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Centrifugation-assisted Immiscible Fluid Filtration (CIFF) for dual-bioanalyte extraction

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Abstract

The extraction of bioanalytes is the first step in many diagnostic and analytical assays. However, most bioanalyte extraction methods require extensive dilution-based washing processes that are not only time consuming and laborious, but can also result in significant sample loss, limiting their applications in rare sample analyses. Here, we present a method that enables the efficient extraction of multiple different bioanalytes from rare samples (down to 10 cells) without washing —Centrifugation-assisted Immiscible Fluid Filtration (CIFF). CIFF utilizes centrifugal force to drive the movement of analyte-bound glass microbeads from an aqueous sample into an immiscible hydrophobic solution to perform an efficient, simple and non-dilutive extraction. The method can be performed using conventional PCR tubes with no requirement of specialized devices, columns, or instruments, making it broadly accessible and cost effective. The CIFF process can effectively remove approx. 99.5% of the aqueous sample in one extraction with only 0.5% residual carryover, whereas a traditional “spin-down and aspirate” operation results in a higher 3.6% carryover. Another unique aspect of CIFF is its ability to perform two different solid-phase bioanalytes extractions simultaneously within a single vessel without fractionating the sample or performing serial extractions. Here we demonstrate efficient mRNA and DNA extraction from low input samples (down to 10 cells) with slightly higher to comparable recovery compared to a traditional column-based extraction technique, and the simultaneous extraction of two different proteins in the same tube using CIFF.

Graphical Abstract

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Author Contributions

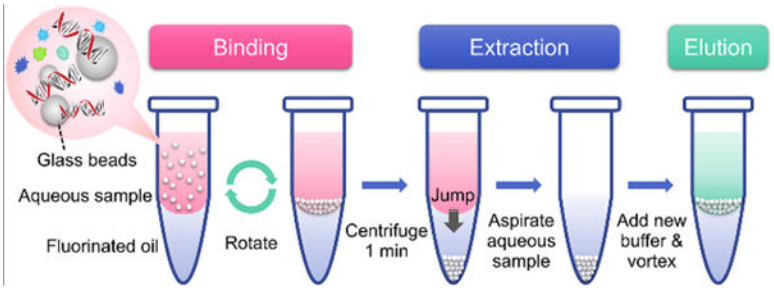
D.S.J. designed and conducted experiments; S.M.B., C.L. assisted in experimental design and data analysis; D.J.B. and J.M.L. oversaw the study; D.S.J., S.M.B., and C.L. wrote and revised the manuscript. All authors reviewed the manuscript. All authors have given approval to the final version of the manuscript.

ASSOCIATED CONTENT

Supporting Information

Derivation of the physics of bead jump (Figure S1), DNA extraction efficiency of CIFF compared to a traditional column-based technique for low input samples (Figure S2), lysis buffer inhibition of reverse transcription and qPCR (Figure S3), and workflow comparison of CIFF with column-based or magnetic bead-based extraction (Figure S4).

The authors declare the following competing financial interest(s): D.S.J. holds equity in Turba LLC; J.M.L. and S.M.B holds equity in Salus Discovery LLC.; D.J.B. holds equity in Bellbrook Labs LLC, Tasso Inc., Stacks to the Future LLC, Lynx Biosciences LLC, Onexio Biosystems LLC, Salus Discovery LLC, and Turba LLC.



Analyte extraction from a complex sample is the ubiquitous first step in most analytical bioassays[1]. The efficiency and recovery of the extraction process can often determine the success, limit of detection, and signal to noise ratio of the bioassay[2]. However, sample extraction is an often-overlooked aspect of bioassay development and could be a critical bottleneck for developing next generation bioassays for applications such as point-of-care diagnostics, as most traditional sample extraction technologies often require extensive liquid handling and washing steps and are not well optimized for processing rare amounts of analytes due to losses associated with the excessive number of liquid handling steps required[3–5]. Thus, advancements in sample extraction technologies will have broad impact across multiple fields such as medical diagnostics and basic research to potentially enable “more data” to be gained per sample.

Although a wide variety of analyte extraction methods have been developed,[1] they can generally be classified into two main approaches: liquid-phase extractions, and solid-phase extractions. Liquid-phase extractions, such as the classic acid guanidinium thiocyanate-phenol-chloroform extraction (AGPC) for nucleic acid extractions[6–8] utilize the differential partitioning and/or denaturation of proteins, lipids and other cellular debris into an organic phase, whereas nucleic acids of interest remain in the aqueous phase. The nucleic acids in the aqueous phase are then separated from the organic phase via centrifugation and can be further precipitated for additional purity. Liquid-phase approaches are generally reliable and yield high sample recovery,[9] owing to the fact that they employ the intrinsic differential solubilities of different types of bioanalytes in aqueous vs. organic solvents. However, it is also time-consuming, requires toxic chemicals, and involves multiple pipetting and tube transfer steps that increase opportunities for sample contamination and mishandling. On the other hand, solid-phase extractions utilize the preferential binding of analytes to a solid support such as a surface-functionalized bead column[10–12] or paramagnetic particles (PMPs).[13, 14] In these platforms, target analytes bind to the solid support in an aqueous solution, whereas non-target biomolecules are eluted through or aspirated from the beads/column to remove contaminants. These approaches are more rapid and simple and do not require toxic organic solvents, as everything can be performed in aqueous solutions. Although results vary depending on the sample source, solid-phase extraction often comes with the tradeoff of lower analyte recovery,[15–17] as it is potentially limited by several factors including the binding incidence and affinity of the analyte to the solid support, removal efficiency of non-target contaminants which share the same aqueous phase as the target analyte, analyte loss during dilutive washing (to effectively remove contaminants), and elution efficiency for freeing the bound analyte from the solid support.

