

## The VerIFAST: an integrated method for cell isolation and extracellular/intracellular staining†

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Isolation and characterization of a specific subset of cells from a large heterogeneous population is necessary for studying rare subpopulations of cells. Existing methods require transfer or wash steps that risk causing loss of the rare cell population of interest. Integrated methods reduce loss, making these methods especially useful for reliable isolation of rare cell populations. In this report, we demonstrate the VerIFAST, a device that builds upon the simplified workflow of the Immiscible Filtration Assisted by Surface Tension (IFAST) to integrate a method for cellular isolation with methods for extra- and intracellular staining. First, a front-end purification step allows cells and unwanted particulates to passively settle out of the operational path of the paramagnetic particles, resulting in good efficiency of capture (>80%) and purity (>70%) with a single virtual wall traverse. Second, a Sieve Chamber is used downstream of the isolation chamber that removes excess unbound paramagnetic particles (PMPs) and performs complex multi-step washing procedures without centrifugation or transfer steps. Further, cellular staining can be performed in the device and is demonstrated for extracellular epithelial cell adhesion molecule (EpCAM), intracellular pan-cytokeratins, and Ki-67.

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### Introduction

The collection and characterization of rare cell populations is critical for many tumor biology and blood-based studies including tumor cell heterogeneity,<sup>1,2</sup> stem cells,<sup>3–5</sup> circulating tumor cells (CTCs),<sup>6–8</sup> T-cells,<sup>9–11</sup> and neutrophils.<sup>12,13</sup> Macroscale methods to isolate and analyze these cells require long, expensive, and laborious procedures that typically result in significant sample loss.<sup>7</sup> Thus, effective devices for cell isolation and downstream analysis will involve integrating these methods to save time, reagents, and ultimately reduce sample loss.<sup>14–16</sup> Microfluidic systems have offered unique solutions to these systems by providing the ability to perform complete analyses on a single device.<sup>17–20</sup> The ability to integrate high purity, high efficiency, and flexible cell isolation with downstream techniques for extracellular and intracellular staining will enable high-content studies of rare or precious samples.

Microfluidic platforms for cellular isolation have been demonstrated that can achieve sensitivity and specificity

unattainable using current macroscale systems.<sup>8</sup> These platforms include functionalized micropost arrays, patterned surfaces, and systems that leverage density, size, or other physical characteristics to isolate cells of interest from non-target cells.<sup>19,20</sup> While successful isolations have been demonstrated with these platforms, limitations with on-chip imaging, cell removal, cell viability, and retention of the original sample for further processing may ultimately limit widespread adoption.<sup>6,19,7</sup>

Immiscible phase filtration (IPF) has shown promise for isolation of nucleic acids,<sup>21–23</sup> proteins,<sup>24,25</sup> and cells/lysates,<sup>26</sup> from a particulate background. Immiscible Filtration Assisted by Surface Tension (IFAST) leverages the dominance of surface tension over gravity at the microscale to establish “virtual walls”<sup>27,28</sup> between the immiscible (*e.g.* oil) and aqueous phases to create a micro scale IPF device. The virtual walls are used to filter contaminants in a single step, thereby eliminating centrifugation, multiple washing steps and multiple PMP capture steps while maintaining cell viability.<sup>29</sup>

This system exhibits multiple advantages over existing methods in its simplicity, efficiency, and ability to isolate a variety of analytes.<sup>30</sup> However, geometric configuration of the IFAST (termed the “Horizontal IFAST” in this manuscript) has revealed specific limitations. First, the Horizontal IFAST has lower efficiency of recovery and overall purity for samples containing large particulates that settle out of solution (*e.g.* debris, precipitates, or other cells). Second, the paramagnetic particles (PMPs) used for the isolation are difficult to remove

