Journal of Visualized Experiments

Quantification of Cellular Densities and Antigenic Properties Using Magnetic Levitation

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62550R1
Full Title:	Quantification of Cellular Densities and Antigenic Properties Using Magnetic Levitation
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TITLE:

Quantification of Cellular Densities and Antigenic Properties Using Magnetic Levitation

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KEYWORDS:

Magnetic levitation, Cell Density, Bead-Cell Complex, Fluorescence Microscopy

SUMMARY:

This paper describes a magnetic levitation-based method that can specifically detect the presence of antigens, either soluble or cell-bound, by quantifying changes in the levitation height of beads with set densities.

ABSTRACT:

The described method was developed based on the principles of magnetic levitation, which separates cells and particles based on their density and magnetic properties. Density is a cell type identifying property, directly related to its metabolic rate, differentiation, and activation status. Magnetic levitation allows a one-step approach to successfully separate, image and characterize circulating blood cells, and to detect anemia, sickle cell disease, and circulating tumor cells based on density and magnetic properties. This approach is also amenable to detecting soluble antigens present in a solution by using a set of low-, and one of high-density beads coated with capture and detection antibodies, respectively. If the antigen is present in solution, it will bridge the two set of beads, generating a new bead-bead complex, which will levitate in between the rows of antibody-coated beads. Increased concentration of the target antigen in solution will generate a larger number of bead-bead complexes when compared to lower concentrations of antigen, thus allowing for quantitative measurements of the target antigen. Magnetic levitation is advantageous to other methods due to its decreased sample preparation time and lack of

dependance on classical readout methods. The image generated is easily captured and analyzed using a standard microscope or mobile device, such as a smartphone or a tablet.

INTRODUCTION:

Magnetic levitation is a technique developed to separate, analyze, and identify cells types[1-3], proteins[4, 5] and opioids[6] based solely on their specific density and paramagnetic properties. Cell density is a unique, intrinsic property of each cell type directly related to its metabolic rate and differentiation status[7-14]. Quantifying subtle and transient changes in cell density during steady state conditions, and during a variety of cell processes, could afford one an unmatched insight into cell physiology and pathophysiology. Changes in cell density are associated with cell differentiation[15,16], cell cycle progression[9,17-19], apoptosis[20-23], and malignant transformation[24-26]. Therefore, quantification of specific changes in cell density, can be used to differentiating between cells of different types, as well as discriminating between cells of the same type undergoing various activation processes. This enables experiments targeting a particular cell sub-population, where dynamic changes in density serves as an indicator of altered cell metabolism[27]. As it has been established that a cell may alter its density in response to a changing environment[7], it is imperative to measure the kinetics of the cell in relation to its density to understand it fully, which current methods may not provide[12]. Magnetic levitation on the other hand, allows for a dynamic evaluation of cells and their properties[28].

Cells are paramagnetic meaning they have no magnetic properties. When suspended in a magnetic solution and introduced to a magnetic field, cells are repelled by the source to a specific levitation height. Diamagnetic levitation of an object confined to the minimum of an inhomogeneous magnetic field is possible when the two following criteria are fulfilled: 1) the magnetic susceptibility of the particle must be smaller than that of the surrounding medium, and 2) the magnetic force must be strong enough to counterbalance the particle's buoyancy force. It has been demonstrated that both criteria can be fulfilled by suspending RBCs in a magnetic buffer and by creating strong magnetic field gradients with small, inexpensive, commercially available permanent magnets[1]. The equilibrium position of a magnetically trapped particle on an axis along the direction of gravity is determined by its density (relative to the density of the buffer), its magnetic susceptibility (relative to the magnetic susceptibility of the buffer), and the signature of the applied magnetic field. As the density and the magnetic properties of the solution are constant throughout the system, the intrinsic density properties of the cells will be the major factor determining the levitation height of the cells, with denser cells levitating lower compared to less dense cells. This approach uses a set of two density reference beads (1.05 and 1.2 g/mL) that allows us to use precise, ratiometric analysis for density measurements. Altering the concentration of the magnetic solution allows one to isolate different cellular populations, such as RBCs from WBCs, as the density of circulating cells is cell specific, removing the need for isolation protocols or other cell manipulation.

The majority of detection methods used in biology research rely on extrapolation of specific binding events into easy to quantify linear signals. These readout methods are often complex and involve specialized equipment and dedicated scientific personnel. An approach aimed at the detection of antigens found either on the plasma membrane of cells or extracellular vesicles or

soluble in plasma, using either one or two antibody coated beads, is herein described. The beads must be of different densities from each other and from those of the interrogated targets. The presence of the target antigen in any given biofluid is translated into a specific, measurable change in the levitation height of an antigen-positive cell that is bound to a detection bead. In the case of soluble antigens or extracellular vesicles, they are bound to both capture and detection beads, forming a bead-bead complex rather than bead-cell complex. The change in levitation height depends on the new density of the bead-cell or bead-bead complexes. In addition to the change in the levitation height of the complexes, which indicates the presence of antigen in the biofluid, the number of complexes is also dependent on the amount of target, making magnetic levitation also a quantitative approach for antigen detection[24].