Previously, our group and others have developed a variety of improved solid-phase sample extraction technologies that our group refers to as exclusion-based sample preparation (ESP), which involves the movement of paramagnetic particles (PMPs) bound to an analyte of interest across an immiscible barrier (oil or air) to effectively "exclude" non-target contaminants from the biosample.[18–28] These technologies enable much faster processing and higher sample recovery compared to traditional PMP extraction techniques that typically involve multiple liquid aspiration and washing steps, which contribute to analyte loss due to prolonged processing, especially for low-binding affinity analytes.[23] The advantages and versatility of ESP technologies were also comprehensively demonstrated for the extraction of nucleic acids, proteins, and even rare cells.[29] One limitation of these approaches however, is that they generally require specially fabricated devices for immobilization of the immiscible phases via physical barriers and a PMP manipulation apparatus for performing parallel extractions, which could impede broad adoption and accessibility. In this study we report a new form of ESP technology that can be accomplished using common laboratory consumables (PCR tubes) and a benchtop centrifuge. Termed "Centrifugation-assisted Immiscible Fluid Filtration" (CIFF), it utilizes the differential density and hydrophilicity of aqueous, oil, and solid-phase capture beads for creating vertical liquid "barrier interfaces" that serve as an analyte exclusion filter under centrifugation. Its basic components comprise an aqueous phase sample, underlaid with a dense oil phase, and an even denser solid-phase capture element (glass microbeads). The glass microbeads reside in the aqueous phase under normal conditions due to their hydrophilicity, but are "extracted" to the oil phase under centrifugal force owing to their higher density. This technique combines some of the advantages of liquid-phase extractions that enable highly efficient exclusion of non-target analytes with the simplicity and efficiency of solid-phase extractions. Further expanding on this concept, we also demonstrated a simultaneous "dual-extraction" using CIFF by overlaying an additional hydrophobic phase that is lighter than the aqueous phase, paired with an even lighter hydrophilic solid phase (buoyant glass microbubbles), which under centrifugal force, are extracted upward to the top of the tube. As the extraction process in CIFF occurs in the vertical axis, this allows parallel extractions to be easily performed in multi-well PCR plates for large scale simultaneous multi-sample processing.

EXPERIMENTAL SECTION

Operation of CIFF.

The components of CIFF comprise a tube-shaped vessel of a hydrophobic material (here we used standard 0.2 mL polypropylene PCR tubes, Eppendorf), a fluorinated oil layer that is denser than the aqueous sample (FC-3283 fluorinated oil, d: 1.82 g/mL, 3M Inc.), and an even denser hydrophilic solid-phase capture element (glass microbeads, d: 2.48 g/mL, Polysciences Inc., USA), and the aqueous sample of interest (d~ 1 g/mL) (Figure 1). The additional benefit of using fluorinated oil is that it is highly inert and exhibits low solubility for both hydrophilic and lipophilic molecules (which account for nearly all biomolecules). Thus, the CIFF process would effectively remove not only hydrophilic contaminants, but also hydrophobic contaminants such as lipids as well. To perform CIFF, 50 μL of fluorinated oil, 100 μL of aqueous sample, and 10 μL of glass microbeads (concentration 1 g/mL), are pipetted into a PCR tube. The high repellency of the three phases ensures that proper phase

separation will occur, even when pipetting vigorously. The tube was then centrifuged in a conventional benchtop centrifuge (Eppendorf Centrifuge 5424) at 10,000 *RCF* for 1 min to "jump" the dense glass microbeads downward into the bottom oil layer (10,000 *RCF* was used as it's sufficiently higher than the minimum bead jump threshold determined in Figure 2B). After jumping, the dense glass microbeads can be collected directly from the bottom oil layer using a pipette. Alternatively, they can be left in the tube whereas the aqueous phase is aspirated and replaced with another aqueous solution of interest (wash buffer, elution buffer, etc.). The glass microbeads can then be easily re-suspended into the aqueous phase by tapping the tube for approx. 1 s on a conventional benchtop vortex, without residual beads left in the oil phase. (Note: to avoid bead contamination with the aqueous sample or premature bead resuspension, the tube should be kept upright after performing CIFF and not tilted to its side or inverted. If contamination still remains a concern, more fluorinated oil can be added to increase the distance between the bead pellet and aqueous phase). This enables multiple solution exchange/processing steps to be performed in the same tube with hardly any bead loss and cross contamination of aqueous solutions, as the fluorinated oil effectively seals off the beads from the aqueous solution after each CIFF operation. This allows us to easily perform binding, washing and elution steps in the same tube.

Quantification of bead residue.

The quantity of residual beads left behind in the aqueous phase (i.e., beads that failed to traverse the aqueous/oil interface) after CIFF was quantified as follows: Glass microbeads were functionalized with goat anti-mouse HRP (Invitrogen) as described below, a commonly-used enzyme that catalyzes the oxidation of a substrate (such as 3,3',5,5'-Tetramethylbenzidine, TMB), changing it from colorless to a blue-colored liquid in the process. The reaction can be stopped by adding 2N H_2SO_4 which changes the solution to a yellow-colored liquid that absorbs at OD 450 nm. Therefore, the number of residual beads, which is proportional to the amount of HRP enzyme present and hence to the absorbance of the TMB substrate, can be quantified. A normal CIFF process was performed in 100 μL DI water as described above, then 90 μL of the water was removed (leaving the residual beads) and added with 100 μL of TMB substrate (1-step ultra TMB-ELISA, Thermo Fisher Scientific), and incubated for 30 min at RT for color development. 50 μL of 2N H_2SO_4 was added to the tube to stop the reaction, then the liquid was transferred to a multi-well plate and the absorbance was scanned at 450 nm on an ELISA plate reader (Molecular Devices). A dilution curve was obtained by serially diluting HRP-conjugated glass microbeads and incubating with the same TMB substrate and stop solution. Carryover percentage was calculated by fitting the absorbance of the glass microbead reacted TMB substrate to the dilution curve.

Quantification of carryover.

