap the at least one fluorescent dye within the erythrocytes.

25 **[36]** In one embodiment, the providing in step (a) comprises obtaining a blood sample from a subject.

**[37]** In one embodiment, the dialyzed solution in (b) has an osmolality ranging from 80-90 mOsm/kg, e.g., an osmolality ranging from 85-87 mOsm/kg.

**[38]** In one embodiment, the method further comprises washing the erythrocytes with an isotonic solution prior to the dialyzing in (b).

**[39]** In one embodiment, the at least one fluorescent dye in (c) is indocyanine green dye having a concentration ranging from 0.25 to 3.0  $\mu\text{moles/mL}$  of dialyzed  
5 solution.

**[40]** One embodiment provides a kit comprising:  
a blood anti-coagulant;  
a dialysis buffer having an osmolality ranging from 50-70 mOsm/kg;  
a dialysis chamber;  
10 at least one fluorescent dye; and  
a resealing solution having an osmolality of at least 1000 mOsm/kg.

**[41]** In one embodiment, the dialysis chamber has suspended from its top a dialysis tube with a molecular weight cut-off ranging from 12,000 to 14,000 Daltons. In one embodiment, the dialysis tube allows introduction of a blood-containing fluid into the  
15 tube, and a dialysis fluid can be introduced outside the suspended tube.

**[42]** Small quantities of erythrocytes, such as would be needed for autologous re-injection into a single individual can be prepared under sterile conditions, using his own blood, in about 1.75 hours time. However, since laboratory sterile conditions are seldom conveniently available to locations where frequent diagnostic and therapeutic  
20 procedures are carried out, a convenient way to prepare or acquire the erythrocytes is a requirement if such procedures are to be broadly applied. One embodiment provides kits (one for each individual subject) each consisting of a series of disposable sterile containers, pre-loaded with appropriate amounts of chemicals. A freshly obtained volume of blood can be transferred, in sequence from one to another, until the  
25 substance-loading steps are completed (see FIGs. 9, 10, and 11). S-IEs prepared in this manner can be stored for use for several days at 4°C, until used.

[43] A small refrigerator containing a small fixed-speed, fixed- time centrifuge can be used. The refrigerator space (accessible from its top, to keep the cold air reasonably contained) can be large enough to hold and pre-cool a kit and to provide a cool sterile workspace, as well as a place to temporarily store prepared S-IEs until used.

5 [44] The advantage of this basic method of preparation is ad hoc introduction of substances or mixtures of substances (i.e., simultaneous introduction of two or more discrete substances, each at a different concentration). Also, autologous re-injection of a subject's cells assures that on contamination of incompatibility of human protein will be encountered. Although no further processing is required, so long as the cells are  
10 stored at proper temperature and that they are used within several days, the cells may be preserved for much longer periods by some of the methods described below. (See Examples 1 and 2)

[45] Accordingly, another embodiment provides a kit, comprising:

a blood anti-coagulant;

15 a plurality of hypotonic solutions;

at least one fluorescent dye; and

a resealing solution having an osmolality of at least 1000 mOsm/kg.

[46] In one embodiment, the kit provides a plurality of hypotonic solutions to successively diluting a blood sample and achieve a final osmolality ranging from 80-90  
20 mOsm/kg, e.g., ranging from 85-87 mOsm/kg. In one embodiment, the plurality of solutions comprise at least three or at least four hypotonic solutions, each having a different osmolality. In one embodiment, the plurality of solutions can have the same or different osmolalities, so long as the maximum osmolality any of the solutions is 300 mOsm/kg and the minimum osmolality of any of the solutions is 50 mOsm/kg.

25 [47] In any kit embodiments disclosed herein, the kit further comprises an isotonic saline washing solution comprising glucose or trehalose, e.g., a 5 mM glucose solution or at least a 50 mM trehalose solution (e.g., a 50 mM trehalose solution).

[48] Another embodiment provides a method, comprising:

(a) combining erythrocytes with a blood anti-coagulant to form a solution having an osmolality of at least 300 mOsm/kg;

(b) successively diluting the solution in (a) with at least three hypotonic solutions to reduce the osmolality to less than 100 mOsm/kg to cause the erythrocyte pores to open;

(c) combining the diluted solution in (b) with at least one fluorescent dye; and

(d) combining the dye-containing solution in (c) with a resealing solution having an osmolality of at least 1000 mOsm/kg to reduce the osmolality, thereby causing the erythrocyte pores to close and entrap the at least one fluorescent dye within the erythrocytes.

**[49]** In one embodiment, the kits or methods disclosed herein include an isotonic resealing solution having an osmolality of at least 2000 mOsm/kg.

**[50]** In one embodiment, after performing any method disclosed herein, after step (d), the method further comprises:

(e) washing the erythrocytes in an isotonic saline washing solution containing glucose or trehalose to remove extracellular dye.

**[51]** In one embodiment, the washing is performed with a saline solution containing 50 mM trehalose.

**[52]** In one embodiment, the kits or methods disclosed herein provide at least one fluorescent dye, such as indocyanine green (ICG) dye. In one embodiment, the dye concentration of a composition comprising the entrapped dye ranges from 0.25 to 1.5 mM. In another embodiment, the dye concentration ranges from 0.3 to 0.4 mM. In one embodiment, this concentration optimizes fluorescence intensity to facilitate visualization of the cells. In yet another embodiment, the dye concentration is greater than 0.4 mM. This concentration can facilitate absorption by the cells of infra-red wavelengths, thereby rendering the cells capable of lysing upon heating.

[53] In one embodiment, the source of erythrocytes is O-type blood. In another embodiment, the erythrocyte source blood of A-type or B-type. In the latter embodiment, the method can further comprise adding  $\alpha$  -  $\beta$  glucosidase to split off the erythrocyte surface A- and B-agglutinogens.

5 [54] In one embodiment, the composition comprising the dye entrapped in the erythrocytes further comprises at least one biocompatible excipient. In one embodiment, the at least one excipient is polysucrose. In one embodiment, the at least one excipient concentration ranges from 0.5% to 5%.

## 2. Pre-prepared Small Volumes of S-IEs for Universal Injection:

10 [55] One embodiment provides a method for producing S-IEs for end-users who want access to S-IEs without having to acquire blood from their subjects. This method can be performed on a large scale and can supply multiple end-users.

[56] In situations where application of the above basic methodology may be inconvenient due to constraints of facilities location or time, it is possible to instead  
15 make use of S-IEs obtained from a source other than the subject. Using the basic methodology, aliquots from large volumes of erythrocytes from human O-negative blood type donors pre-loaded with various substances or mixtures of substances, can be slowly frozen and maintained at cryogenic temperatures until needed for universal  
20 injection. Such an approach can be used to create substance-loaded cells that can be universally injected into human subjects, while obviating the necessity to first obtain the subject's own blood and the time required for perpetration. This approach is particularly suited to preparations of cells loaded with substances likely to be used frequently in numerous subjects, such as for performance of standardised diagnostic or therapeutic procedures, where ad hoc use of substances is not contemplated.

25 [57] Alternatively, the pool of subjects that forms the source of erythrocytes for bulk preparation of universally injectable substance-loaded erythrocytes can be expanded beyond those having only type O-negative by using  $\alpha$  -  $\beta$  glucosidase to split off the cell surface A- and B-agglutinogens at the beginning of the cell-loading process.

There appears to be nothing definitive in the literature to indicate the threshold level at which any cell membrane surface antigens not removed by washing or other treatment would induce an immunologic reaction. Since it is anticipated, especially with regard to diagnostic use, that the maximum volume of processed cells re-injected will be on the order of only 1 or 2 ml, there is minimal likelihood of inducing an immunologic reaction.

### 3. Preparation of Off-the-Shelf S-IEs for Universal Injection

[58] Freeze-drying S-IEs prepared for universal injection into which the desired substances have been inserted at desired concentrations results in an off-the-shelf preparation that is easily stored at room temperature for prolonged periods (in excess of a year). Reconstitution requires only addition of sterile water, making such a product the most convenient form of injectable S-IEs. This would appear to be the most convenient method for rendering the substance-loaded cells stable and easily reconstituted.

[59] Past attempts to develop a method for freeze-drying erythrocytes such that reconstitution of the cells results in erythrocytes with an intact cytoskeleton and with biologically active hemoglobin (i.e., completely biologically viable cells) have proven illusive; the fact that such a preparation is not readily available underscores this. Generally, when erythrocytes have been lyophilized by previous methods, for example in either an aqueous or phosphate-buffered saline (PBS) solution, the reconstituted cells are damaged to the extent that they are not capable of metabolizing (that is, the cell hemoglobin cannot carry oxygen), and/or the yield of intact, non-deformed cells is unacceptably low. A factor in causing the damage may arise in the freezing process, where ice crystals form internal and external to the cells, thereby disrupting cell structure. Attempts to obviate this problem seem mainly to have included procedures that utilize cooling rates that are faster than ice crystal formation (see for example, United States Patent 5656498), or inclusion in the cell suspension fluid a monosaccharide which may or may not diffuse into the cells, the presence of which suppresses ice crystallization during cooling (see for example, United States Patent

5340592). However, it appears that these approaches have failed to produce sufficiently high yields of functional cells upon reconstitution to be useful in the clinical arena.

[60] In the methods disclosed herein, containing composition comprising the entrapped dye is frozen or freeze-dried. Accordingly, disclosed herein are frozen or freeze dried (lyophilized) compositions comprising a freeze dried (lyophilized) composition comprising at least one fluorescent dye entrapped within erythrocytes.

[61] In one embodiment, the erythrocytes in (d) further entraps a substance (e.g., while in a hypotonic state) that inhibits the destructive formation of ice crystals during cooling. In one embodiment, the substance is a monosaccharide, e.g., trehalose. Accordingly, in one embodiment, erythrocytes can further entrap trehalose or at least one therapeutically effective agent..

[62] One embodiment relates to the recognition that obviating the problems encountered by these various previous attempts lies in recognition of two advantages associated with the methods of use and production of substance-loaded erythrocytes with respect to use and production methods aimed at long-term storage of metabolically functional blood. The first is that production of metabolically normal O<sub>2</sub>-carrying cells upon reconstitution is not important for the diagnostic and treatment applications of S-IEs; in fact, replacement of a portion of the cells' hemoglobin content is part of the loading process. Secondly, the methods of S-IE production all involve opening of pores in the cells' membranes to allow equilibrium between the cells' internal contents and the solution in which the cells are suspended. This latter situation readily facilitates controlled introduction of an adequate amount of monosaccharide (preferably, trehalose) to prevent ice crystal formation during the freeze-drying process; this is accomplished by substituting the 50 mM trehalose for the 5 mM glucose in the washing buffer solution composition described in the Example 1 Ad-hoc Preparation of ICG dye-loaded Human Erythrocytes for Autologous Re-injection. Once this is done, the finished substance-loaded cells are diluted in to a 50% "hematocrit" in the same trehalose washing buffer, polysaccharide (5%) is added as an excipient to reduce cell clumping

upon reconstitution, when 1 ml of distilled water is added to the freeze-dried pellet in the stoppered vial. As demonstrated in FIG. 4, the reconstituted ICG-loaded human erythrocytes are equivalent in fluorescence intensity to that they possesses prior to freeze-drying.