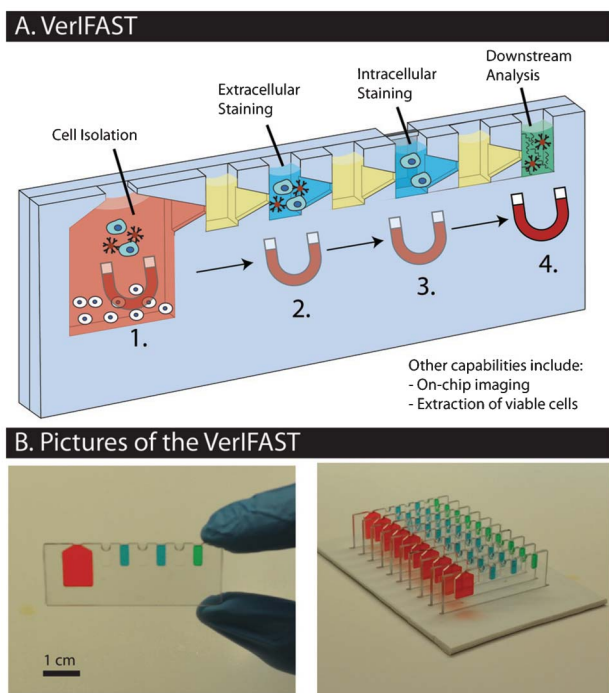
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**Fig. 1** A) The VerIFAST utilizes novel methods to enable cell isolation, extracellular staining, intracellular staining, and downstream analysis in a single, simple device. B) Images of the VerIFAST devices.

from the cells, impairing downstream applications such as fluorescent imaging. Finally, while cellular staining is possible in the Horizontal IFAST, it can be prohibitive due to the number of wells necessary for a staining process (*i.e.* two wells necessary for each step, see supplementary information).

Here we present the VerIFAST (Fig. 1), a significant improvement upon the Horizontal IFAST for the capture and molecular analysis of rare cell populations. The VerIFAST is an improvement upon previous IFAST technology with three important additional features: 1) large particle contaminants settle out of the operational path of the IFAST, resulting in increased purity, 2) excess PMPs can be easily removed for improved visualization and imaging, and 3) complex cellular staining protocols (*e.g.* fixation, permeabilization, staining, washing) can be performed directly in a single well within the device. The VerIFAST combines features of the previously described IFAST<sup>21</sup> and density-mediated immiscible phase filtration,<sup>23,24</sup> resulting in increased performance, operational robustness, and reduced reagent volumes. In the VerIFAST, the PMPs are pulled along the sidewall instead of across the bottom surface, allowing non-target cells to settle out of the operational path of the analyte-bound PMPs. We demonstrate that the VerIFAST enables isolation of rare cells from a heterogeneous background of peripheral blood mononuclear cells (PBMCs) in an elegant integrated front-end processing step. Further, by leveraging the vertical orientation and the ability to fabricate a microporous membrane within the device, the VerIFAST allows for the creation of a chamber (termed the

Sieve Chamber) in which cells and reagents are contained in two adjacent wells separated by a membrane. The Sieve Chamber is designed to have a microporous membrane large enough to allow PMPs to sieve through the membrane, yet small enough to prevent cells of interest from passing through the filter. Furthermore, addition and removal of fluid can be performed within the Sieve Chamber without perturbing the sample, yet when necessary the sample can, at any point, be removed from the device. The VerIFAST is able to take cell capture beyond enumeration endpoints, as cells are able to be isolated from a background in a method that maintains viability, and has an integrated method appended to perform fixation and permeabilization for more intricate intracellular analyses.

## Methods & materials

### Device preparation

The VerIFAST device was fabricated in 2 mm thick polystyrene (PS, Goodfellow, UK) via micromachining (PCNC770, Tormach, USA). The well height and widths can be varied, but were constrained such that the input well had a 200  $\mu\text{L}$  capacity and each successive well had a 30  $\mu\text{L}$  capacity. Each well is connected by a funnel that is a constant 200  $\mu\text{m}$  deep and has a height that tapers from 2 mm to 0.8 mm between the inlet and outlet, respectively. The alternative device for intra-cellular staining employs an 8  $\mu\text{m}$  microporous membrane (Part PET8025100, Sterlitech, USA) and a second layer of PS. The second PS layer, containing one 30  $\mu\text{L}$  well, was solvent bonded (Weak solvent based chip lamination, Zhou, 2010) to the original PS piece, with the membrane sandwiched between. For both the single layered device and the membrane device, an adhesive backing was applied to both sides of the device to contain the fluids (MicroAmp, Applied Biosystems, USA).