To quantitatively measure the amount of liquid carryover from the aqueous phase from CIFF, we prepared a solution of acridine orange at 500 μg/mL in deionized water and also deionized water containing 0.1% Triton X-100. In each experiment, 10 mg of glass beads (30–50 μm diameter, Polysciences Inc.) was added to a 0.2 mL PCR tube containing 100 μL of the acridine orange solution with or without Triton X-100 and underlaid with 50 μL of FC-3283 fluorinated oil (3M Inc.). After centrifugation and CIFF, the glass beads were

collected, resuspended in 100 μL deionized water, and spun down. The fluorescence intensity of the resuspended bead supernatant was measured using a NanoDrop 3300 fluorospectrometer (Thermo Fisher Scientific) at 530 nm, and the percent carryover was calculated by fitting the fluorescence intensity to a serially diluted calibration curve of acridine orange solution.

Oligo(dT) glass microbead surface functionalization.

Functionalization of glass microbeads with oligo(dT) was performed as follows: 2 g of glass microbeads (30–50 μm) were placed in a 15 mL conical tube and washed with 10 mL of 1:1 methanol/HCl at RT for 30 min to remove potential organic contaminants on the bead surface. The beads were spun down and washed once with deionized water, followed by incubation with 5 mL of concentrated sulfuric acid to activate the glass microbead surface. The beads were then washed 3 times using deionized water and 3 more times using 99% ethanol, then incubated with 4 % (v/v) (3-mercaptopropyl)trimethoxysilane (Sigma-Aldrich) in 99% ethanol for 45 min at RT to change the surface functionality to a thiol moiety. After washing 3 times, the beads were incubated with N-γ-maleimidobutyryl-oxysuccinimide ester (GMBS, 0.25 mM in DMSO, Sigma-Aldrich), a heterobifunctional thiol to amine crosslinker for 30 min at RT, washed again for 3 times using ethanol and 3 times using PBS, then incubated with 10 μg/mL streptavidin in PBS for at least 1 hour at RT to covalently functionalize the glass microbeads with streptavidin. The streptavidin conjugated glass microbeads can be directly stored at 4 °C for a prolonged period of time before use. Oligo(dT) surface functionality was introduced by incubating the glass microbeads in 1 nmol/mL biotinylated Oligo(dT) probe (Promega) for 30 min at RT. Before use in mRNA extraction, the microbeads were first washed in mRNA lysis/binding buffer (100 mM Tris-HCl (pH 7.5), 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM dithiothreitol (DTT)).

DNA extraction.

DNA extraction using CIFF was performed as follows. In brief, cells in 20 μL of PBS was added 2 μL of Proteinase K and 20 μL of Buffer AL (Qiagen), vortexed for 15 s, then incubated at RT for 30 min to allow for complete cell lysis. 20 μL of 99% ethanol was then added to the tube and vortexed, followed by addition of 10 μL of washed unmodified glass microbeads (in Buffer AL) at a concentration of 1 g/mL, then rotated at RT for 3 min to capture the released DNA from cells onto the beads. After binding, the bead/aqueous mixture was transferred to a PCR tube containing 50 μL of FC-3283 fluorinated oil then centrifuged at 10,000 *RCF* for 1 min to perform CIFF. The aqueous sample was then aspirated out using a pipette leaving the fluorinated oil layer with beads. Washing (optional, for more complete removal of lysis buffer) was performed by adding 100 μL of washing Buffer AW1 (Qiagen) to the tube, vortexing briefly (approx. 1 s) to resuspend the beads back into the aqueous phase, and performing CIFF again by centrifuging at 10,000 *RCF* for 1 min. Buffer AW1 was then aspirated, and the washing process was repeated using Buffer AW2 (Qiagen). Buffer AW2 was then aspirated, and the DNA was eluted from the beads by adding in 100 μL of elution buffer (Buffer AE, Qiagen) to the tube and vortexing to resuspend the beads in the elution buffer. The beads were then removed by centrifuging them into the fluorinated oil phase, leaving the pure DNA eluent in the aqueous phase, which can be completely recovered without bead contamination. For comparison, DNA

extraction using a traditional column-based technique (Qiagen QIAamp DNA Mini Kit) was performed in accordance to manufacturer's protocols.

qPCR analysis of extracted DNA.

Following DNA extraction, 5 μL of the 100 μL eluted DNA sample was mixed with 10 μL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories), 3 μL of nuclease free water and 2 μL of primers for Long interspersed nuclear element-1 (LINE1, Forward primer: 5'-CGCAGAAGACGGGTGATTTC-3', Reverse primer: 5'-CCGTCACCCCTTTCTTTGAC-3', Integrated DNA Technologies) in a 96 well PCR plate and sealed with adhesive optically transparent PCR tape. The solution was pre-incubated for 5 min at 98 °C, then amplified for 50 cycles (98 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s) on a LightCycler 480 (Roche) real-time thermocycler. Threshold cycle (C_T) values were calculated using the built-in second derivative algorithm from the LightCycler 480 software.

mRNA extraction.

THP-1 cells at various concentrations were lysed in 100 μL of lysis/binding buffer for 5 min at RT, then added to a PCR tube containing 50 μL of FC-3283 fluorinated oil. 10 μL of washed oligo(dT) functionalized glass microbeads at a concentration of 1 g/mL was added to the tube, then rocked at RT for 10 min to capture the released mRNA from cells onto the beads. After binding, the tubes were centrifuged at 10,000 *RCF* for 1 min to perform CIFF. The aqueous sample was then aspirated out using a pipette leaving the fluorinated oil layer. A single wash (optional, for more complete removal of lysis buffer which could inhibit downstream PCR activity) was performed by adding 100 μL of RNA washing buffer (10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA) to the tube, vortexing briefly (approx. 1 s) to resuspend the beads back into the aqueous phase, and performing CIFF again by centrifuging at 10,000 *RCF* for 1 min. The wash buffer was then aspirated, and the mRNA was eluted from the beads by adding in 30 μL of elution buffer (10 mM Tris-HCl, pH 7.5) to the tube and vortexing to resuspend the beads in the elution buffer. The beads were then removed by centrifuging them into the fluorinated oil phase, so what remains in the aqueous phase is now pure mRNA eluent which can be completely recovered without bead contamination. As a comparison, mRNA extraction was also performed using matched samples with the same beads and reagents (except without the fluorinated oil), and were washed three times using a conventional "wash, spin-down, aspirate" method inside a 0.2 mL PCR tube instead of doing CIFF.