PROTOCOL:

The experimental protocol used in this study was approved by the Beth Israel Deaconess Medical Center Institutional Review Board (IRB).

1. Instrument setup

NOTE: Imaging levitating cells requires two rare earth neodymium magnets magnetized on the z-axis to be placed with the same pole facing each other to generate a magnetic field. The distance between the magnets can be customized depending on the intensity of the magnetic field and the density of the targets. In this case the magnets are separated by a 1mm space sufficient for insertion of a 50 mm long 1 x 1 mm squared glass capillary tube. The device was 3-D printed using an AutoCAD design, which is available upon request.

1.1. Lay microscope on its side, perfectly horizontal.

NOTE: A microscope placed in a standard upright position is not directly suitable for imaging levitating objects due to the positions of the magnets with respect to condenser and objective. This limitation can be bypassed by laying the microscope on its side, perfectly horizontal allowing the condenser to focus the light into the capillary, and the objective lens to image the cell levitating in between the magnets, while maintain Kohler illumination requirements.

1.2. Support and level the stand on a breadboard table using 2 or 3 lab jacks.

1.3. To limit vibrations, support the breadboard table with rubber dampening feet.

1.4. Remove the stage and replace it with a compact lab jack for adjusting the height of the levitation device (*y-axis*), and two single-axis translational stages; one for adjusting the focus (*z-axis*), and the second one for scanning the capillary tube.

1.5. Attach the magnetic levitation device to the lab jack using two mini-series optical posts.

2. Binding of Antibody to Carboxy-Microparticles/Beads (Modified from a protocol by PolyAn)

NOTE: Only low-density beads (1.05 g/mL) need to be coated for Rh(+) detection, but both high- and low-density beads are coated for the detection of extracellular vesicles.

2.1. Take out a 1 mg equivalent of bead suspension and add into 0.5 mL of Activation Buffer (50 mM MES (MW195.2, 9.72 mg in 1 mL)) pH 5.0 and 0.001% Polysorbate-20).

2.2. Add 12 µL of freshly made 1.5 M EDC (MW 191.7, 0.28755 g in 1 mL) and 12 µL of 0.3 M Sulfo-NHS (MW 217.14, 0.0651 g in 1 mL) in ice-cold water.

2.3. Shake vigorously for 1 h at room temperature to activate the carboxyl groups on beads.

2.4. Stop the activation after 1 h by adding 0.5 mL of Coupling Buffer (10x PBS, or 0.1 M phosphate pH 7-9).

2.5. Pellet the beads by centrifuging at the 20,000 x *g* for 10 min, or, if the beads cannot be pelleted, use a 0.45 µm centrifuge tube filter. Aspirate the supernatant or flow-through.

2.6. Wash the beads with 1 mL of 10x PBS 3 times as in step 2.5.

2.7. Calculate 25 µg of antibody per mg beads and mix desired antibody with activated beads to a 0.7-1.0 mg/mL final antibody concentration in 10X PBS.

2.8. Roll tubes gently at room temperature overnight for coupling.

2.9. Repeat step 2.5 and wash the beads twice with 1 mL of 10x PBS.

2.10. Wash with 0.5 mL of 1 M ethanolamine (98% stock = 16.2 M) in buffer pH 8.0 with 0.02% Polysorbate-20 for 1 h at room temperature while gently shaking.

2.11. Repeat step 2.5 and wash the beads once in 1 mL of DPBS. The beads can be stored in 200 µL of DPBS at 4 °C until needed.

NOTE: The protocol can be paused here.

3. Collection and Preparation of Blood for Rh(+) Detection

3.1. Using a one-click lancing device, prick the finger of an Rh(+) donor and collect 10 µL of blood into 1 mL of DPBS.

3.2. 3.2. Stain the Rh+ cells with a fluorescent plasma membrane stain. Optionally, add 1 µL of fluorescent dye to the 1 mL suspension of Rh+ cells (1:1000 dilution).

3.3. Incubate the cells with the fluorescent dye at 37 °C for 15 min.

3.4. Pellet the cells by spinning at 5,600 x *g* for 15 s and wash 3 times using 1 mL of DPBS. Resuspend in 1 mL of HBSS++.

3.5. Using the one-click lancing device, prick the finger of an Rh(-) donor and collect 2 µL of blood.

NOTE: If preparing more than 2 conditions, collect enough blood to add 1 µL of Rh(-) blood to each tube.

3.6. Prepare the necessary experimental tubes: Beads alone, IgG control, sample.

3.6.1. Beads alone: add 174 µL of HBSS++, 1 µL of IgG control beads, 1 µL of high-density beads (1.2 g/mL), and 24 µL of 500 mM Gd^{3+} (60 mM).