## 5 **Diagnostic Application of Fluorescent Dye-loaded Erythrocytes**

[63] Medical angiographic imaging typically involves administration of a detectable substance to a subject, as described, e.g., in U.S. Patent Nos. 6,915,154 and 6,351,663, the disclosures of which are incorporated herein by reference. In some instances the detectable substance may also be a therapeutic agent, as described in, 10 e.g., U.S. Patent Publication No. 2004/0206364, the disclosure of which is incorporated herein by reference.

[64] Aside from their role in supplying the metabolic requirements of the tissues they serve, among the naturally occurring particles in blood, as well as the artificial ones that have been introduced into blood circulation for various purposes, 15 erythrocytes are well suited for use as substance carriers. They are the largest naturally occurring blood-borne particles, and from a hemodynamic point of view, they are essentially as passive as serum, adding only resistance to blood flow because of their size and mass. (By comparison, leukocytes and thrombocytes are biologically active, so their movements in flowing blood are influenced by other than serum fluid 20 dynamics. For example, leukocytes, which are part of the immune system, exhibit a drag-and-roll behavior as they pass the endothelial surface of vessel walls.) Moreover, despite their large volumes, S-IEs retain the capability of the original erythrocytes to deform, allowing them to pass through small diameter capillaries; this feature overcomes the size limitation inherent in use of artificial carriers (such as liposomes) 25 whose geometry always is rigid. These attributes of erythrocytes make them ideal for transporting the fluorescent dyes used in angiography. Unlike conventional angiograms showing dye-stained plasma movement, which often show a poorly defined wave front that transits only once, angiograms showing individual fluorescent erythrocytes can

provide continuous information about blood flow speed and direction in a multitude of vessels simultaneously. There are several aspects of creating and using dye-loaded erythrocytes (e.g., ICG-loaded erythrocytes) for angiography:

[65] 1. Dye-loaded erythrocytes, rather than dye-stained ones, are  
5 desirable because dye binding to the outer erythrocyte membrane appears to be weak (i.e., not covalent binding). Energy imparted to those cells "stained" with dye as a result of turbulent blood flow, for example, is sufficient to break those weak bonds. Then dye molecules become available to bond with other nearby proteins, quickly resulting in plasma and blood vessel wall staining that diminishes contrast between the erythrocytes  
10 and their background, while at the same time diminishing the brightness of the erythrocytes themselves. Moreover, it is not possible to bind enough dye to the cell membranes to achieve the peak fluorescence intensity needed for adequate visualization of individual cells (see FIGs. 1A and 1B). On the other hand, the dye-loading process permits control of the amount of dye inserted into erythrocytes, such  
15 that the dye concentration inside the cells is very near—but does not exceed—the concentration at which fluorescence quenching occurs(cf. 1A and 1B to 1D). In the case of ICG, this is achieved by addition of 1 $\mu$ mol of ICG dye to each millilitre of the dialysed erythrocyte solution at that point in the substance-loading procedure when the volumes of the cells have increased to the extent that pores in the cells' membranes  
20 have opened (see FIG. 2). With the pores open, trans-membrane exchange of large molecules occurs; haemoglobin inside the cells moves to the outside until a dynamic equilibrium between inward and outward haemoglobin movement is reached. ICG dye added to the solution in which the cells are suspended binds with hemoglobin molecules, and in time, those molecules move into the cells and reach concentration  
25 equilibrium with those outside the cells. When the pores in the cells are closed and dye/haemoglobin molecules outside the cells are washed away, the remaining dye-loaded cells fluoresce optimally when stimulated by appropriate excitation light energy.

[66] 2. Once filled with dye-stained plasma, as in conventional fluorescent dye angiography, blood vessels exhibit only steady-state fluorescence, inhibiting

visualization of any blood-borne particles, even if the particles themselves were fluorescent. However, it is possible to visualize individual erythrocytes in capillaries if they, rather than the plasma, are emitting fluorescence and if separation between adjacent erythrocytes exceeds the diffraction limited resolution of the optics of the eye (about 7  $\mu\text{m}$  for the perifoveal capillaries and 11  $\mu\text{m}$  for the choriocapillaris (see Flower RW. Optimizing treatment of choroidal neovascularization feeder vessels associated with age-related macular degeneration. *Am J. Ophthalmol.* 2002; 134:228-239. <sup>8</sup>). These conditions were met by entrapping ICG dye in erythrocytes, as described above, and re-injecting a small bolus of them. During transit to the ocular vasculatures, they are diluted in circulating blood to the extent that individual ICG-loaded cells were separated by more than 11  $\mu\text{m}$ , making visualization of them possible. It has been discovered that using erythrocytes loaded so as to produce optimal fluorescence produces the strongest fluorescence signal for any given level of excitation light irradiance. For reasons of safety, it is desirable to remain as far below the maximum permissible exposure (MPE) for a given tissue as possible.

[67] 3. Visualization of circulating dye-loaded erythrocytes requires use of a device that irradiates the tissue field-of-view of interest with light energy at an appropriate wavelength (optimally, 805 nm for ICG). This can be efficiently done when that energy is applied as high-frequency trains of high-peak-power, short duration pulses of light, each of which illuminate the entire field-of-view of interest. This configuration results in a high image signal-to-noise ratio by requiring the lowest signal amplification, thereby minimizing introduction of electronic noise associated with amplification. Additionally, acquiring images during very short time periods in synchrony with short duration pulses of illumination acts essentially to freeze motion within each image, thereby obviating blurring associated with movement of erythrocytes within the field of view (FOV).

[68] The efficacy of using dye-loaded erythrocytes has been successfully demonstrated in both rabbit and non-human primate eyes<sup>9</sup>. (see Flower R, Peiretti E, Magnani M, Rossi L, Serafini S, Gryczynski Z, Gryczynski I., Observation of erythrocyte

dynamics in the retinal capillaries and choriocapillars using ICG-loaded erythrocyte ghost cells. Invest. Ophthalmol. Vis. Sci. 2008; 49: 5510-5516, the disclosure of which is incorporated herein by reference). Moreover, the results reported validated the prediction by Bek that all the clinical findings concerning edema in diabetic maculopathy can be explained as a result of disturbances in retinal vasomotion<sup>10</sup>. Results have also been obtained from the first human subjects in a preliminary clinical study to demonstrate feasibility of autologous re-injection of ICG-loaded erythrocytes for angiography (see FIG. 5). The human study protocol required injecting very small volumes of ICG-loaded cells in the first subjects and gradually increasing the volume in subsequent ones, until reaching a level equivalent to that used in the monkeys. Consequently, there are significant differences between the appearance of the published monkey angiograms, where the methodology had been optimized, and these from these first human subjects. The main reason is that the fraction of circulating fluorescent erythrocytes in these first human subjects was about 4-times less than in the monkeys.

[69] Nevertheless, these first human angiograms support some of the findings in the more extensive monkey studies. FIGs. 5A and 5B are from the normal right-eye of an age-related macular edema subject; FIG. 5B shows a single ICG-loaded erythrocyte angiogram image of the 10°-area field of view. The distribution of stalled erythrocytes is relatively even, consistent with what was reported for the normal monkey eye<sup>9</sup>. In the diseased left eye (FIGs. 5C and 5D), the image from the angiogram is centered over the choroidal neovascular lesion (CNV). The distribution of stalled erythrocytes surrounding the CNV is similar to that of the normal fellow eye, but in the CNV no erythrocytes are apparent. When watching the actual angiogram movie, however, a few erythrocytes eventually do pass through the CNV area; but they do so at about half the speed as those erythrocytes which are not stalled in the area surrounding the CNV or those in the normal eye. (This may be the result of both plasma skimming and of higher CNV resistance to blood flow.) Minimally, the first human angiograms demonstrate that ICG-loaded erythrocytes can be acquired and that apparently the

presence of vasomotion—or lack of it—can be determined thereby. Based on Bek's work <sup>10</sup>, visualization of erythrocytes can be used as a predictor for retinal edema onset and for monitoring early efficacy of treatment. Also, clearly ability to track individual erythrocytes makes possible routine quantifiable assessment of blood flow in any vessel.

### **Exogenous Substances for EGC Fluorescence Enhancement**

**[70]** In one embodiment, the methods disclosed herein further comprise the step of adding metallic colloids prior to step (d). In one embodiment, the metallic colloids are silver.

**[71]** Metallic silver colloids in close proximity to weakly fluorescing dyes can markedly increase fluorescence yields. It has been shown that if ICG molecules are brought into close proximity by first binding silver colloids to amino groups that have been plated on a glass surface and then binding ICG/albumin conjugates to the same surfaces, fluorescence yields from the ICG increased more than 30-fold <sup>11</sup>. Subsequently, experiments were carried to determine if the same enhancement effect would occur in vivo in ICG angiograms made in rabbit eyes.

**[72]** Samples of ICG dye alone and of dye/Ag-colloid suspension, containing identical concentrations of dye (0.250 mg/ml) were injected into rabbit ear veins, and ICG fluorescence angiograms recorded. The fundus of the eye was uniformly illuminated by pulsed laser diode light at 805 nm wavelength, and as the dye transited through the ocular vasculatures, the 835 nm wavelength fluorescence light emitted by the dye was digitally recorded. Comparison of dye only and dye/colloid-suspension angiograms from the same eyes were made, using images from the same phase of vascular dye filling in each case. As indicated by the example angiogram images in FIG. 6, the fluorescence intensity of the dye/suspension ranged from only two to three times greater than that from dye alone.

**[73]** The disparity between fluorescence enhancement reported in the experiments made using glass surfaces on which the dye/colloid conjugates were

bound with amino groups (30-fold) and those observed in the in vivo angiograms (2- to 3-fold) apparently was due to the fact that the colloid fluorescence-enhancement effect is manifest in very thin layers of the dye/suspension and suppressed in thick ones. This is demonstrated by comparing fluorescence from thick (1 cm) and thin (100  $\mu\text{m}$ ) layers of both samples, as shown in FIG. 7. In this figure, the thick samples are at the top, and the thin ones at the bottom; 0.250 mg/ml ICG in HAS is on the left, and 0.250 mg/ml ICG in HAS with 4.684 mg/ml Ag colloid on the right. The array of samples was uniformly illuminated with 805-nm wavelength light, and the ICG fluorescence image was captured. In the case of the thick samples, the ICG-only on the left is significantly brighter, while in the case of the thin samples, the dye/colloid suspension is significantly brighter. In part, the explanation for these relationships is that colloids in suspension scatter light, as shown in FIG. 8, wherein the same array of samples in FIG. 7 is uniformly illuminated with 810 nm wavelength light and an image made from the reflected light. Note that both the thick and thin dye/colloid samples reflected—rather than absorbed or transmitted—the incident light; that is, the observed light interactions with the dye/suspensions arise as a surface phenomenon. Consequently, more dye molecules in the ICG-only thick sample interacted with light than did those in the dye/suspension thick sample. In the case of the two thin samples, however, the fluorescence enhancement in the dye/suspension sample resulted in brighter fluorescence from the ICG dye molecules present than from the same density of molecules in the dye-only sample. This was because more of the incident light was transmitted by the dye-only sample than by the dye/colloid-suspension one.