RT-qPCR analysis of extracted mRNA.

After extraction, 10 μL of the 30 μL eluted mRNA samples were mixed with 10 μL 2X RT buffer and 1 μL of 20X RT enzyme from a reverse transcription kit (High Capacity RNA-to-cDNA Kit, Thermo Fisher Scientific) in an 8-well 0.2 mL PCR strip tube (USA Scientific) and reverse transcribed at 37 °C for 1 h followed by heating to 95 °C for 5 min on a thermocycler (Techne, TC-412), as per the manufacturer's recommendations. 2.5 μL of the converted cDNA was mixed with 5 μL of LightCycler 480 Probes Master (Roche), 2 μL of nuclease free water and 0.5 μL of manufacturer preformulated primer-hydrolysis probe (FAM/MGB) mix for Human large ribosomal protein (RPLP0, Applied Biosystems, catalog no. 4333761) in a 96 well PCR plate (dot scientific, USA) and sealed with adhesive optically

transparent PCR tape. The solution was pre-incubated for 10 min at 95 °C, then amplified for 45 cycles (95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s) on a LightCycler 480 (Roche) real-time thermocycler. Threshold cycle (C_T) values were calculated using the built-in second derivative algorithm from the LightCycler 480 software.

Glass microbead and microbubble protein functionalization.

Functionalization of glass microbeads and microbubbles with antibodies was performed using a previously reported protocol.[30] In brief, S38 XHS glass microbubbles and glass microbeads were placed in a 15 mL conical tube and washed with 10 mL of 1:1 methanol/HCl at RT for 30 min to remove potential organic contaminants on the bead surface. The beads were spun down then washed 3 times using deionized water and 3 more times using 99% ethanol, then incubated with 10% (v/v) (3-Aminopropyl)triethoxysilane (Sigma-Aldrich) in 99% ethanol for 60 min at RT to functionalize the glass surface with amine groups. The beads/bubbles were washed with ethanol three times, then incubated with 2.5% (v/v) glutaraldehyde in PBS for 1 hour at RT. This was followed by washing three times with PBS, and incubation with proteins of interest (mouse IgG and rabbit IgG isotype control antibodies, Invitrogen, and goat anti-mouse HRP, Invitrogen) to covalently attach the proteins to the bead surface. The bead surface was blocked with 1% BSA in PBS to reduce non-specific binding.

Dual-CIFF.

Dual simultaneous bead-based extractions are a unique property enabled by CIFF. Operation of dual-CIFF is similar to single CIFF except that an additional buoyant hydrophilic solid phase (S38 XHS glass microbubbles, d: 0.38 g/mL, 3M Inc.) was added to the aqueous phase and overlaid with an additional lighter hydrophobic phase (silicone oil, d: 0.91 g/mL, Sigma-Aldrich) (Figure 5). 50 μL of FC-3283 fluorinated oil, 100 μL of aqueous sample with glass microbeads and glass microbubbles, and up to 100 μL of silicone oil were added to a 0.2 mL PCR tube. The dual-CIFF system was then centrifuged in a conventional benchtop centrifuge (Eppendorf Centrifuge 5424) at 10,000 *RCF* for 1 min to “jump” the dense glass microbeads downward or glass microbubbles upward into their respective top or bottom oil layers. After centrifugation, the top glass microbubbles can be collected using a large-orifice pipette tip (Fisher scientific) together with the silicone oil, whereas the dense glass microbeads can be collected directly from the bottom using a pipette or, as described previously, left in the tube whereas the aqueous phase is removed.

RESULTS AND DISCUSSION

Operational theory.

The physics underlying the operational principle of CIFF is governed by a competing balance between gravitational inertial forces with lipophobic resistance between the glass beads and the fluorinated oil phase. In brief, successful “jumping” of glass beads into the hydrophobic phase occurs when the gravitational force applied to the glass bead aggregate is higher than the lipophobic resistance keeping them from entering the oil phase. This is governed by multiple variables including relative centrifugal force *RCF* (a dimensionless unit defined as a multiple of gravitational acceleration at the Earth’s surface), mass of the

bead cluster (M_{beads}), radius of the tube (r_{tube}), radius of the beads (r_{bead}), as well as the interfacial tension of the glass beads with oil and hydrophilicity of the bead surface (Figure S1). A more detailed derivation of the theory governing this phenomenon can be found in SI, but here we present a simplified version based on the following assumptions: the beads spread across the full diameter of the tube (typically observed in our experiments), the thickness of the glass bead cluster is constant across the whole tube, the interfacial tension between the glass beads and oil phase is constant, the hydrophilicity, density, and size of the bead is constant, and beads are packed uniformly and tightly across the entirety of the tube. From these approximations we estimate that the required relative centrifugal force *RCF* for a given mass of beads (M_{beads}) in milligrams is:

$$RCF \approx C/M_{beads}$$

Where C is a constant estimated to be ≈ 12000 · mg for the described CIFF system.

By plugging in this value, we obtain the blue solid curve in Figure 2B which shows the same relative relationship of $RCF \approx C/M_{beads}$ as the obtained experimental values (green circular dots, which translate to an estimated C of ≈ 30000 · mg). We believe the discrepancy between the predicted curve and the experimental data is due to difficulties in accurately estimating constant C from the above listed assumptions (see detailed discussion in SI).

Characterization of CIFF.

To determine the threshold centrifugal force required to perform the “jumping” of glass microbeads into the fluorinated oil phase for a given number of beads, we screened four bead amounts ranging from 5 mg to 20 mg per 0.2 mL PCR tube for their respective threshold “jumping” centrifugal force. Results show that the threshold centrifugal force for successful jumping of beads from the aqueous to the fluorinated oil phase is inversely correlated with the number of beads, with 5 mg beads requiring up to 6000 *RCF* of force to traverse the oil/aqueous liquid barrier but 20 mg of beads requiring only approx. 1500 *RCF* of force (Figure 2B). However, although the amount of residual beads after CIFF is similar regardless of input (approx. 0.06 mg), the percentage of residual beads relative to input is significantly higher using 5 mg of beads (Figure 2C), thus 10 mg of beads was chosen for its lower residual bead amount (which would result in higher recovery) and lower centrifugal force threshold for successful jumping of beads.