3.6.2. IgG control: add 172 µL of HBSS++, 1 µL of IgG control beads, 1 µL of high-density beads, 1 µL of Rh- blood, 1 µL of stained Rh+ blood suspension, and 24 µL of 500 mM Gd^{3+}.

3.6.3. Sample tube**:** add 172 µL of HBSS++, 1 µL of anti-RhD coated beads, 1 µL of high-density beads, 1 µL of Rh- blood, 1 µL of stained Rh(+) blood suspension, and 24 µL of 500 mM Gd^{3+}.

NOTE: High density beads are added to Rh samples for reference.

4. Isolation of PMNs for Cell Separation Demonstration

4.1. Isolation of Neutrophils

4.1.1. Draw 40 mL of venous blood into a 60 mL syringe containing 6 mL of sodium citrate/citric acid (0.15 M, pH 5.5) and 14 mL of 6% Dextran-70.

4.1.2. Wait for 50 min for blood to sediment.

4.1.3. Slowly layer the buffy coat cells on top of 20 mL of Ficoll-Paque by pushing the top 18 mL through the blood collection tubing into a 50 mL tube, avoiding contamination with sedimented RBCs. It is recommended to use a fresh blood collection set, to minimize the contamination with residual RBCs left over in the original blood collection tube.

4.1.4. Pellet the buffy coat cells by centrifugation for 20 min at 3,000 x *g*. Neutrophils and contaminating RBCs will pellet at the bottom of the tube. PBMC will form a white layer on top of Ficoll-Paque.

4.1.5. Transfer the neutrophils to a new 50 mL tube.

4.1.6. Lyse any residual RBCs by incubating the neutrophils with 20 mL of 0.2% cold NaCl solution for 25 seconds, followed by an additional 20 mL of 1.6% NaCl. The final concertation of NaCl should be 0.9% (isotonic).

4.1.7. Centrifuge the suspension for 10 min at 3,000 x *g*.

4.1.8. Remove the supernatant and resuspend the neutrophils to the desired concentration.

4.2. Isolation of Lymphocytes

4.2.1. Plate the PBMCs in RPMI with 5% heat inactivated serum in 6-well culture plates.

4.2.2. Incubate the plates for 1 h at 37 °C. Monocytes will adhere to the plate, lymphocytes will be freely floating.

4.2.3. Remove the buffer containing the lymphocytes and wash it twice with RPMI.

4.2.4. Resuspend the lymphocytes at the desired concentration.

4.3. **Label RBCs, PMN, and Lymphocytes.**

4.3.1. Label each cell type with a different fluorescent dye. Make sure each dye fluoresces in a different channel. Follow the manufacturer's instructions for each of the chosen dyes.

5. Generation of RBC Extracellular Vesicles via the Compliment Method

5.1. Obtain whole blood through venipuncture using EDTA tubes.

5.2. Pass blood through a white blood cell filter, then centrifuge 3 times at 500 x *g* for 10 min each to isolate red blood cells. Use HBSS++ as a washing buffer.

5.3. Make aliquots of 100 μL packed RBCs in 1.5 mL tubes and make up the volume to 1 mL by adding HBSS++.

5.4. Add C5b,6 solution to a 0.18 μg/mL final concentration in HBSS++, then vortex.

5.5. Put on a slow shaker at room temperature for 15 min.

5.6. Add C7 protein to a final concentration of 0.2 μg/mL. Mix by inverting the tube gently a few times. Do not vortex from now on.

5.7. Put on a slow shaker at room temperature for 5 min.

5.8. Add C8 protein to a final concentration of 0.2 μg/mL, and C9 protein to 0.45 μg/mL. Mix by inverting the tubes gently a few times.

5.9. Incubate at 37 °C for 30 min.

5.10. Centrifuge at 2,500 x *g* for 10 min.

5.11. Collect the EV-containing supernatant in a new tube(s), try not to be too close to the cell pellet at the bottom.

5.12. Repeat steps 5.9-5.10 if needed.

6. Analyzing Cells on the Magnetic Levitation Device

6.1. Perform instrument startup according to manufacturer settings.

6.2. Load 50 μL of sample into a capillary tube until the tube is filled. Seal the ends of the capillary tube with capillary sealant making sure there are no air bubbles present.

6.3. Load the capillary tube into the holder between the top and bottom magnets. Adjust the stage and focus for optimal viewing.

NOTE: Cells/beads can take anywhere from 5-20 min to settle at their magnetic equilibrium position based on their density and the concentration of Gd^{3+}.

REPRESENTATIVE RESULTS:

Magnetic levitation focuses objects of different densities at different levitation heights depending on the object's density, its magnetic signature, the concentration of paramagnetic solution, and the strength of the magnetic field created by two strong, rare-earth magnets. As the two magnets are placed on top of each other, levitating samples can only be viewed, while maintaining Köhler illumination, by using a microscope turned on its side (**Figure 1**). The final levitation height reached by each cell type can easily be modified by changing the concentration of the paramagnetic solution. **Figure 2** illustrates the separation of the different circulating blood cells by using various concentrations of gadolinium. The two bead types with different densities (1.05 and 1.2 g/mL) were used to provide density levitation heights and size references. As the levitation height of a given cell type depends on its intrinsic density, magnetic levitation provides a direct means of isolating cells of interest without any significant manipulation[24].