[74] Based on the above observations, achieving significant ICG fluorescence enhancement in angiography through association of the dye with metallic colloids requires not only that the dye molecules be held in close association with the colloids, but that the dye/colloid conjugation be contained in thin layers. These conditions can be met by containing the dye/colloid conjugates within S-IEs, wherein the small amount of hemoglobin remaining in the cells is sufficient to act as the ICG protein-binding site. Thus, significant and useful enhancement of the dye fluorescence from ICG-loaded

EGCs can be achieved by encapsulation of silver metallic colloid during the S-IE loading process.

### **Therapeutic Application of Substance-loaded EGCs**

**[75]** In one embodiment, the erythrocytes entrap at least one therapeutically effective agent. Since S-IEs essentially are closed-volume circulating containers in which the concentration of inserted molecules can be precisely controlled, S-IEs can be used to transport their contents, sequestered from the blood, to a given vascular location, where their presence can be detected and their contents released. Such a scenario assumes that the vascular location is visually accessible, that the S-IEs contain a detectable marker (such as a fluorescent dye) in addition to the transported substance, and that a means to release the S-IEs' contents exists.

**[76]** Controlled substance release in targeted vascular areas by this method makes possible use of substances that would be prohibited if delivery were by conventional means. This is because when encapsulated in S-IEs and injected in a bolus of packed cells, the aggregate substance volume introduced is very small. So long as that aggregate volume is on the order of 1/1 00th of the threshold volume for producing a pharmacological effect (therapeutic or toxic), it generally is considered micro-dosing. (In Canada, Europe, and the U.S., now human experimental IND studies often can proceed on the basis of just small animal data. This puts such studies in a range more likely to be affordable by smaller companies.) Therefore, the spectrum of dyes that might be considered potentially usable is greatly expanded when administered as micro-doses.

**[77]** S-IEs maintain the shape, behavior, and longevity of the erythrocytes from which they are derived, so they are able to deform in order to pass through small diameter capillaries, making them ideal vesicles for carrying and delivering substances intravascularly. Re-injected substance-loaded erythrocytes can circulate for up to about 120 days (the average lifetime of erythrocytes), during which time their contents will be released by one means or another. Because any population of S-IEs behaves the same

as any population of normal erythrocytes with respect to eryptosis, unless externally triggered to do so, only a small fraction of injected cell contents will be released at any particular time, complete release being spread-out over approximately 120 days. And since only very small volumes of cells are injected, levels of molecules released into the body at any point in time—or even in the aggregate—should always be orders of magnitude below effect or toxic thresholds. Moreover, since the distribution of S-IEs eventually would be uniform throughout the total circulating blood volume, further diluting the concentration of gradually released substance to possibly undetectable (or at least, trace) levels.

[78] During initial transit through the circulation, an injected packed S-IE bolus remains relatively intact. Within a few minutes following injection, the S-IEs become fairly distributed throughout the body, and unless somehow targeted in a specific area of the body, thereafter substance release is gradual over a 120-day period. In vasculatures that can be directly observed by optical means, release of the drug content of circulating S-IEs can be triggered by application of light energy, especially during initial and early transit. Such light energy could easily be focused to cover any desirable area, small enough to cover a single cell or large enough to cover the vasculature of an entire organ.

[79] Direct visualization facilitates control over the amount of substance released, by taking into account that, for any given area of vasculature anywhere in the body, the density of circulating S-IEs will be greatest during initial transit and will rapidly diminish with passage of time until only a few cells will be present in each capillary of the visualized area. For example, if the light energy were applied to a certain vascular area during the initial passage of the injected cells, released substance concentration would be high; if applied a few minutes later, the concentration would be much less, but always in proportion to the ratio of S-IEs to normal circulating erythrocytes. Even though the aggregate volume of injected substance encapsulated in S-IEs may be at the micro-dose level, when released only within the blood volume of a small area of vasculature, substance concentration could be very high.

[80] Accordingly, in combination with the methods disclosed herein wherein step (d) results in a first population of dye-entrapped erythrocytes, after step (d) the method further comprises:

(e) repeating steps (a) and (b) with a second sample of erythrocytes;

5 (f) repeating step (c) with at least one fluorescent dye at a concentration higher than that of step (c);

(g) repeating step (d) with the product of (f) to form a second population of dye-entrapped erythrocytes; and

(h) combining the first and second populations.

10 [81] In one embodiment, the method further comprising adding at least one therapeutically effective agent at step (e) or (f), wherein the second population further comprises agent-entrapped erythrocytes. Numerous therapeutically effective agents can be envisioned for pre-loading. Exemplary drugs include ganciclovir (antiretroviral),  
triamcinolone acetonide (steroid); fluocinolone and dexamethasone (ocular-specific  
15 steroids); pegaptanib sodium and rhuFab V2 (Anti-VEGF, anti-angiogenic); verteporfin (benzoporphyrin derivative) (Photodynamic:therapy); and carboplatin and topotecan (chemotherapeutic).

[82] In one embodiment, the at least one dye in the first population has a concentration ranging from 0.3 to 0.4 mM, and the at least one dye in the second  
20 population has a concentration greater than 0.4 mM,

wherein upon illumination with 805 nm laser energy, the erythrocytes of the first population will fluoresce, and

wherein increasing the laser energy heats the erythrocytes of the second population due to their enhanced absorption, causing them to lyse and release the  
25 entrapped therapeutic agent.

[83] Ability to release an encapsulated substance from S-IEs requires ability to detect or observe presence of the cells in the target area, so that release-stimulating

light energy can be applied precisely at the time the desired concentration of cells is present. This is accomplished by including a fluorescent dye (such as ICG) in the cells, along with the therapeutic substance. A software algorithm applied to the video images of the vasculature of interest can be used to track in real time the number-and hence, density-of S-IEs present in the vascular area of interest, and when the desired level is reached, it can automatically trigger application of the release-stimulating light energy. Knowing the density of cells present prior to triggering release may be necessary to assure that not so many cells simultaneously rupture that significant reduction or occlusion of blood flow occurs.

[84] Accordingly, one embodiment provides a software algorithm applied to the video images of a vascular area of interest that can track in real time the number-and hence, density-of S-IEs present in the vascular area of interest. In one embodiment, when a predetermined level of S-IEs density is reached, application of light energy capable of causing the substance-loaded erythrocytes to release their contents will be triggered.

[85] In another embodiment, a software algorithm takes into account the respective volumes of two different substance-loaded erythrocyte populations injected as a mixed bolus, the total volume of circulating blood, and the time elapsed following injection, will, based on the detection of S-IEs of one type, calculate the density of the other type present.

[86] In one embodiment, where the type of S-IE detected is loaded with ICG dye, and the second type of S-IE present is loaded with a therapeutic agent as well as the highest possible concentration of ICG, the latter substance being entrapped to facilitate absorption of infra-red wavelengths, thereby heating and lysing the cells.

[87] The applied light can interact with the S-IEs in a number of ways to cause release of the cells' contents. For example, the light can be selectively absorbed by the cells, thereby raising their temperatures to the point they rupture, or it might react with a specific molecule associated with the cell membrane that will compromise the membrane 's structural integrity, causing rupture. Selective absorption of light energy

can be accomplished by applying a wavelength that is efficiently absorbed by some substance associated with the substance-loaded cells. For instance, assuming those cells also contain a fluorescent dye to facilitate visualizing them, then a higher power level of the same fluorescence-stimulating light can be applied to just the target vascular area rather than the entire field of view. Of course, if this approach is used, then, it becomes desirable to have as much of the dye as possible present in the S-IEs for efficient light absorption.

[88] If, however, the dye in question undergoes fluorescence concentration quenching, then there is an optimum concentration of its molecules, in any given volume and for any given illumination level at which optimum fluorescence occurs. Above or below that optimum concentration, reduced fluorescence intensity occurs, and exceeding the maximum permissible exposure (MPE) cannot compensate for that. (For example, the specific methodology employed to insert ICG into S-IEs is such that only the optimum concentration of dye molecules is present in the loaded cells, about 0.03 mg/ml; above that level fluorescence quenching occurs.) Thus, the optimum inter-cellular dye concentration for stimulating cell fluorescence is not necessarily high enough to act as an absorber of laser energy for purposes of heating the cells to the level that they lyse and release their contents; optimum absorption calls for the highest possible dye concentration. The solution to this dilemma is to inject a mixture of two species of S-IEs: one containing just the fluorescent dye at the optimum concentration for maximum fluorescence, and the second one containing both the therapeutic substance and the dye at the maximum concentration possible. The mixture would have fewer of the former type cells than the latter, since the purpose of the first type is only to indicate presence of the loaded S-IEs and to facilitate determining their concentration within the vascular area of interest.

## EXAMPLES

**Example 1****Ad-hoc Preparation of ICG dye-loaded Human EGCs for Autologous Re-injection**

[89] ICG is encapsulated in human erythrocytes by a procedure of hypotonic dialysis, isotonic resealing and re-annealing. These steps are carried out using the various sterile containers (pre-loaded with appropriate fluids) included in a kit, as depicted in FIG. 9. Some of the containers are centrifuge tubes, and all containers and vials are disposable. FIG. 9 is a diagram of the sterile containers used in substance loading of erythrocytes. A: one 10-ml vacutainer containing an anticoagulant for acquisition of a blood sample. B: represents two 15-ml and two 50-ml centrifuge vacutainer tubes, each containing a pre-measured amount of isotonic saline washing buffer. C: a container having a rubber stopper top to which a length of dialysis tubing (sealed at its bottom) is affixed, such that an erythrocyte-containing solution can be introduced into the dialysis tube via an injection needle inserted through the rubber stopper. Outside the dialysis tube a small amount of washing solution keeps the dialysis tube membrane moist. D: a sealed container containing the volume of hypotonic dialysis buffer into which the dialysis tube containing the washed erythrocyte solution is lowered. In the center of this container's cover is a covered port into the rubber stopper of C fits. E: a rubber stoppered vial containing a pre-measured quantity of lyophilized ICG dye and a sealed vial containing a pre-measured volume of distilled H<sub>2</sub>O for reconstituting the dye. F: a rubber-stoppered vial containing a pre-measured amount of resealing solution. G: a rubber-stoppered vacutainer for storing the finished cells. H; one of a number of long (6") sterile needles for transferring cells from container to container.