As Triton X-100 is a common surfactant known to decrease the interfacial tension between oil and aqueous interfaces,[31, 32] we also tested whether addition of Triton X-100 might reduce the required centrifugal force during CIFF. Results show that the concentration of Triton X-100 in the aqueous phase (ranging from 0.0001% to 1%) is indeed inversely correlated with the threshold centrifugal force required for jumping the beads, although at higher concentrations of Triton X-100 (0.1% and 1%), it also resulted in formation of emulsions and incomplete bead jump (Figure 2D, blue triangles). The addition of Triton X-100 also resulted in a higher amount of aqueous sample carryover as revealed by acridine orange quantification (Figure 2E), which is likely caused by the lowered interfacial tension resulting in reduced filtration efficiency when the beads traverse the aqueous/oil barrier.

Worth noting though, is that the CIFF process successfully removes approx. 99.5% of the aqueous sample in one operation with only 0.5% residual carryover, whereas a traditional "spin-down and aspirate" operation results in a much higher 3.6% carryover (Figure 2E). We acknowledge that other physical parameters may also affect the "jumping" and purification efficiency of the CIFF process (bead density, bead surface hydrophobicity, oil density, oil hydrophobicity, etc.), but these parameters are not easily varied in practice as they're intrinsic properties to the material itself (glass and fluorinated oil) and hence were not explored in this study.

DNA extraction.

After showing that CIFF extraction can successfully remove approx. 99.5% of liquid from a 100 μL aqueous sample, we next attempted to perform DNA extraction using CIFF. DNA is known to bind to the surface of silica/glass under high chaotropic salt (such as guanidine hydrochloride or guanidine thiocyanate) concentrations, and elute under low salt conditions, which is the principle of most column and bead-based DNA extraction methods. Here we employed unmodified glass beads paired with a lysis buffer containing guanidinium salts (Buffer AL, Qiagen) for performing CIFF DNA extraction and compared extraction efficiency with or without washing. DNA extraction yield was quantified using qPCR with primers directed against LINE1 (Long interspersed nuclear element-1). Starting with 100,000 THP-1 cells, we showed that CIFF can efficiently extract DNA from cells without washing, followed by subsequent qPCR detection. Results from the qPCR for showed that CIFF yielded better recovery (lower Ct values) for the same amount of input sample without washing compared to CIFF with washing using standard DNA column washing buffers (Buffer AW1 and AW2, Qiagen) (Figure 3A). We also showed that CIFF can successfully extract DNA from low input samples (10 to 10,000 LNCaP cells) with comparable to slightly higher recovery compared to a traditional column-based technique (Qiagen QIAamp DNA Mini Kit) as determined by qPCR (Figure S2). Although no washing yielded better results in this application, additional washing could be employed for samples containing excess amounts of PCR inhibitors or when the eluted DNA must be highly concentrated (small elution volume).

mRNA extraction.

In addition to DNA extraction, we also attempted mRNA extraction using this method, as mRNA extraction is one of the most commonly practiced extractions in biology labs. mRNA extraction efficiency of CIFF was quantified using a housekeeping gene primer RPLP0 (ribosomal protein lateral stalk subunit P0). We initially attempted to perform qPCR directly from a single CIFF extracted sample without any washing, but results showed that PCR threshold cycle (C_T) values were higher (meaning less PCR efficiency) for the single-CIFF extracted sample without washing, compared to two sequential CIFF extractions with a single wash in between, suggesting that the tiny residual carryover from the aqueous phase (which contain the strongly protein denaturing 1% lithium dodecyl sulfate (LiDS)) could inhibit the downstream PCR reaction (Figure 3B). To investigate this possibility, we performed a lysis/binding buffer spike-in contamination experiment for RT and qPCR reactions and found slight inhibition to occur with as low as 0.078% Lysis/Binding buffer contamination for RT reactions, gradual inhibition all the way to 0.625%, and complete

inhibition starting at 1.25%. For qPCR, we did not observe significant inhibition all the way to 0.313%, but complete inhibition starting at 0.625% (Figure S3). Judging from this data, the 0.5% carryover in CIFF would indeed result in inhibition of both RT and qPCR for undiluted samples, thus necessitating the one additional wash step if maximum sensitivity is desired. Thus, we performed subsequent RT-qPCR assays by two sequential CIFF operations with a single wash in between to further effectively remove contaminating lysis/binding buffer. Worth noting however, is that we were aiming for maximum sensitivity with the RT-qPCR analysis (down to 10 cells), so the eluted mRNA sample was not diluted prior to use and hence the concentration of the lysis buffer carryover would be higher in the final reaction. With larger samples, dilution of mRNA can be performed to omit the additional wash. We also showed that the CIFF process yields mRNA recovery rates at least comparable or slightly better (lower C_T values) compared to a traditional 3X “wash, spin-down, aspirate” operation using the same matched glass microbeads and samples (Figure 3C). This suggests that the non-dilutive extraction and fewer washing operations of CIFF provide an advantage in sample recovery and operational speed compared to traditional washing-based extraction techniques.

Simultaneous dual-CIFF.