The main purpose of this protocol was to demonstrate the ability of magnetic levitation to detect the presence of membrane bound antigens, in this case the Rh factor, on circulating red blood cells. For this experiment, low density beads were coated with either IgG control antibody or anti-RhD. Samples were then prepared by spiking blood from an Rh(-) donor with blood from an Rh(+) at a ratio of 1:1000. Anti-RhD(+) coated beads or the control IgG coated beads were added to the blood sample and incubated for 10 minutes. **Figure 3A** shows the IgG control sample, which did

not generate any bead-red cell complexes. Next, the identity of the red blood cells captures by the Rh(+)-positive beads was verified by pre-staining the Rh(+) cells with a fluorescent plasma membrane stain, then imaged using fluorescence microscopy (**Figure 3B**). A positive detection event is shown in **Figure 3C**. The binding of the anti-Rh-bead to the Rh(+) cells creates a bead-cell complex with a density in between that of the beads and the cell, therefore levitating at a height situated in between the unbound capture beads and negative RBCs. A close up of the bead-cell complexes was imaged under fluorescent light to confirm the presence of the Rh(+) cells labeled with a fluorescent plasma membrane stain. (**Figure 3D**). **Figure 3E** shows bead-bead complexes forming between high- and low- density beads coated with antibodies for CR1 and CD47, which indicate the presence of extracellular vesicles. A schematic of the bead-cell complex is shown in **Figure 3F**.

FIGURE AND TABLE LEGENDS:

Figure 1. Principles of Magnetic Levitation: (**A**) Schematics of magnetic field. (**B**) A research grade microscope tipped on its side to allow side-imaging the targets levitating in the capillary tube. (**C**) Angled view of the magnetic levitation apparatus under the microscope objective. (**D**) Magnetic levitation apparatus frontal view. (**E**) Schematics of the magnetic levitation apparatus with a capillary tube mounted between two magnets, front and side view.

Figure 2. Demonstration of Cell-Specific Magnetic Equilibrium: (**A**) Low- and high-density beads (1.05 and 1.2 g/mL) alone at 60 mM Gd^{3+} (viewed on a 10x objective). (**B**) PMNs levitating above red blood cells at 21 mM Gd^{3+}, with out of focus platelets circulating (viewed on a 10x objective). (**C**) Whole blood levitating at 60 mM Gd^{3+}, which is a concentration that focuses on RBCs (viewed on a 10x objective). (**D**) This figure has been modified from [Tasoglu, S. *et al.* Levitational Image Cytometry with Temporal Resolution. *Advanced Materials.* **27** (26), 3901-3908, doi:10.1002/adma.201405660 (2015).] Density separation of RBCs (red), PMNs (green) and lymphocytes (blue) at 40 mM Gd^{3+}. Each cell population levitated at a height based on their intrinsic densities (viewed on a 10x objective).

Figure 3. Detection of Rh Factor in a Blood Sample: (**A**) Rh(-) blood spiked with Rh(+) blood and IgG control beads. No bead-cell complexes are formed (viewed on a 10x objective). (**B**) IgG control sample under fluorescent light highlighting the Rh(+) cells (viewed on a 10x objective). (**C**) Rh(-) blood spiked with Rh(+) blood and anti-RhD coated beads, showing the formation of bead-cell complexes (viewed on a 10x objective). (**D**) A close-up view of a bead-cell complex under fluorescent light (viewed on a 10x objective). (**E**) Anti-CR1 and anti-CD47 coated beads forming complexes, indicating the presence of RBC derived extracellular vesicles (viewed on a 10x objective). (**F**) Diagram depicting the bead-cell complex.

DISCUSSION:

Gradient centrifugation is currently the standard technique for isolating subcellular components based on their unique densities. This approach, however, requires the use of specialized gradient media as well as centrifuge equipment. The magnetic levitation approach presented here allows detailed investigation of the morphological and functional properties of circulating cells, with minimum, if any manipulation of the cells, providing a near *in vivo* access to circulating cells.

However, when using magnetic levitation several points are worth mentioning. Firstly, the microscope used for imaging must be dedicated to this method, as the setup is time consuming, and requires the microscope to be partially disassembled, positioned perfectly horizontally with the optics precisely aligned with the magnets and the capillary tube. Secondly, the lab jack and the translational stages used to adjust the movements of the capillary glass and the focus require exact positioning and free movement on each of the three axes. Likely the most critical alignment of the entire setup, is mounting the magnetic levitation device in the translational stages such that the top and bottom magnets are perfectly aligned with the gravity vector and perfectly horizontal. Any deviation from this, would create an angle between the magnetic force and gravity which would push the cells toward either sides of the capillary tube, disrupting the levitation process, and making the results unreliable.