[90] Nine (9) ml of fresh blood, to which an anti-coagulant (acid-citrate-dextrose or ethylenediaminetetraacetic acid) is added, are obtained under sterile conditions and centrifuged at 2000 g for 5 min to obtain at least 4.0 ml of erythrocytes

(see FIG. 10, A). Blood collection is done using a 10-ml vacutainer\* pre-loaded with anti-coagulant. This same tube is then inserted into the centrifuge, and the blood is separated, with the erythrocytes at the bottom. Using a long needle connected to a syringe, the 4-ml erythrocyte sample is extracted from the bottom, so as to exclude any  
5 white cells in the overlying buffy coat and serum. (\* Note: "vacutainer", as used throughout, refers to a variation of the sterilised rubber-stoppered evacuated test tube-like container commonly used for venipuncture. Each is constructed to withstand centrifugation, is sterilised, pre-filled with an appropriate fluid, and evacuated of air to form a vacuum.)

10 [91] The erythrocytes are then washed twice in 10 mM HEPES (pH 7.4) containing 154 mM NaCl and 5 mM glucose (washing buffer) to remove leukocytes and platelets, centrifuging at 2000 g for 5 minutes each time. This is done by injecting the erythrocytes in the syringe, from the above step, into the first of two 15-ml vacutainer pre-loaded with 10 ml of washing solution and centrifuged, again at 2000 g for 4 min..  
15 Once the erythrocytes are again concentrated in the tube bottom, they are removed using the long needle and syringe (see FIG. 10, B) and transferred to the second 15-ml vacutainer pre-loaded with washing solution, centrifugation is repeated and the concentrated erythrocytes again collected in a syringe.

[92] The erythrocytes then are suspended at 70% hematocrit (Ht) in the  
20 washing buffer solution inside a dialysis tube having a cut-off of 12-14 kD; this is done by injecting the syringe contents from the above step through the top and into the bottle containing the dialysis tube (see FIG. 11, A). The erythrocyte-containing dialysis tube is then removed from its bottle by lifting off the bottle top (to which the dialysis tube is attached) and transferring it to the large container (FIG. 11,B). The small lid of the large  
25 container is removed, and the dialysis bag is inserted through the opening and pressing it down to seal the container (see FIG. 11, C). Inside the large container, one volume of RBCs is dialysed for 90 min against 50 volumes of dialysis buffer (10 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM  $\text{NaHCO}_3$  and 20 mM glucose, pH 7.4) containing 3 mM reduced glutathione and 2 mM ATP. The osmolality of the buffer is about 60 mOsm, whereas that of the RBC

solution reached about 87 mOsm at the end of the dialysis time. All these procedures are performed at 4°C.

[93] The dialysis tube is then removed from the large container and returned to its original bottle, at which time 1 µmol of ICG dye is then added to each millilitre of the dialysed RBC solution (see FIG. 11,D), and the mixture is incubated for 30 min at 70°C  
5 under gentle agitation. (The correct amount of lyophilised ICG is provided in a small vial to which a per-measured volume is added.)

[94] The erythrocytes are resealed by adding 0.1 vol of, 100 mM inosine, 20 mM ATP, 4 mM MgCl<sub>2</sub>, glucose anhydrous 100 mM, sodium pyruvate 100 mM, 190 mM  
10 NaCl, 1666 mM KCl and 33 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4) per volume of dialysed erythrocyte solution (this solution is pre-measured and provided in a sterile ampoule). The dialysis bag in its bottle is placed in a small bath at 37°C, and the cells are incubated for 25 min.

[95] The resealed cells are washed 4 times in the washing buffer (50 ml each time) and centrifuged each time for 10 min at 500 g; there is a cell recovery of about  
15 40%. Typically about 32 ml of whole blood yields 16 ml of packed (70 Ht) ICG-loaded erythrocytes. This is done according to the same procedure shown in FIG. 10, B, and finally the processed ICG-loaded erythrocytes are injected into the provided sterile storage vial and stored at 4°C until used for angiography on the subject from whom the cells were obtained.

## 20 **Example 2**

### **A Non-dialysis Alternative to the Method in Example 1**

[96] ICG encapsulation in human erythrocytes by a procedure of hypotonic dialysis, isotonic resealing and re-annealing similar to the method described in Example 1 can be carried out without recourse to a dialysis step. This is done by decreasing the  
25 tonicity of the solution in which erythrocytes are suspended from 300 mOsm/kg to 87 mOsm/kg in four stages to open pores in the cells' membranes. These stages are all carried out in the same 50-ml centrifuge tube, at the completion of which ICG dye can

be introduced to the cell suspension solution. As in Example 1, a kit consisting of various sterile containers and pre-measured fluids is utilised. In this case, the a kit has different components than indicated in the one depicted in FIG. 9; this kit consists of:

- 5                   1 10-ml vacutainer \* containing an anti-coagulant (acid-citrate-dextrose)
- 2 15-ml vacutainers, each containing 10 ml sterilised washing buffer
- 1 50-ml vacutainer containing 4 ml of 198 mOsm/kg sterilised washing  
buffer
- 1 vial containing 10 ml of 198 mOsm/kg sterilised washing buffer
- 1 vial containing 20 ml of 99 mOsm/kg sterilised washing buffer
- 10               1 vial containing 29.8 mg of lyophilised ICG dye
- 1 syringe pre-loaded with 1.5 ml sterilised distilled H<sub>2</sub>O for reconstitution  
of the ICG
- 1 vial containing 3.8 ml of sterilised resealing solution
- 5 long (about 4 inch) sterile needles
- 15               3 50-ml vacutainers, each containing 40 ml normotonic sterilised washing  
buffer
- 1 5-ml rubber-stoppered, evacuated vial containing 1 ml of normotonic  
washing buffer

( \* Note: "vacutainer", as used throughout, refers to a variation of the sterilised rubber-stoppered evacuated test tube-like container commonly used for venipuncture. Each is constructed to withstand centrifugation, is sterilised, pre-filled with an appropriate fluid, and evacuated of air to form a vacuum.)

[97]   Nine (9) ml of fresh blood, to which an anti-coagulant (acid-citrate-dextrose or ethylenediaminetetraacetic acid) is added, are obtained under sterile  
25   conditions and centhugged at 2000 g for 5 min to obtain at least 4.0 ml of erythrocytes (see FIG. 10, A). Blood collection is done using a 10-ml vacutainer \* pre-loaded with anti-coagulant. This same tube is then inserted into the centrifuge, and the blood is separated, with the erythrocytes at the bottom. Using one of the two supplied long

needle connected to a syringe (see FIG. 9, H), the 4-ml erythrocyte sample is extracted from the bottom, so as to exclude any white cells in the overlying buffy coat and serum.

**[98]** The erythrocytes are then washed twice in 10 mM HEPES (pH 7.4) containing 154 mM NaCl and 5 mM glucose (washing buffer) to remove leukocytes and platelets, centrifuging at 2000 g for 5 minutes each time. This is done by injecting the erythrocytes in the syringe, from the above step, into the first of two 15-ml vacutainer pre-loaded with 10 ml of washing solution and centrifuged, again at 2000 g for 4 min.. Once the erythrocytes are again concentrated in the tube bottom, they are removed using one of the long needles and a syringe (see FIG. 10, B) and transferred to the second 15-ml vacutainer pre-loaded with washing solution, centrifugation is repeated and the concentrated erythrocytes again collected in a syringe.

**[99]** Three (3) ml of the twice-washed erythrocytes are added to a 50-ml vacutainer containing 4 ml of 198 mOsm/kg washing buffer and gently agitated for 20 min.

**[100]** The pre-measured 10 ml volume of 99 mOsm/kg washing buffer is added to a 50-ml vacutainer and gently agitated for an additional 20 min.

**[101]** The pre-measured 20 ml volume of 49 mOsm/kg washing buffer is added to a 50-ml vacutainer and gently agitated for a final 20 min.; this will produce a mixture at about 87 mOsm/kg, causing pores in the erythrocytes to opened.

**[102]** The pre-measured 1.5 ml volume of distilled H<sub>2</sub>O is added to the vial containing 29.8 mg vial of lyophilised ICG to reconstituted it, and the liquid dye is then added to the contents of the 50ml vacutainer, resulting in a dye concentration of 1µmol/ml. The 50-ml vacutainer is gently agitated in a 37°C bath for 20 min.

**[103]** The erythrocytes are resealed by adding the pre-measured 3.8 ml volume of, 100 mM inosine, 20 mM ATP, 4 mM MgCl<sub>2</sub>, glucose anhydrous 100 mM, sodium pyruvate 100 mM, 190 mM NaCl, 1666 mM KCl and 33 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4) to the solution in the 50-ml vacutainer to make the mixture normotonic; it is agitated and kept in the 37°C bath for 20 min.

[104] The 50-ml tube is centrifuged at 2000 g for 5 min, after which one of the supplied long needles and a syringe are used to remove the 3-ml volume of cells at the bottom of the tube and transfer them to a fresh 50-ml stoppered tube containing 40 ml of washing buffer. This tube is agitated and centrifuged at 2000 g for 4 min.

5 [105] The procedure of paragraph [052] is repeated twice more, after which 1-2 ml of the thrice-washed dye-loaded erythrocytes at the bottom of the tube are removed with the last of the long needles and a syringe and deposited in a sterile 5-ml rubber-stoppered, evacuated vial containing 1-2 ml of the normotonic washing buffer. These cells at about 50% Ht can be stored at 4°C for up to several days prior to re-injection for  
10 angiography.

#### References:

1. DeLoach R. Encapsulation of exogenous agents in erythrocytes and the circulating survival of carrier erythrocytes. *J Appl Biochem.* 1983; 5:149-57.
2. De Loach JR Sprandel U: Red blood cells as carriers for drugs. Basel  
15 Karger 1985; De Flora A PNAS 1986; 83:7029.
3. Ropars C, Chassaing M, Nicolau C, Red Blood Cells as carriers for drugs. Potential therapeutic applications. Oxford, Pergamon Press, 1987.
4. Rossi L, Brandi G, Schiavano GF, Scarfi S, Millo E, Damonte G, Benatti U, De Flora A, Magnani M. Heterodimer-loaded RBCs as bioreactors for slow delivery of  
20 the antiviral drug azidothymidine and the antimycobacterial drug ethambutol. *AIDS Res Hum Retrovir* 1999; 15:345-353.
5. Rossi L, Serafini S, Cenehni L, Picardi F, Bigi L, Panzani I, Magnani M. RBC-mediated delivery of dexamethasone in patients with chronic obstructive pulmonary disease. *Biotechnol Appl Biochem.* 2001 ; 33:85-89.
- 25 6. Rossi L, Castro M, D'Orio F, Damonte G, Serafini S, Bigi L, Panzani I, Novelli G, Dallapiccola B, Panunzi S, Di Carlo P, Bella S, Magnani M. Low doses of

dexamethasone constantly delivered by autologous RBCs slow the progression of lung disease in cystic fibrosis patients. *Blood Cells Mol Dis.* 2004; 33:57-63.