Although paramagnetic particles are an immensely powerful and versatile tool for performing analyte extractions and have proven to be faster and more affordable than fluorescence labeling-based techniques for performing cell separations (fluorescence-activated cell sorting), it lags behind optical based techniques in one critical aspect: there is only one “flavor” of magnetic force, which means only one target extraction can be performed at one time whereas the multiple different wavelengths of fluorescence dyes and colors affords multi-biomarker labeling and sorting simultaneously. One unique aspect of the CIFF method however, is that although gravitational force also only has one “flavor”, it pulls more strongly on objects with greater mass and density compared to lighter objects. This allows for movement of matter with different densities in two opposite directions in a liquid (sinking or floating) under a single force field (gravitational pull). Harnessing this property, we demonstrated that CIFF can simultaneously extract two analytes of interest from the same sample in opposite directions by harnessing the differential densities (in the order of decreasing density) of glass microbeads, fluorinated oil, aqueous liquid, silicone oil, and glass microbubbles. While microbubbles are hollow[30, 33] (i.e. density lower than water) and microbeads are solid (i.e. density higher than water), both share the same surface chemical properties of soda-lime-borosilicate glass and are equally suitable for chemical modifications. Under centrifugation, glass microbeads extract to the bottom of the tube under centrifugation, whereas glass microbubbles float to the very top of the tube via buoyancy. As a proof of concept, glass microbeads and glass microbubbles were respectively conjugated with rabbit IgG and mouse IgG isotype control antibodies for capturing different targets. They were then incubated with a mixed solution containing Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 647 anti-mouse IgG, then simultaneously extracted using dual-CIFF.

After extraction, the beads and bubbles were taken out using a pipette and respectively imaged on an epifluorescence microscope (Nikon *Ti* Eclipse) at 485/525 nm (Excitation/

Emission) and 648/684 nm. Results from the overlaid fluorescence images show that the CIFF process was able to highly effectively extract the proteins of interest from the aqueous sample without significant carryover or cross-contamination of the two bead types, resulting in a very clean fluorescence signal for both beads (Figure 4).

CONCLUSION

To summarize, we have demonstrated a simple and efficient sample extraction method termed CIFF for performing solid-phase analyte extractions with better contaminant removal, comparable or better sample recovery than conventional washing, and much fewer operating steps (Figure S4). CIFF is compatible with a broad range of solid-phase analyte extractions as long as the analyte resides in an aqueous phase (such as DNA, RNA and proteins) or solvent that is of a different density and is immiscible with the oil phase. It does not require specialized equipment, is very cost effective, and is amenable to parallel processing. CIFF is also unique in its ability to perform two different solid-phase bioanalyte extractions simultaneously within a single vessel, which cuts back on required processing time, especially for analytes that require prolonged incubation. It would be of interest to further expand this concept for performing simultaneous analyte extractions in future bioassay development to enable more information to be garnered per sample. In addition, we believe that the physical properties of CIFF make it intrinsically well suited for performing rare sample extractions due to its low sample volume, efficient processing, and very few washing steps. The relatively low sample volume and low requirement of washing in CIFF could enable better sample recovery and downstream analysis efficiency. Despite its advantages, we acknowledge that the CIFF technique has a few drawbacks. One is the requirement of a benchtop centrifuge to perform the process, which limits the technology's use in resource-poor settings with limited access to electricity. Another is the use of fluorinated oils in the process, which are known to have high recalcitrance to degradation leading to long environmental persistence,[34] although the inert properties of fluorinated oils could potentially allow it to be recycled and reused after the extraction process, reducing environmental impact.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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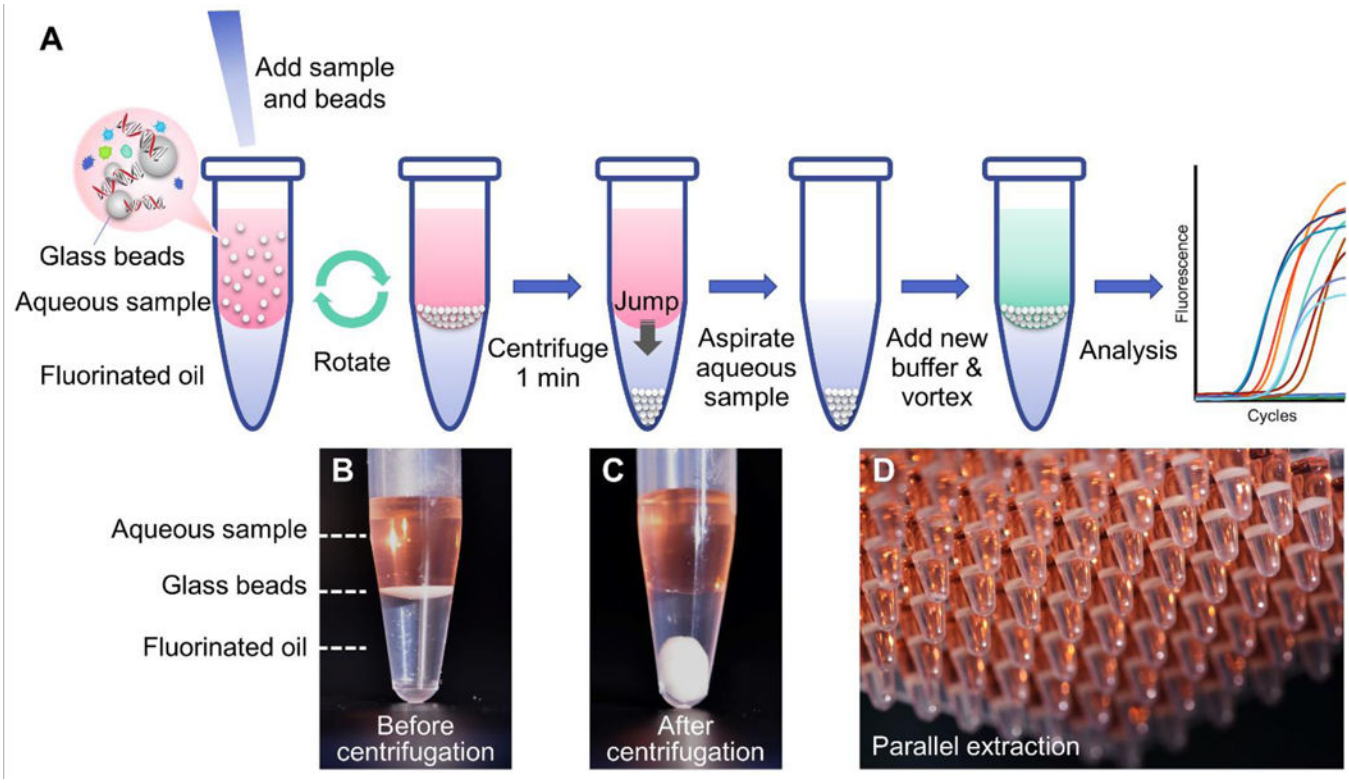

Figure 1.