The optimal concentration of the gadolinium solution used for levitating cells needs to be adjusted for the cell type and the goal of the experiment. Changes in the concentration of the gadolinium solution will significantly alter the levitation height of the cells being analyzed, and therefore needs to be kept constant. If the concentration is too low, depending on the density of the target cells, they may not levitate at all, while if it is too high, will limit the range of detectable changes that can be accurately quantified.

Samples will create stable bands if the only forces acting on them are gravity and magnetic repulsion. The presence of even a millimeter-size bubble in the capillary tube will create small circular currents at the air-liquid interface, which will disturb the levitating cells, making any analysis impossible. When loading a sample into a capillary tube, and then after sealing, one must make sure that no air is present, by examining the capillary under a stereomicroscope or low power (4x) objective.

Levitation of cells at a set height depends on their magnetic signature staying the same over time. If the paramagnetic gadolinium ions from the levitation media enters the cells, either through pinocytosis or increased membrane permeability, such as during apoptosis, the increased cell density measured based on the density reference beads will be erroneous. Fortunately, most of circulating cells have poor pinocytic capabilities[26], making this method a suitable approach for studying cells over long periods of time.

ACKNOWLEDGMENTS:
The authors would like to thank Dr. Getulio Pereira for his help with extracellular vesicle work.

This work was supported by the following National Institute of Health grants to ICG: RO1CA218500, UG3HL147353, and UG3TR002881.

DISCLOSURES:
The authors have no conflicts to disclose.

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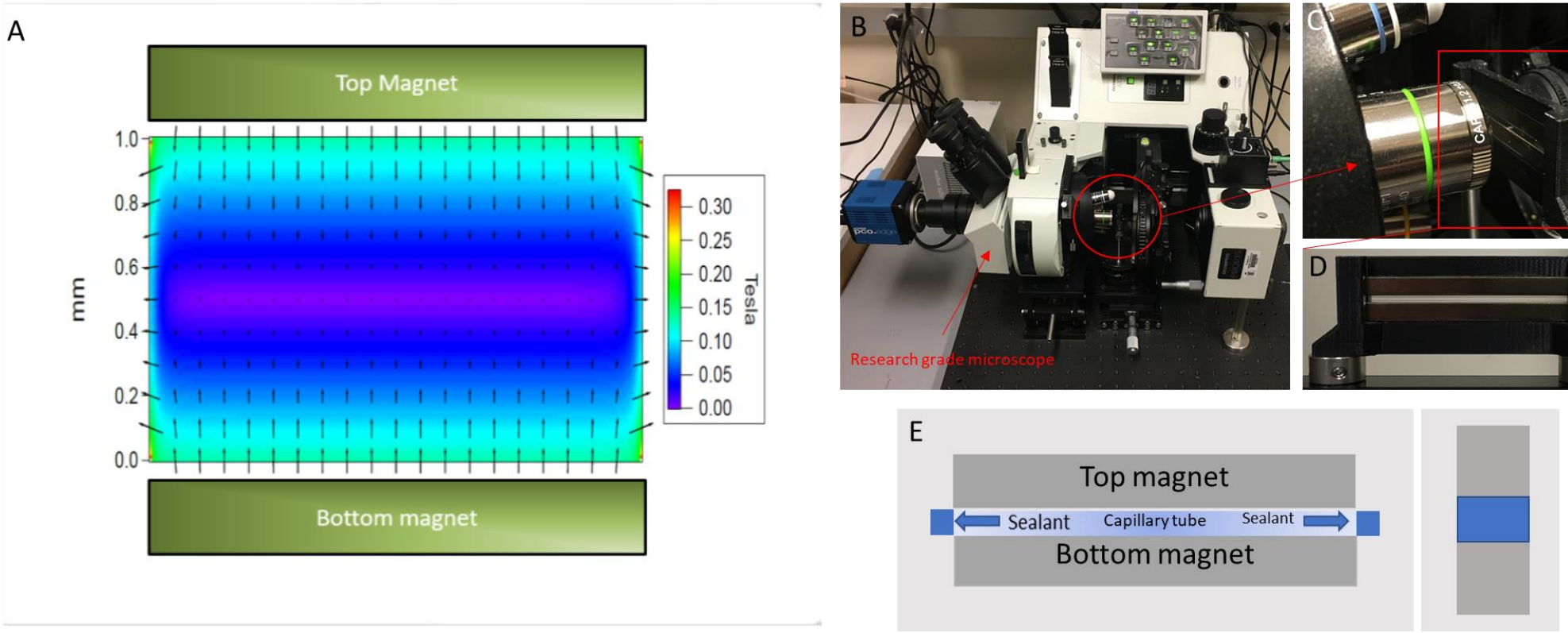
Figure 1