### Paramagnetic particle preparation

The paramagnetic particles (PMPs) used for these experiments were Dynabeads<sup>®</sup> M-280 coupled with Streptavidin (Life Technologies, USA). To remove the stock buffer solution from the PMPs, the PMPs were washed and re-suspended with 0.01% Tween-20 in phosphate buffered solution (PBS). 5  $\mu\text{M}$  of biotinylated antibody (R&D Systems, USA) for the epithelial cell adhesion molecule (EpCAM) was added to the PMP solution. The PMPs and antibodies were mixed on a shaker for 30 min at 800 rpm to allow binding, then was washed three times and re-suspended with 0.1% BSA in PBS.

### Cell culture

Target cells used in these experiments were human lymph node carcinoma of the prostate (LNCaP) immortalized cell lines. Cells were maintained at 37  $^{\circ}\text{C}$  and 5%  $\text{CO}_2$  in MEM-alpha culture medium supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin (Gibco, USA), and 100  $\mu\text{g mL}^{-1}$  streptomycin (Gibco, USA). Before running quantification experiments, cells were incubated for ten minutes with calcein AM (Life Technologies, USA) at a concentration of 2  $\mu\text{M}$

in serum-free MEM-alpha culture media. The cells were centrifuged and washed three times with 0.1% BSA in PBS, then counted with a hemocytometer and re-suspended in 0.1% BSA in PBS.

Peripheral Blood Mononuclear Cells (PBMCs) were collected from healthy volunteers under a University of Wisconsin IRB-approved protocol. PBMCs were isolated by density centrifugation, counted in a hemocytometer, and used as non-target cells for cell purification experiments. PBMCs were treated with CellTracker Red CMTPX (Life Technologies, USA) at a concentration of 2.5  $\mu\text{g}$  of CellTracker in 1 mL of serum-free MEM-alpha cell culture medium for 30 min. Following incubation, the PBMCs were washed three times then resuspended with 0.1% BSA in PBS.