7. Annese V, Latiano A, Rossi L, Lombardi G, Dallapiccola B, Serafini S, Damonte G, Andriulli A, Magnani M. RBCs-mediated delivery of dexamethasone in steroid-dependent IBD patients-a pilot uncontrolled study. *Am J Gastroenterol.* 2005; 100:1 370-1 375.

8. Flower RW. Optimizing treatment of choroidal neovascularization feeder vessels associated with age-related macular degeneration. *Am J. Ophthalmol.* 2002; 134:228-239.

9. Flower R, Peiretti E, Magnani M, Rossi L, Serafini S, Gryczynski Z, Gryczynski I., Observation of erythrocyte dynamics in the retinal capillaries and chohocapillahs using ICG-loaded erythrocyte ghost cells. *Invest. Ophthalmol. Vis. Sci.* 2008; 49: 551 0-551 6.

10. Bek T. Diabetic maculopathy caused by disturbances in retinal vasomotion: a new hypothesis. *Acta Ophthalmol Scand.* 1999; 77:376-380.

11. Geddes CD, Haishi C, Gryczynski I, Gryczynski Z, Jiyu F, Lakowicz JR. Metal-Enhanced Fluorescence (MEF) Due to Silver Colloids on a Planar Surface: Potential Applications of Indocyanine Green to in Vivo Imaging. *J Phys. Chem. A.* 2003: 107:3443-3449.

20

CLAIMS

1. A kit comprising:
  - a blood anti-coagulant;
  - a dialysis buffer having an osmolality ranging from 50-70 mOsm/kg;
  - 5 a dialysis chamber;
  - at least one fluorescent dye; and
  - a resealing solution having an osmolality of at least 1000 mOsm/kg.
2. The kit of claim 1, wherein the dialysis chamber has suspended from its top a dialysis tube with a molecular weight cut-off ranging from 12,000 to 14,000  
10 Daltons.
3. The kit of claim 2, wherein the dialysis tube allows introduction of a blood-containing fluid into the tube, and a dialysis fluid can be introduced outside the suspended tube.
4. A method comprising:
  - 15 (a) providing erythrocytes in a blood anti-coagulant solution;
  - (b) dialyzing the erythrocyte-containing solution in (a) against a dialysis buffer having an osmolality ranging from 50-70 mOsm/kg to cause pores in the erythrocyte to open;
  - (c) combining the dialyzed solution in (b) with at least one fluorescent  
20 dye; and
  - (d) combining the dye-containing solution in (c) with a resealing solution having an osmolality of at least 1000 mOsm/kg to reduce the osmolality, thereby causing the erythrocyte pores to close and entrap the at least one fluorescent dye within the erythrocytes.

5. The method of claim 4, wherein the providing in step (a) comprises obtaining a blood sample from a subject.

6. The method of claim 4 or 5, further comprising washing the erythrocytes with an isotonic solution prior to the dialyzing in (b).

5 7. The method of any one of claims 4 to 6, wherein the at least one fluorescent dye in (c) is indocyanine green dye having a concentration ranging from 0.25 to 3.0  $\mu\text{moles/mL}$  of dialyzed solution.

8. The method of any one of claims 4 to 7, wherein the dialyzed solution in (b) has an osmolality ranging from 80-90 mOsm/kg.

10 9. A kit comprising:  
a blood anti-coagulant;  
a plurality of hypotonic solutions;  
at least one fluorescent dye; and  
a resealing solution having an osmolality of at least 1000 mOsm/kg.

15 10. The kit of claim 9, wherein the plurality of hypotonic solutions comprises at least three hypotonic solutions, each having a different osmolality, wherein the maximum osmolality is 300 mOsm/kg and the minimum osmolality is 50 mOsm/kg.

11. The kit of claim 10, wherein the plurality of hypotonic solutions comprises at least four hypotonic solutions.

20 12. The kit of any one of claims 1-3, and 9-11, further comprising an isotonic saline washing solution comprising glucose or trehalose.

13. A method comprising:  
(a) combining erythrocytes with a blood anti-coagulant to form a solution having an osmolality of at least 300 mOsm/kg;

(b) successively diluting the solution in (a) with at least three hypotonic solutions to reduce the osmolality to less than 100 mOsm/kg to cause the erythrocyte pores to open;

5 (c) combining the diluted solution in (b) with at least one fluorescent dye; and

(d) combining the dye-containing solution in (c) with a resealing solution having an osmolality of at least 1000 mOsm/kg to reduce the osmolality, thereby causing the erythrocyte pores to close and entrap the at least one fluorescent dye within the erythrocytes.

10 14. The method of any one of claims 4 to 6 and 13, wherein after step (d), the method further comprises:

(e) washing the erythrocytes in an isotonic saline washing solution containing glucose or trehalose to remove extracellular dye.

15 15. The method of any one of claims 4 to 6, 13, and 14, wherein the washing solution contains trehalose having a concentration of at least 50 mM.

16. The kit or method of any preceding claim, wherein the fluorescent dye is indocyanine green (ICG) dye.

17. The kit or method of claim 16, wherein the concentration of the entrapped dye in (d) ranges from 0.25 to 1.5 mM.

20 18. The kit or method of claim 16, wherein the concentration of the entrapped dye in (d) ranges from 0.3 to 0.4 mM.

19. The kit or method of claim 16, wherein the concentration of the entrapped dye in (d) is greater than 0.4 mM.

25 20. The method of any one of claims 4 to 8 and 13 to 19, further comprising the step of adding at least one therapeutically effective agent prior to step (d) to entrap the at least one agent within the erythrocytes.

21. The kit or method of any preceding claim, wherein the resealing solution has an osmolality of at least 2000 mOsm/kg.

22. The kit or method of any one of claims 5 to 8 and 13 to 21, wherein a source of the erythrocytes in (a) is O-type blood.

5 23. The kit or method of any one of claims 5 to 8 and 13 to 21, wherein a source of the erythrocytes in (a) is A-type or B-type blood.

24. The method of claim 23, wherein after step (a), the method further comprises adding  $\alpha$  -  $\beta$  glucosidase to split off the erythrocyte surface A- and B-agglutinogens.

10 25. The method of any one of claims 4 to 8, 13, and 15 to 24, wherein after step (d), the entrapped dye is frozen or freeze-dried.

26. The method of claim 14, wherein after step (e), the entrapped dye is frozen or freeze-dried.

15 27. The method of any one of claims 4 to 8 and 13 to 26, wherein the erythrocytes in (d) further entraps a substance that inhibits the destructive formation of ice crystals during cooling.

28. The method of claim 27, wherein the substance is a monosaccharide.

29. The method of claim 27, wherein the monosaccharide is trehalose.

20 30. The method of any one of claims 4 to 8, 13, and 15 to 29, wherein after step (d), the method further comprises adding at least one biocompatible excipient.

31. The method of claim 14, wherein after step (e), the method further comprises adding at least one biocompatible excipient.

32. The method of claim 30 or 31, wherein the at least one excipient is polysucrose.

33. The method of any one of claims 30 to 32, wherein the at least one excipient concentration ranges from 0.5% to 5%.

34. The method of any one of claims 4 to 8 and 13 to 33, further comprising the step of adding metallic colloids prior to step (d).

5 35. The method of claim 34, wherein the metallic colloids are silver.

36. The method of any one of claims 4 to 8 and 13 to 35, wherein step (d) results in a first population of dye-entrapped erythrocytes, and wherein after step (d), the method further comprises:

(e) repeating steps (a) and (b) with a second sample of erythrocytes;

10 (f) repeating step (c) with the product of (e) and at least one fluorescent dye at a concentration higher than that of step (c);

(g) repeating step (d) with the product of (f) to form a second population of dye-entrapped erythrocytes; and

(h) combining the first and second populations.

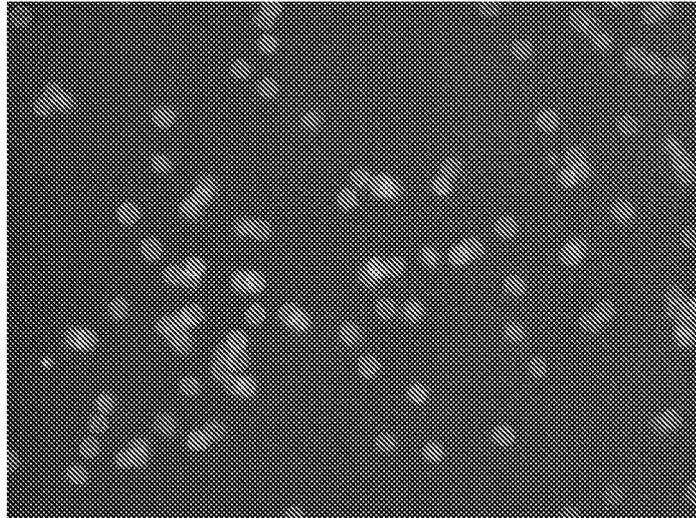
15 37. The method of claim 36, further comprising adding at least one therapeutically effective agent at step (e) or (f), wherein the second population further comprises agent-entrapped erythrocytes.

20 38. The method of claim 37, wherein the at least one dye in the first population has a concentration ranging from 0.3 to 0.4 mM, and the at least one dye in the second population has a concentration greater than 0.4 mM,

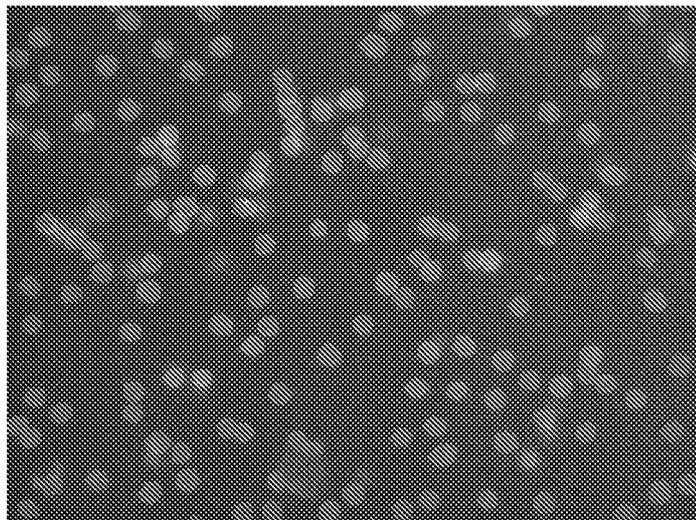
wherein upon illumination with 805 nm laser energy, the erythrocytes of the first population fluoresce, and

25 wherein increasing the laser energy heats the erythrocytes of the second population due to their enhanced absorption, causing them to lyse and release the entrapped therapeutic agent.