Principle of Centrifugation-assisted Immiscible Fluid Filtration (CIFF). (A) CIFF comprises of two immiscible liquid phases, an aqueous phase (d: 1 g/mL) and a hydrophobic phase that is denser than the aqueous phase (FC-3283 fluorinated oil, d: 1.82 g/mL) underlying the bottom of the aqueous sample. Functionalized glass microbeads (d: 2.48 g/mL) are mixed with the aqueous sample to bind analytes of interest, then loaded into a reservoir (here we used a 0.2 mL PCR tube for demonstration). Due to the hydrophilicity of the glass microbeads, they remain trapped inside the aqueous phase even after vigorous mixing (B). Under centrifugation however, the increased centrifugal force allows the glass microbeads to overcome the lipophobic resistance between the glass beads and oil phase and partition according to their density into the bottom oil phase, which effectively removes non-target molecules behind in the aqueous phase (C). (D) Parallel CIFF processes can be performed using conventional multi-well PCR plates.

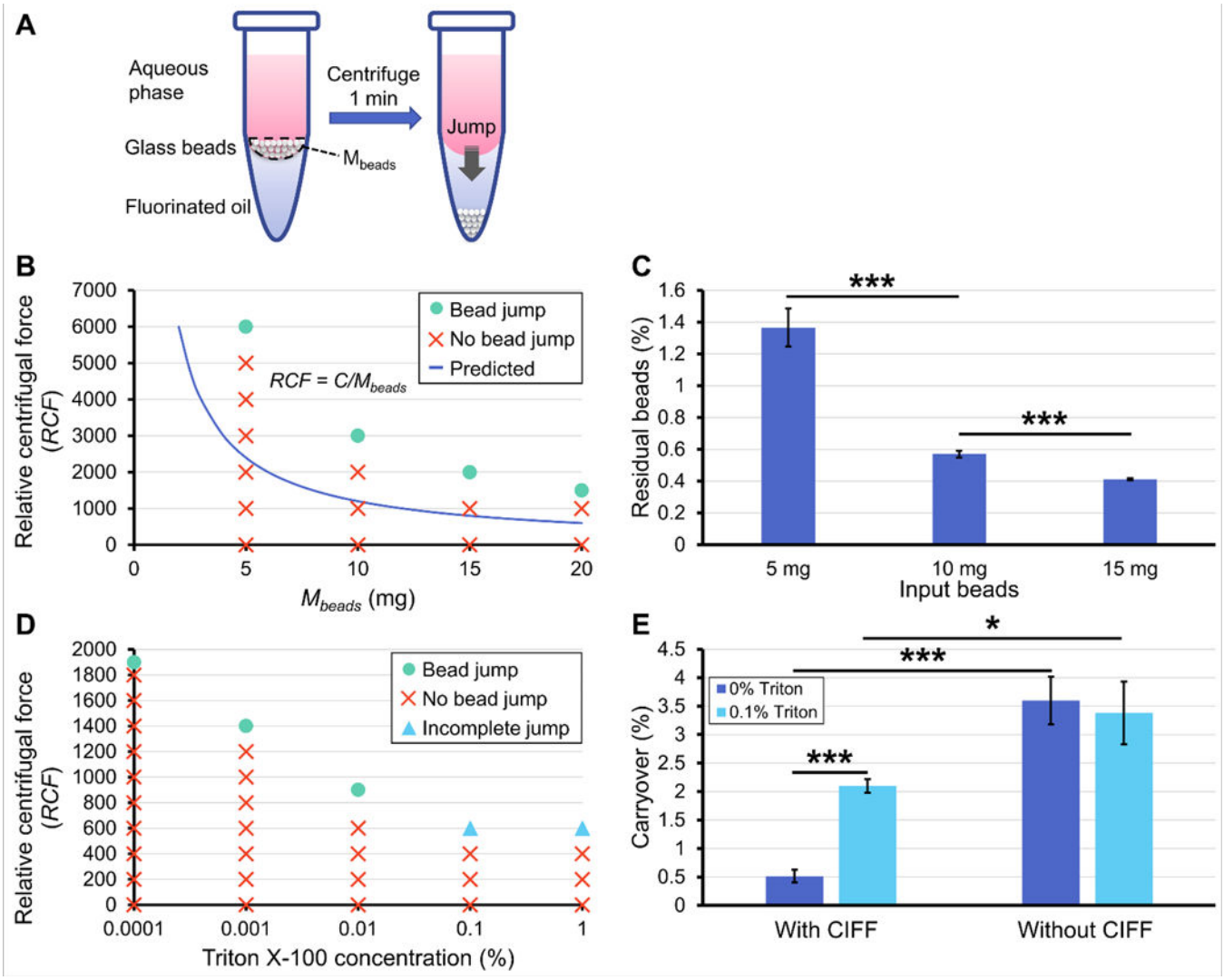

Figure 2.

Characterization of CIFF. (A) Schematic of the CIFF process. (B) Relation of centrifugal force and amount of glass beads to the “jumping” of beads in CIFF. The smaller the amounts of added beads, the higher the centrifugal force required for the beads to overcome the resistance from jumping into the oil phase. (C) Percentage of residual glass microbeads (relative to input) left in the aqueous phase after performing CIFF for different amounts of input beads. Beads were all spun at 10,000 RCF for this measurement. (D) Relation of centrifugal force and Triton X-100 concentration in the aqueous phase to the “jumping” of 10 mg beads in the CIFF process. (E) Percentage of aqueous phase carryover of 10 mg beads after performing CIFF with or without 0.1% Triton X-100 in the aqueous phase. This was compared to a traditional “spin down beads, aspirate supernatant, and resuspend” approach (without CIFF). Error bars denote the standard deviation from 3 technical replicates. Statistical significance is represented by * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