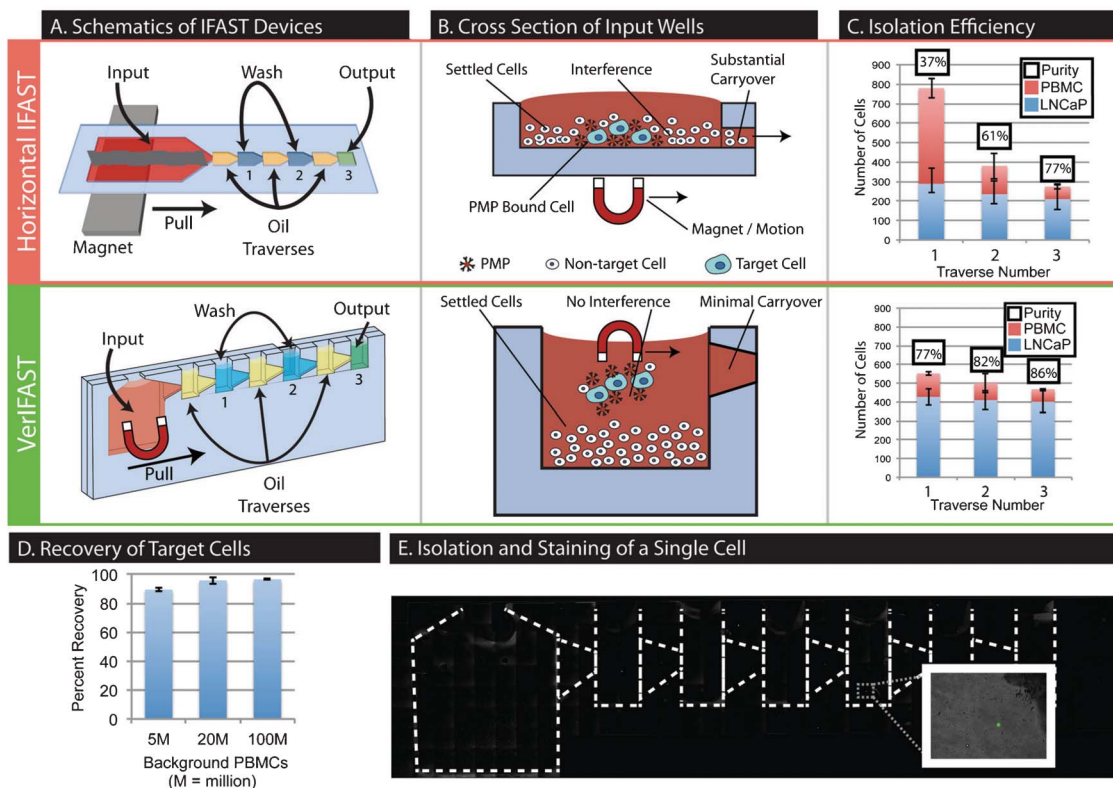
### Experimental setup

250  $\mu\text{g}$  of PMPs and assay-dependent numbers of LNCaPs and PMBCs were added into a mixture of 5 mM EDTA, 0.001% Tween-20, and supplemented with 0.1% BSA in PBS. This mix was tumbled for 30 min in a 4 °C cold room to bind PMPs to cells. The mixture was added to the input well of the device for each experiment, with alternating wells of olive oil (Unilever) and PBS (Life Technologies, USA) through the device. Olive oil was chosen due to its robust interfacial energy with aqueous solutions,<sup>21</sup> see supplemental.

To count the number of cells that were either pulled through or left behind, the device was imaged using an inverted fluorescent microscope (Olympus IX-70). The device was placed on its side, and imaged for calcein AM (492 nm excitation) and the CellTracker (572 nm excitation).

### Staining protocols

Antibodies used for in-line staining endpoints in the VerIFAST was a PE-bound EpCAM antibody (Abcam, USA). Staining was achieved in the wells of the IFAST device by adding the antibody at a 1 : 50 dilution into a solution of 0.1% BSA in PBS. Cells were pulled into the staining well and refrigerated at 4 °C for 30 min prior to being pulled into a final well for imaging containing 0.1% BSA in PBS. Primary antibodies used for intracellular staining were a pan-cytokeratin with tagged FITC antibody (Abcam, USA) at a 1 : 50 dilution and a Ki-67 antibody (Abcam, USA) at a 1 : 50 dilution, both in 0.1% BSA in PBS. The secondary antibody used in conjunction with the Ki-67 was an APC-bound goat anti-rabbit antibody (1 : 100). Nuclear staining was achieved using 0.02 mg mL<sup>-1</sup> Hoescht stain. Staining was achieved in the membrane well in an eight step process including wash steps. Supplemental Table 1 illustrates the steps involved in the intracellular staining protocol.



**Fig. 2** A) Schematics of the Horizontal IFAST and the VerIFAST. B) Cross sectional view of the IFAST devices. In the Horizontal IFAST cells settle to the bottom and interfere with the beads, causing high levels of non-target cell carryover. In the VerIFAST, cells settle out of the path of the PMPs, significantly reducing the amount of carryover. C) Purity level of target cells (LNCaPs) after isolation from a background of 5 million non-target cells (PBMCs) D) Percent recovery of target cells isolated from varying levels of non-target cells. E) Stitched image of the VerIFAST after isolating one LNCaP tumor cell from a background of 20 million PBMCs. Isolation was through to the 5th well, keeping the 7th well open for further analytical techniques (e.g. RNA, DNA, proteins).