1/14



**FIG. 1A**

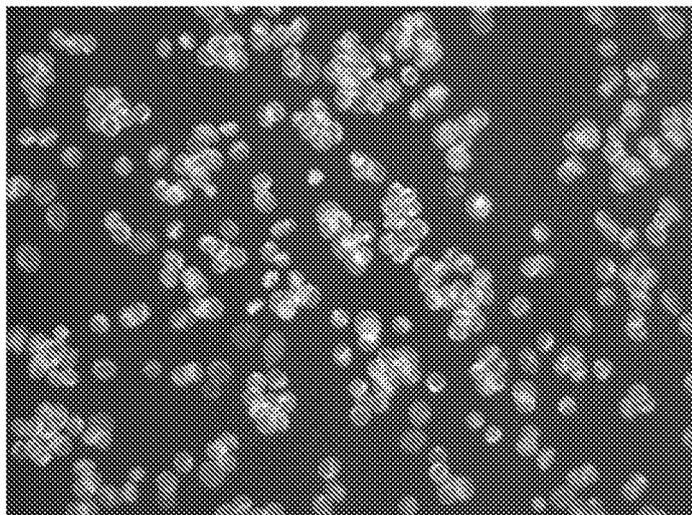


**FIG. 1B**

2/14

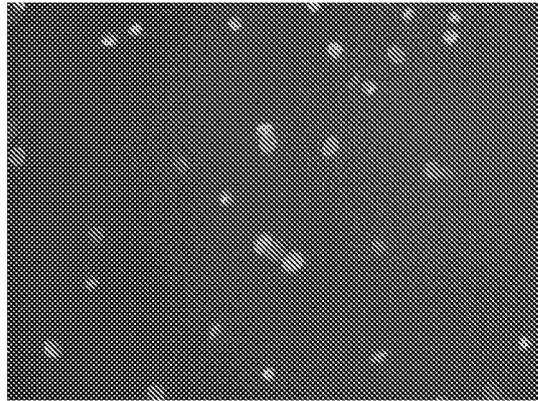


**FIG. 1C**

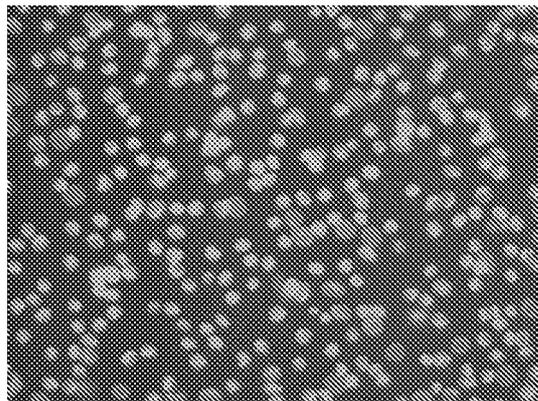


**FIG. 1D**

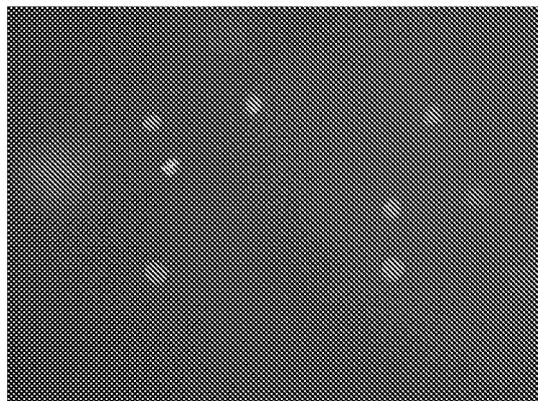
3/14



**FIG. 2A**

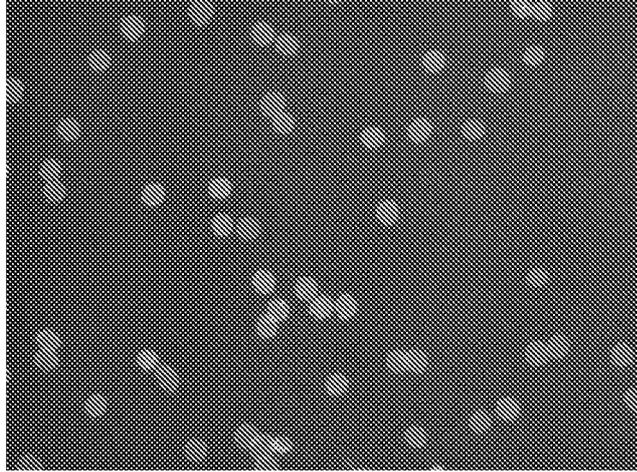


**FIG. 2B**

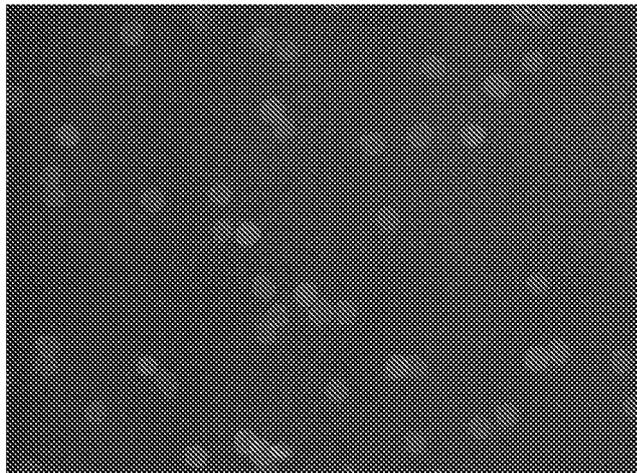


**FIG. 2C**

4/14

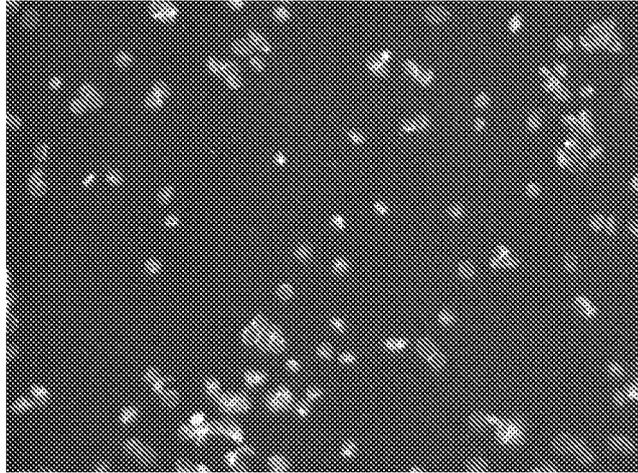


**FIG. 3A**

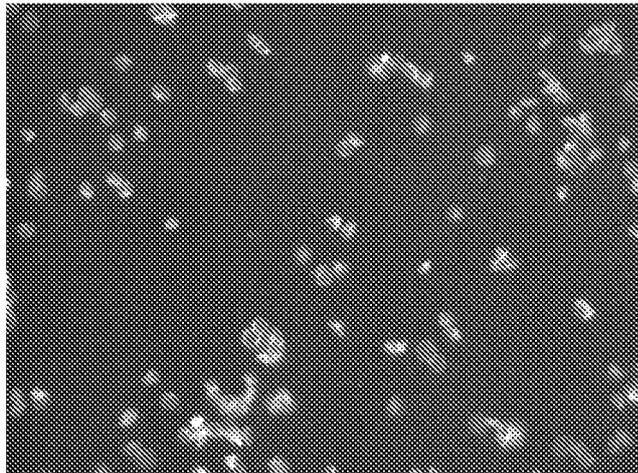