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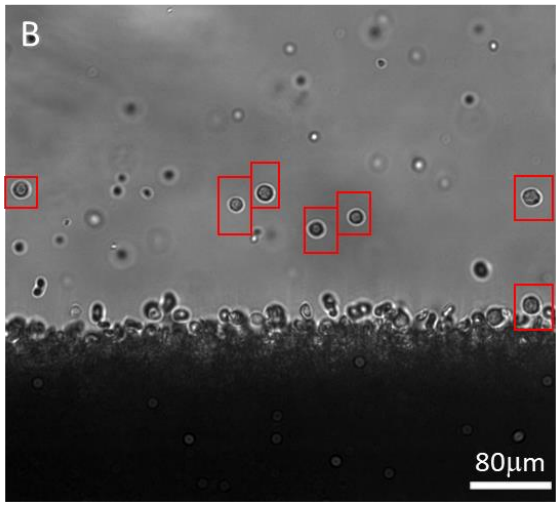
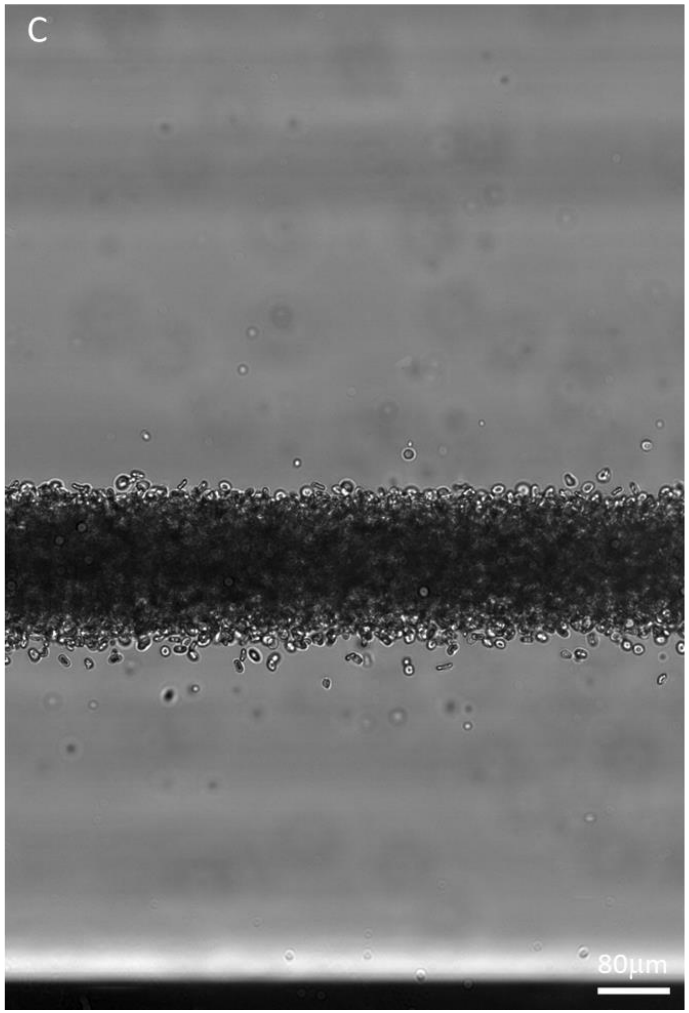
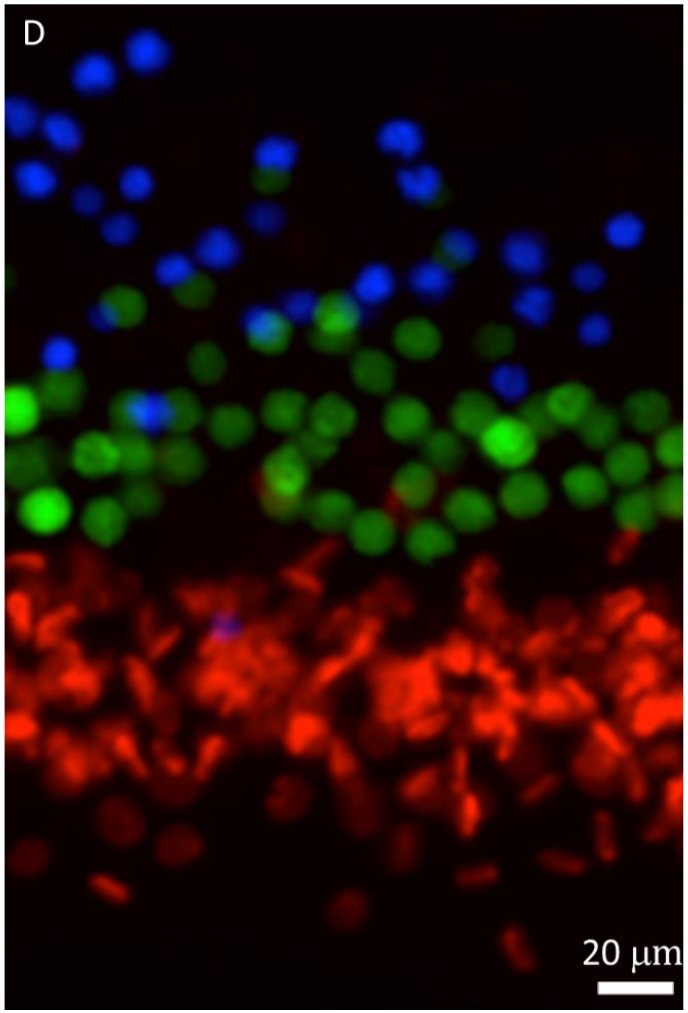