## Results and discussion

### Settling chamber

While the VerIFAST operates according to similar principles as the Horizontal IFAST, the VerIFAST enables isolation of rare or low quantity cells more effectively. Due to the orientation and design of the Horizontal IFAST, PMPs are pulled along the bottom surface of the device (Fig. 2A). Since the cells settle to the bottom of the device, the PMPs push unwanted cells through the oil phases, thereby decreasing the efficiency of the isolation (Fig. 2B). The VerIFAST mitigates this issue by utilizing an alternative orientation and design of the input well, allowing cells to settle out of the path of the PMPs. (Fig. 2A and B) The input well where the sample is loaded is deep and is followed by a series of alternating immiscible phases (e.g. oil) and aqueous phases (e.g. stains, elution buffers, washes). By utilizing a well that is both deep and narrow, two goals are accomplished. First, the aspect ratio of the opening holds fluids in the device such that the device can be inverted and turned without losing fluid. Second, PMPs are pulled along the side of the device, instead of the bottom, which allows non-target cells to settle out of the way of the PMPs. Therefore, the PMPs do not pull non-target cells through the oil. This is particularly important since non-target cells pulled through the oil would necessitate additional traverses to achieve high levels of purity. The purity levels and corresponding number of traverses were characterized by isolating LNCaPs from a background of PBMCs (Fig. 2C). In the Horizontal IFAST device, three oil traverses were necessary to achieve 77% purity (99.998% non-target cell removal), which was due to the effects previously described. The VerIFAST is capable of achieving the same level of purity in one traverse. Furthermore, because target cells are lost with every traverse, the VerIFAST has a higher efficiency of isolation by achieving higher levels of purity in fewer traverses. The robustness of the VerIFAST method was demonstrated with the isolation and visualization of a single cell placed into a background to 20 million PBMCs (Fig. 2D). This technique can also remove target cells from high numbers of background cells without impacting the recovery rate of the device, enabling this method to be used following a batch process for cellular enrichment, as is the case with a Ficoll-Paque density centrifugation. Specifically, target LNCaPs were removed from various amounts of background PBMCs (5–100 million cells in 200  $\mu$ l), and the isolation efficiency remained high (>80%) for each level of background cells (Fig. 2E).

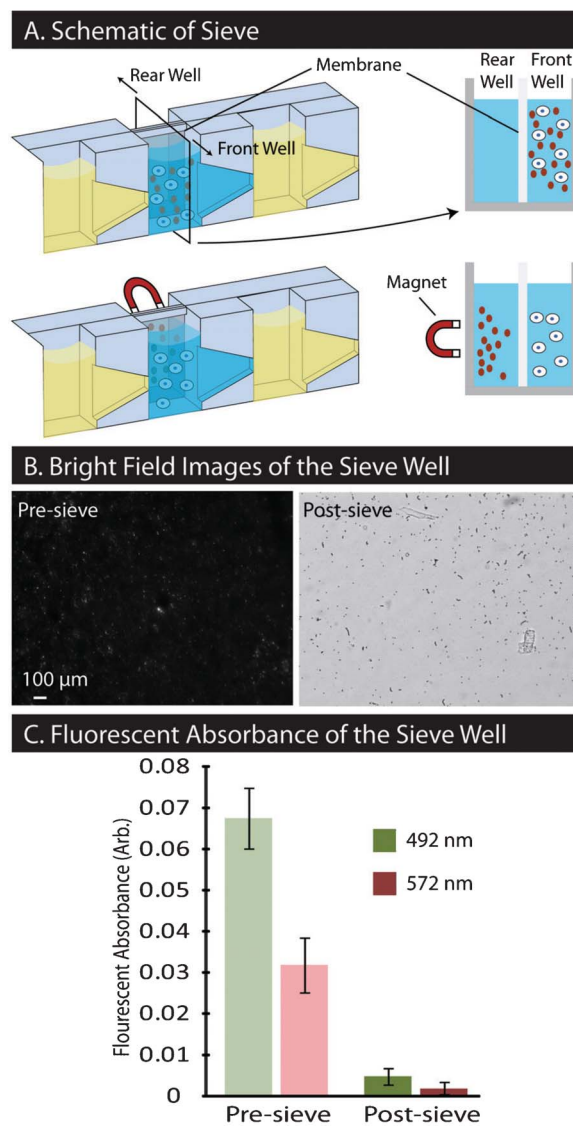
### In-line staining

Extracellular surface stains are important to many cellular analyses, as these stains can be performed without the need for cell fixation and enable visualization of many surface proteins (*i.e.* EpCAM, CD4, *etc.*). We demonstrate the ability of the VerIFAST device to perform simple in-line extracellular staining for the surface protein EpCAM. This process is completed by simply pulling cells into a well containing antibody conjugated to a fluorophore (Fig. 1D). The immiscible barriers on each side of the well contain the antibody and cells in an aqueous solution for the duration of the incubation (30 min at 4  $^{\circ}$ C). After the incubation, the magnet is used to