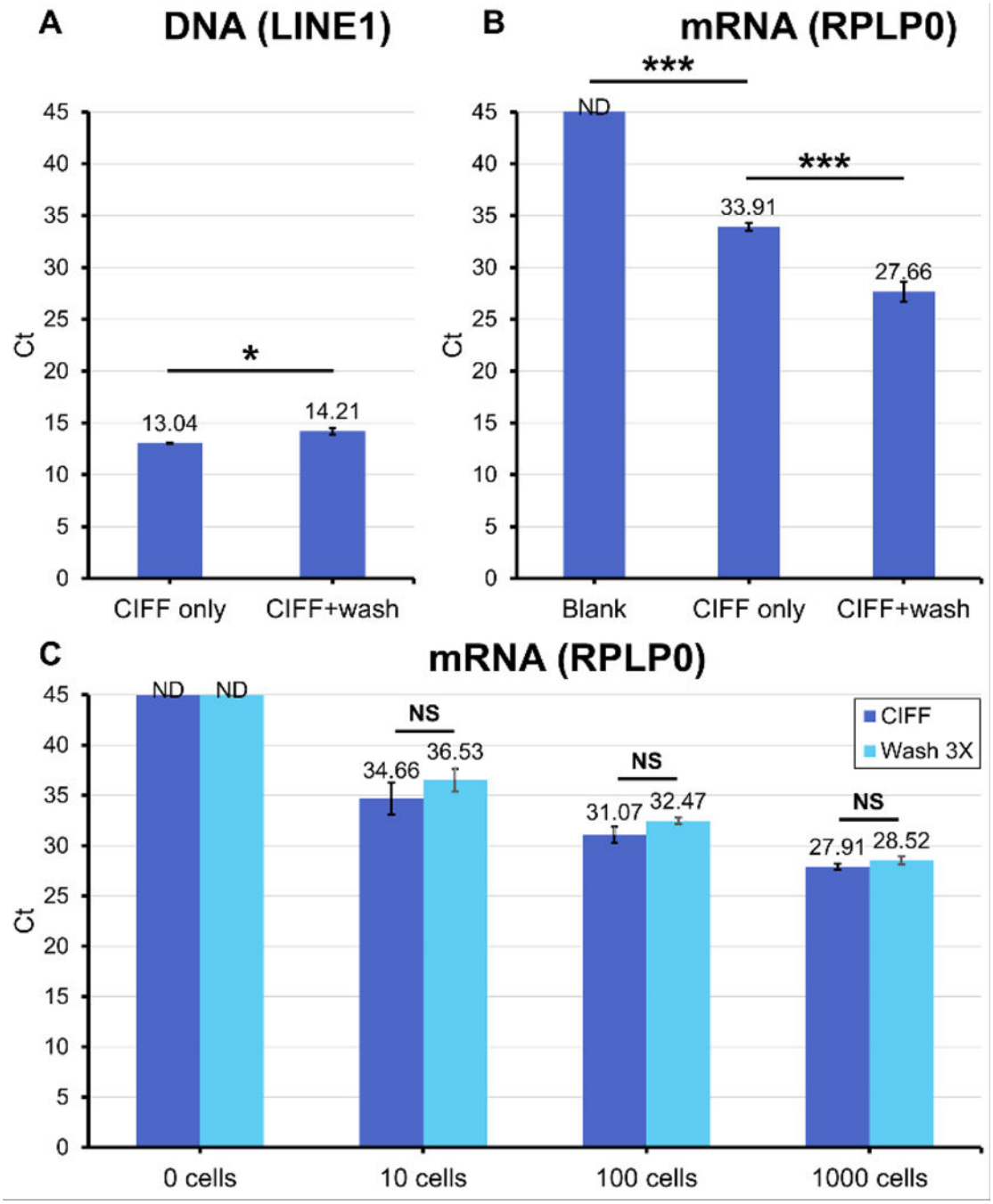

Figure 3.

DNA and RNA extraction performance in CIFF. (A) qPCR performance of LINE1 DNA extracted using CIFF only, compared to CIFF plus two additional washes with buffer AW1 and AW2 (Qiagen). (B) RT-qPCR performance of RPLP0 mRNA extracted using CIFF only, compared to CIFF plus one single wash with RNA washing buffer. The RNA sample was not diluted prior to RT-qPCR, thus one extra wash was necessary to further remove residual lysis/binding buffer contamination which causes inhibition of RT-qPCR (see Figure S2). (C) mRNA extraction performance of CIFF with one single wash compared to a conventional

manual 3-wash operation. ND: Not detected. Error bars denote the standard deviation from 2 technical replicates. Statistical significance is represented by * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$, NS = not significant.

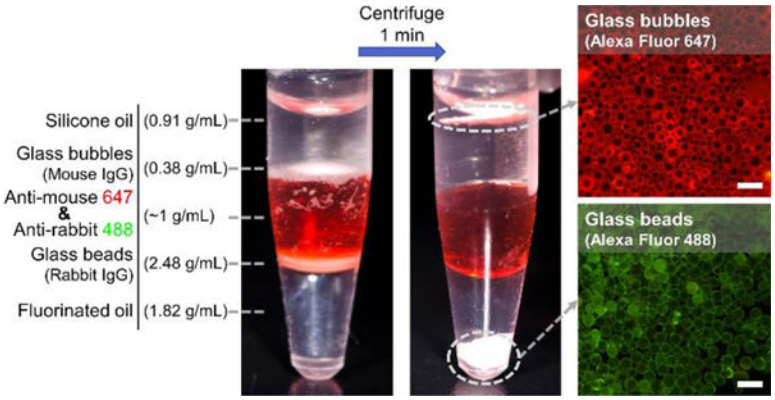

Figure 4.

Simultaneous extraction of two different proteins (Alexa Fluor 647 anti-mouse IgG and Alexa Fluor 488 anti-rabbit IgG) from the same aqueous phase using dual-CIFF. The differential densities of mouse IgG-conjugated glass microbubbles and rabbit IgG-conjugated glass microbeads allow them to travel in opposite directions (floating vs. sinking) under centrifugation and simultaneously extract different target analytes to their respective top and bottom oil layers. Scale bar: 100 μm.