**FIG. 3B**

5/14

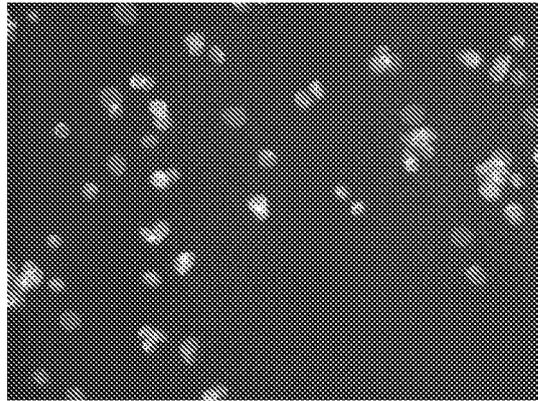


**FIG. 3C**

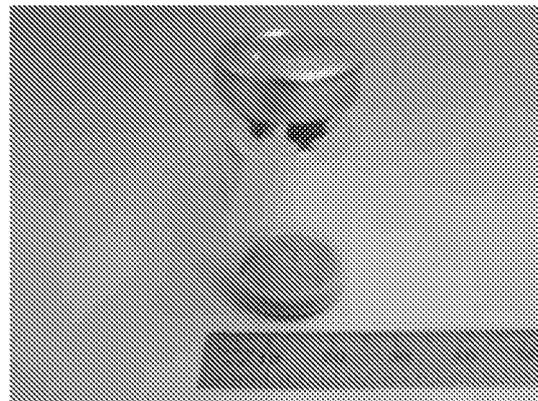


**FIG. 3D**

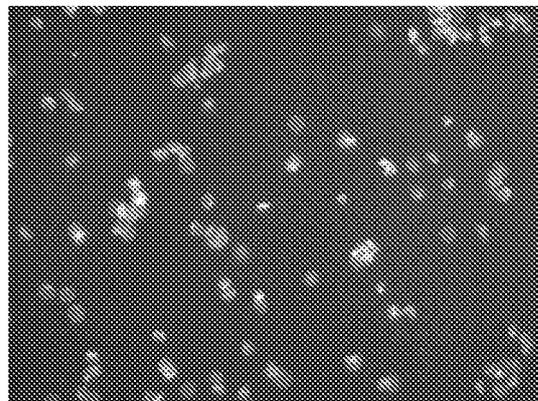
6/14



**FIG. 4A**

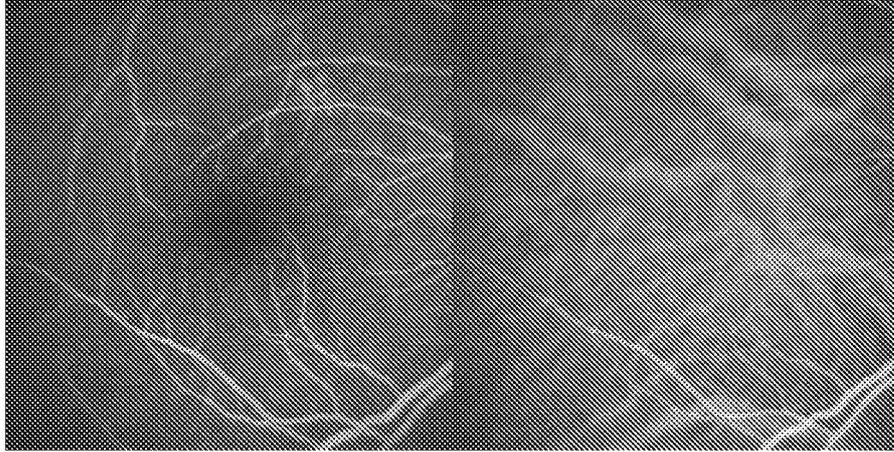


**FIG. 4B**

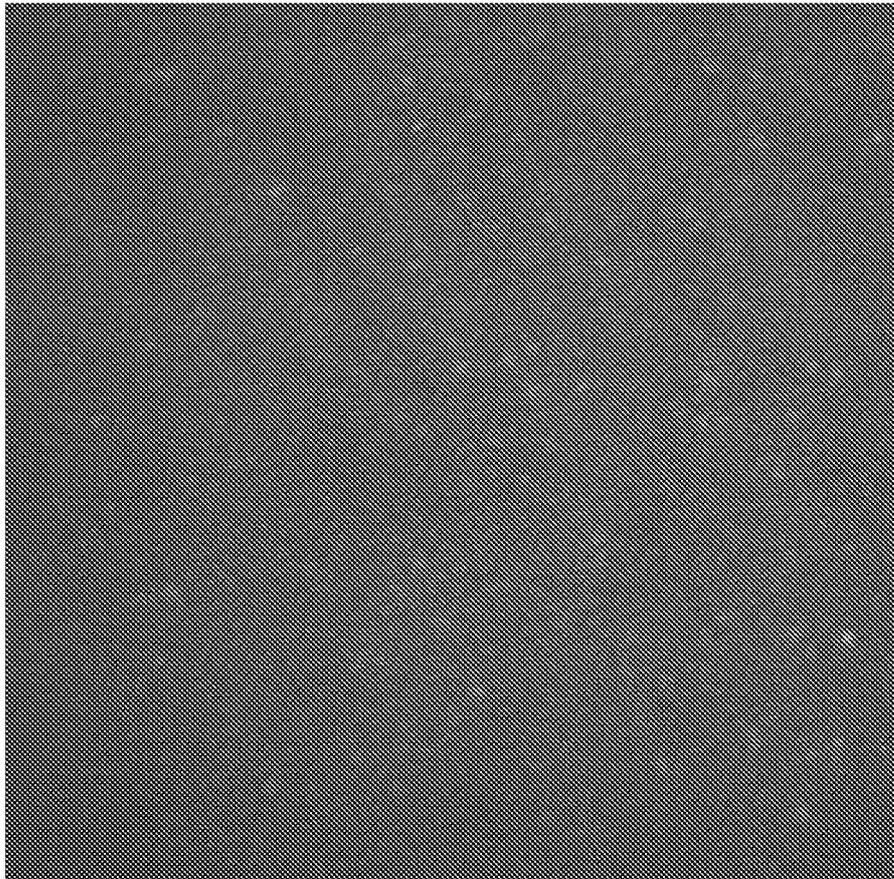


**FIG. 4C**

7/14

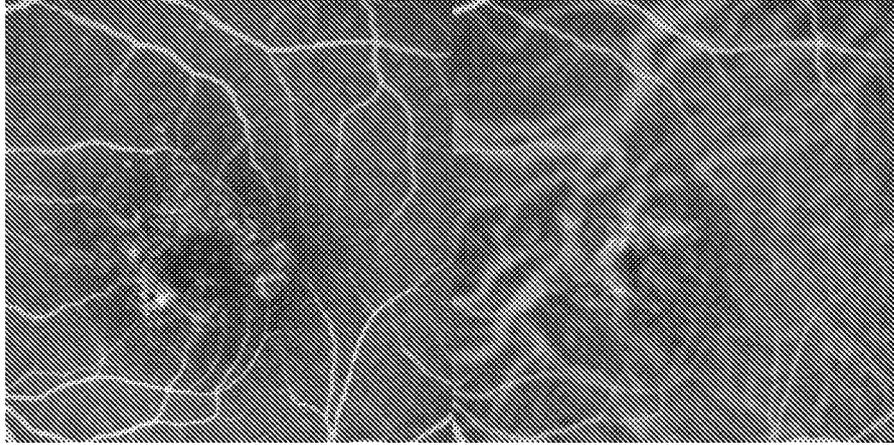


**FIG. 5A**

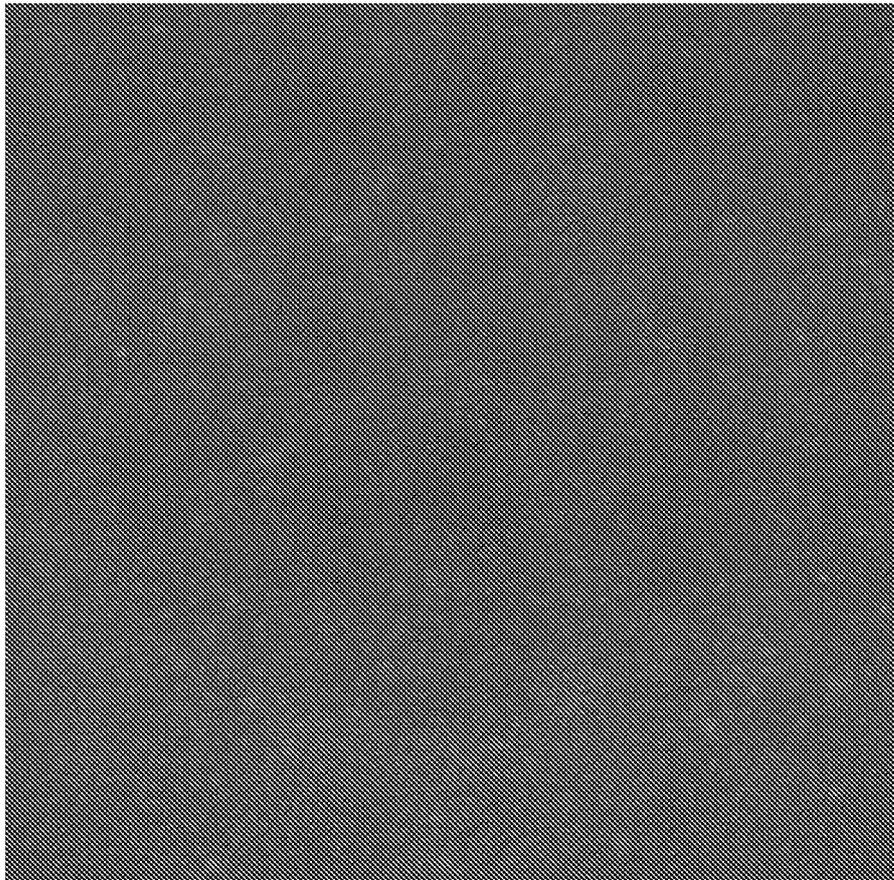