B
80µm


C
80µm


D
20 µm

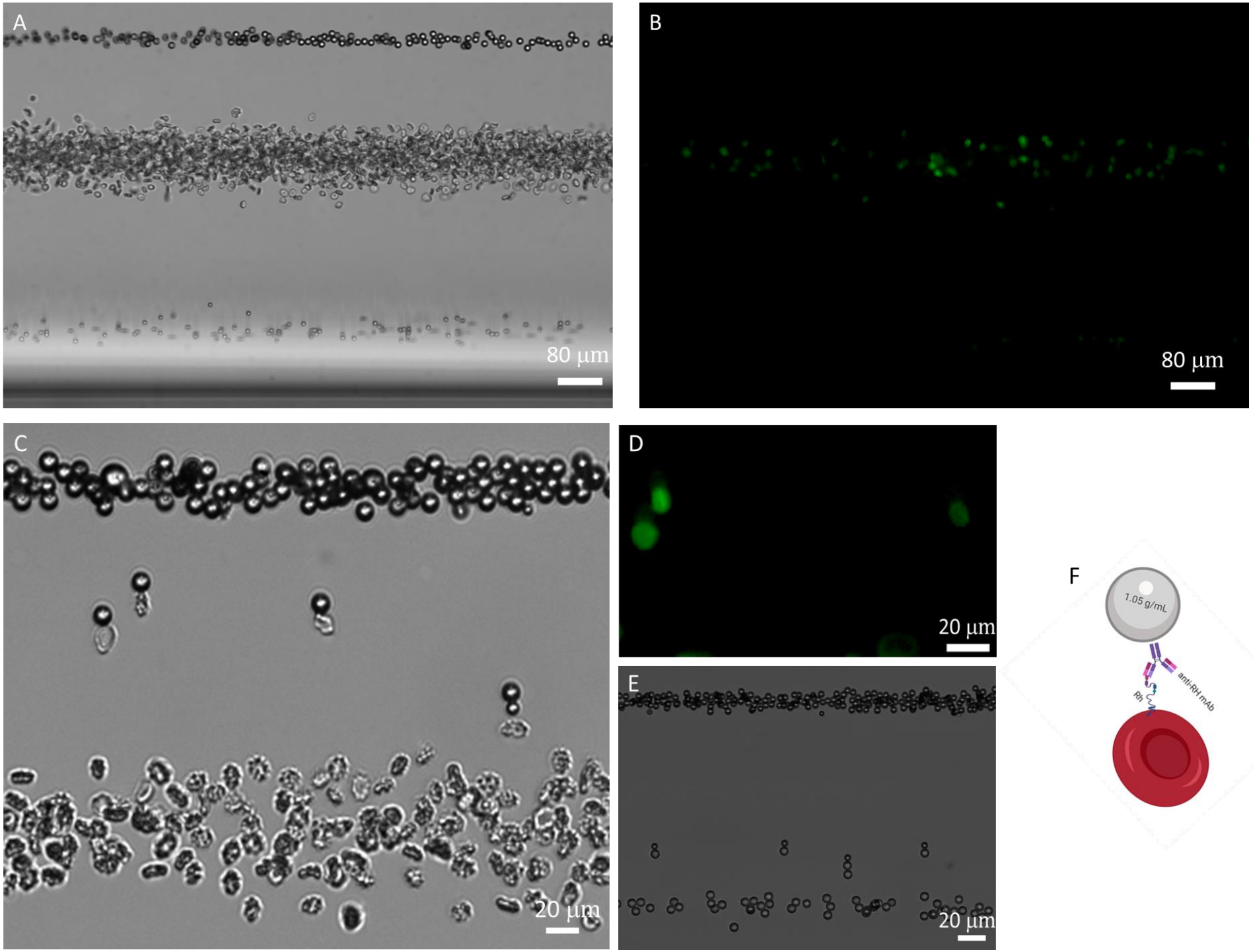

A
80 μm
B
80 μm
C
20 μm
D
20 μm
E
20 μm
F
1.05 g/mL
anti-RH mAb
Rh

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
2-(N-Morpholino)ethanesulfonic acid hydrate	Sigma Aldrich	M-2933
50x2.5x1 mm magnets, Nickel (Ni-Cu-Ni) plated, grade N52, magnetized through 5mm (0.197") thickness	K&J Magnetics	Custom
Capillary Tube Sealant (Critoseal)	Leica Microsystems	267620
Centrifuge tube filters (Corning Costar Spin-X)	Sigma Aldrich	CLS8163
Compact Lab Jack	Thorlabs	LJ750
DPBS, no calcium, no magnesium	Gibco	14190-144
Ethanolamine	Sigma Aldrich	E9508-100ML
Fluorescent Plasma Membrane Stain (CellMask Green)	Invitrogen	C37608
Gadoteridol Injection	ProHance	NDC 0270-1111-03
HBSS++	Gibco	14025-092
Human C5b,6 complex	Complement Technology, Inc	A122
Human C7 protein	Complement Technology, Inc	A124
Human C8 protein	Complement Technology, Inc	A125
Human C9 protein	Complement Technology, Inc	A126
Mini Series Post Collar	Thorlabs	MSR2
N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride	Sigma Aldrich	E1769-10G
Normal Rabbit IgG Control	R&D Systems	AB-105-C
Phosphate Buffered Saline (10X Solution, pH 7.4)	Boston Bioproducts	BM-220
Polysorbate 20 (Tween 20)	Sigma Aldrich	P7949-500ML
Polystyrene Carboxyl Polymer	Bangs Laboratories	PC06004

Rabbit RhD Polyclonal Antibody	Invitrogen	PA5-112694
Research Grade Microscope	Olympus	Provis AX-70
Rubber Dampening Feet	Thorlabs	RDF1
Square Boro Tubing	VitroTubes	8100-050
Sulfo-NHS	Thermoscientific	24510
Translational Stage	Thorlabs	PT1

Comments/Description
(MES); component of activation buffer
Magnets used for the magnetic levitation device
Used to cap the ends of the capillary tubes
Used to wash beads
Used for adjusting the magnetic levitation device
Solution for bead suspensions
Used during a wash step for beads
Used to stain Rh+ cells
Gadolinium (Gd^{3}+); magnetic solution used to suspend cells
Solution for sample preparation
Used to generate RBC Evs
Used to generate RBC Evs
Used to generate RBC Evs
Used to generate RBC Evs
Used to secure magnetic levitation device to lab jacks
(EDC); used in antibody coupling reaction
Used to coat beads as a control condition
Component of coupling buffer, used for washing steps
Component of activation buffer
Top density beads (1.05 g/mL), used for antibody coupling

Used to coat beads for the dectection of Rh factor in red blood cells
Microscoped used to mount magnetic levitation device and view levitating cells
Used to support the breadboard table
Capillary tube used for loading sample into Maglev
Used in antibody coupling reaction
Used for focusing and for scanning capillary tube

Reviewers' comments:

Reviewer #1:

This is a nice piece of work showing the critical role of magnetic levitation process in cell separation according to their densities. I believe many scientists from different backgrounds will find the paper useful for their multidisciplinary research. My only minor suggestion to the authors is that their message might be more effective if they improve the opening paragraphs by mentioning the wide spectrum of magnetic levitation application in medicine such as identification of opioid drugs, protein separation, and disease detection.