**FIG. 5B**

8/14

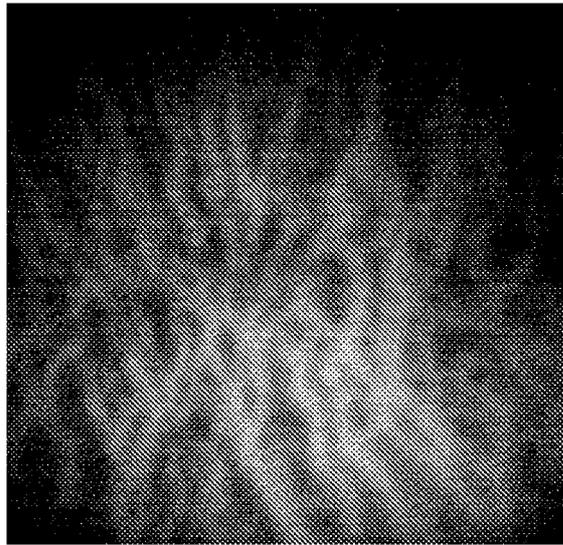


**FIG. 5C**

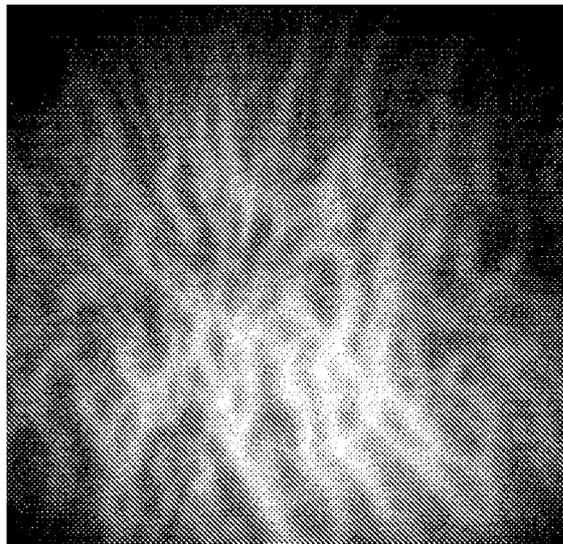


**FIG. 5D**

9/14

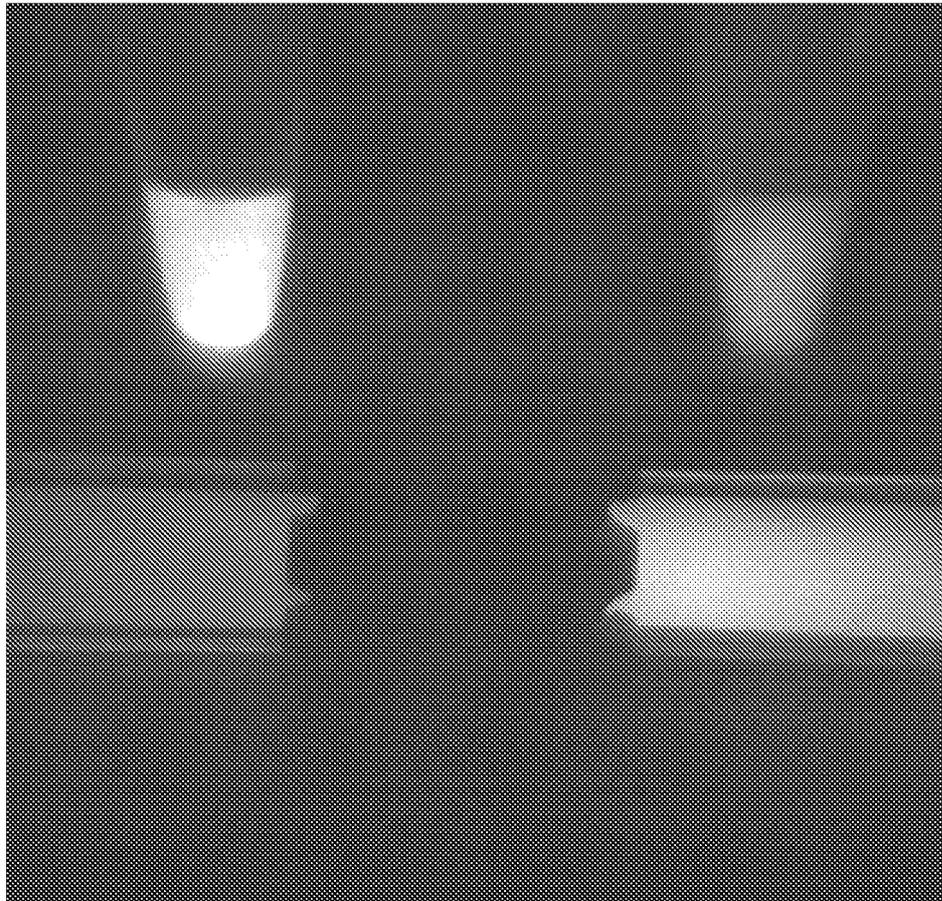


**FIG. 6A**



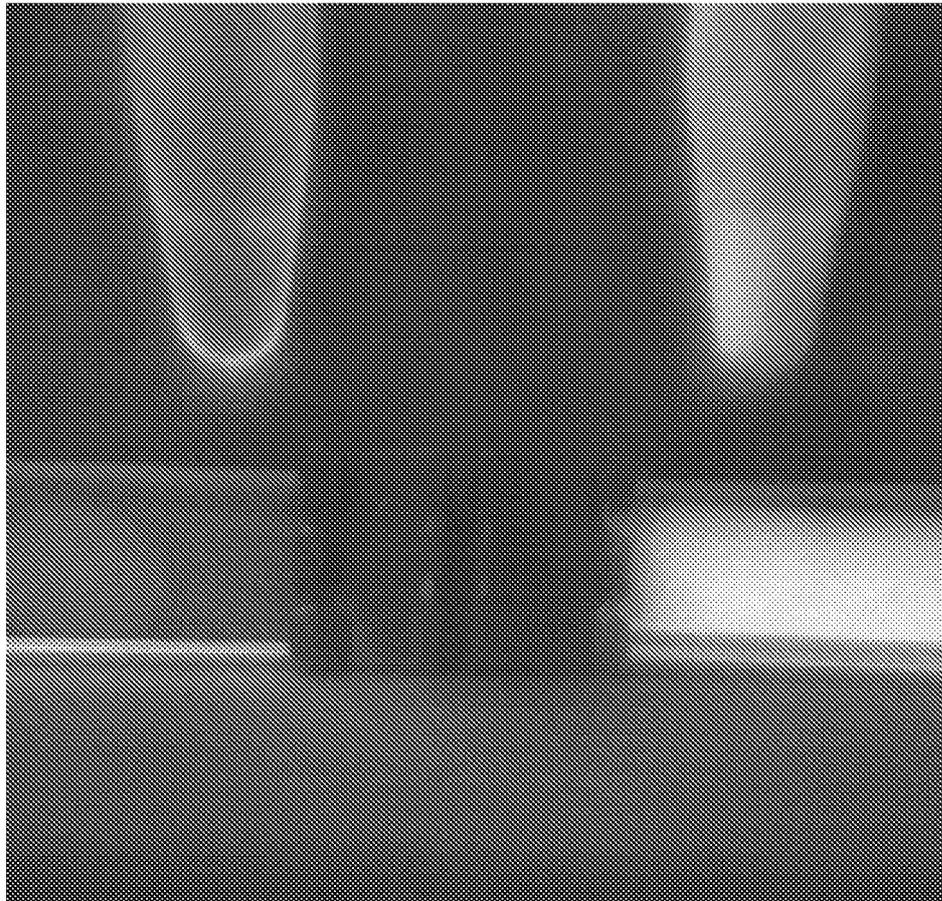
**FIG. 6B**

10/14



**FIG. 7**

11/14



**FIG. 8**

12/14

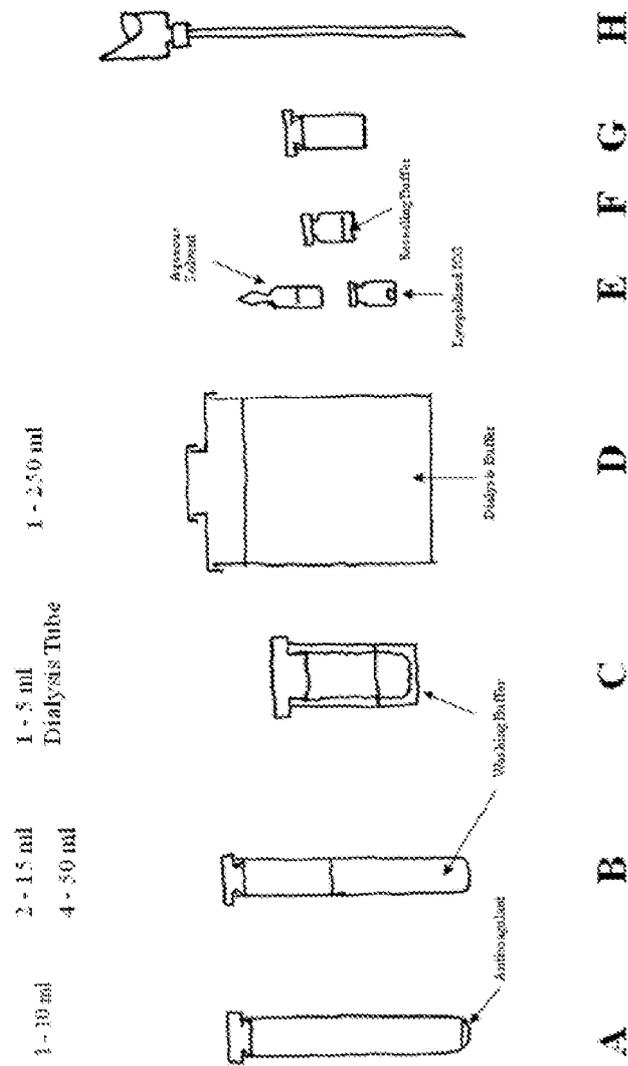
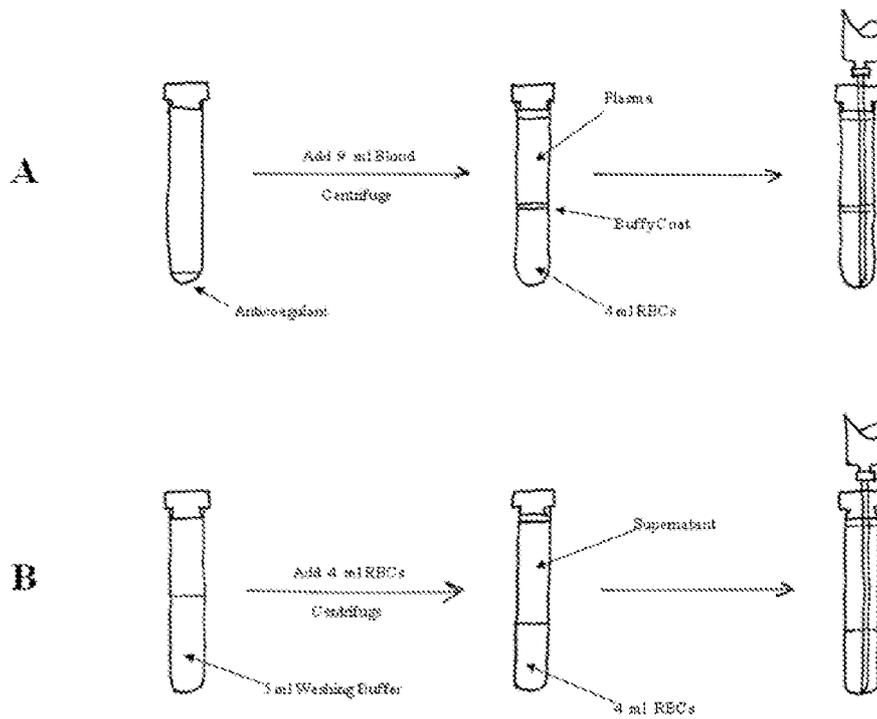


FIG. 9



**FIG. 10**

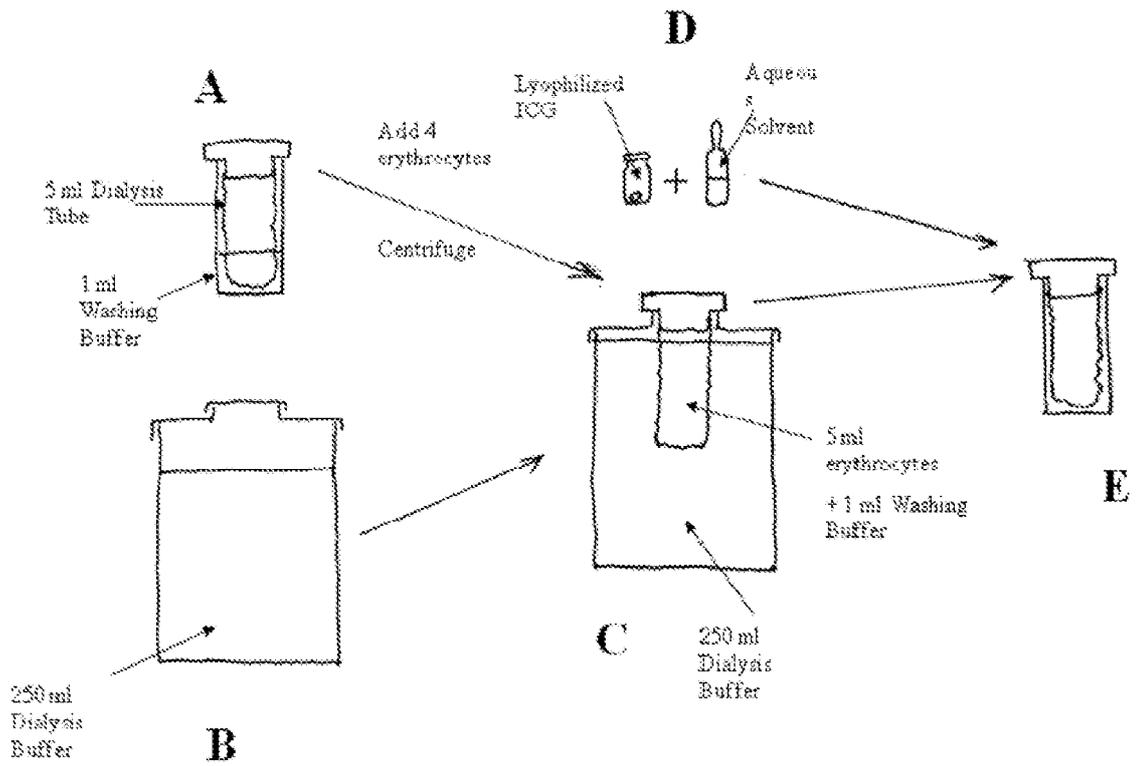


FIG. 11