I'd publish this interesting paper.

Response: We thank the review for the suggestion; we have now incorporated into the manuscript the necessary references.

Reviewer #2:

Manuscript Summary:

In this article, the interaction between antibody-coated beads and target antigen on cells was investigated in a magnetic levitation platform to identify the cell of interest by altering cell levitation height. As a concept, Rh(-) blood cells were identified in the presented platform. The presented protocol will be very useful for the researcher who would like to start magnetic levitation-based assays. Hence, the reviewer recommends the publication of the manuscript after addressing the following issues presented below.

Major Concerns:

-# 1Other magnetic levitation methods to identify target antigens (DOI: 10.1039/C7LC00402H, 10.1021/acs.analchem.0c02479, etc.) should be discussed in the manuscript.

Response: We have now included the reference along with additional ones in the manuscript.

#2 Figure 2 experiments were not explained in the protocols. They should be given.

Response: We have now added the necessary explanation in the text.

#3 Figure 2a, the usage of two different beads should be explained

Response: We have now included the explanation for the necessity of using the two density reference beads.

- In Figure 2, how were PMNs validated? It should be discussed.

Response: Isolation of PMN was performed using published protocols, which we now reference.

- *Authors could discuss the binding efficiency of beads to target cells and also the identification efficiency of target cells in the platform.*

Response: This is an important observation, given the possibility of steric hindrance between the capture beads and the targets, the density of the capture antibody and the density of the antigens present on the intended cell, as well as the actual size of the target. We have now, in addition to discussing the points the reviewer raised, added another figure showing that the method is suitable for detecting extracellular vesicles, which are larger than soluble mediators such as cytokines, but smaller than a cell.

- *Authors mentioned that bubbles inside the channels could affect measurements. Authors should discuss how they eliminated the bubbles in the capillary.*

Response: We have yet to find a way to remove bubbles form once the capillaries are sealed with putty. Therefore, our efforts are geared toward preventing the formation of bubbles by sealing the capillary only once the liquid at the end of the capillary has a slight convex shape (bulging out). We have now re-written the discussion part to better explained our method.

Minor Concerns:
- *Scale bars can be given on the micrographs.*

Response: We have now added the scale bars.