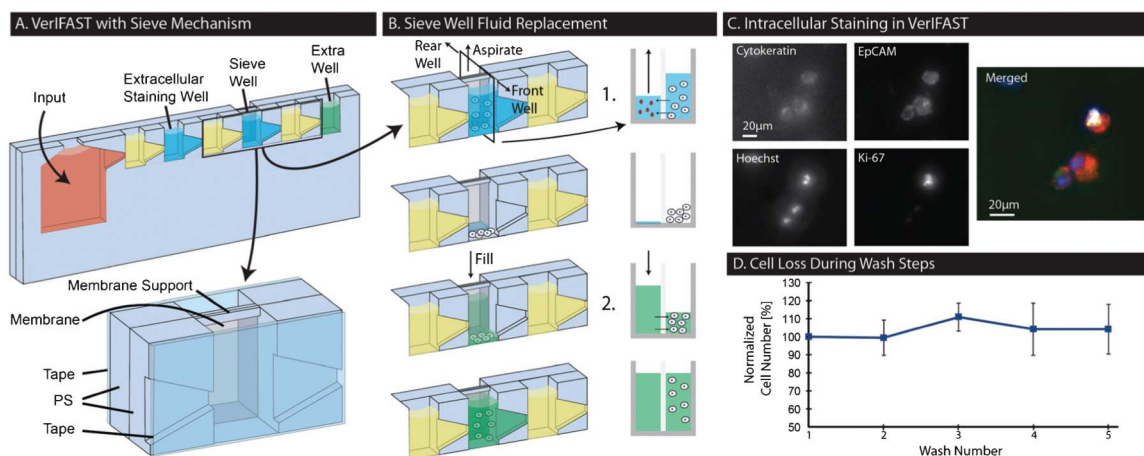
pull the PMP-bound cells through the oil phase, leaving behind unbound antibody in a single wash step. Oil well traverses have been previously demonstrated to maintain cell viability.<sup>30</sup>

### Sieve chamber

Isolating, staining, and imaging cells within a single device can be advantageous for low or rare populations of cells as it eliminates the need for wasteful transfer or centrifugation steps. The VerIFAST mitigates cell loss by enabling cell isolation *via* PMPs and immiscible phases with a simplified workflow. However, the PMPs involved in the isolation can



**Fig. 3** A) When a magnet is placed on the backside of the device, PMPs pull through a microporous membrane, separating the PMPs from the cells. B) Bright field images of the sieve well before and after PMPs are removed *via* the sieve method. C) Fluorescent absorbance of the fluid contents of the sieve well before and after the PMPs were removed. Absorbance was measured at two different wavelengths (492 nm and 572 nm) and the presence of beads in solution contributed to an approximate 7-fold decrease in absorbance upon beads being sieved from the device.



**Fig. 4** A) Schematic and enlarged view of the VerIFAST with the sieve mechanism. The extra well is for optional downstream extractions/isolations. B) Fluid replacement is completed in two simple steps, namely aspirating unwanted fluid and dispensing new fluid into the device. C) Images of cells that have been stained in the VerIFAST for both extra-cellular (EpCAM) and intracellular (Cytokeratin, Ki-67, Hoechst) markers. D) Repeatability of the quantity of cells remaining in the sieve well after a specified number of washes ( $n = 3$ ).

cause issues with downstream analyses or experimentation. We have overcome this obstacle by integrating a sieve chamber into the VerIFAST to facilitate removal of background PMPs from a cell suspension.

The Sieve Chamber (Fig. 3A) utilizes a polycarbonate microporous membrane (8  $\mu\text{m}$  pores) that is bonded between two wells. The front well is coplanar with the other wells in the VerIFAST and is the recipient of the isolated cells. The rear well is adjacent and of identical size to the front well and the same size, but separated by the microporous membrane. After the PMP bound cells are pulled into the first well, the magnet is removed and reapplied to the backside of the device, against the rear well. This magnetic force pulls the PMPs (3  $\mu\text{m}$  diameter) through the membrane pores (8  $\mu\text{m}$  diameter) while the target cells (average 15  $\mu\text{m}$  diameter<sup>30</sup>) are physically too large to get through the membrane. The pore size of the membrane is particularly important, as the pores must be larger in diameter than the PMPs yet smaller than the diameter of the target cells. This process removes excess unbound PMPs from the solution.

Unbound PMP removal is necessary for imaging purposes. The optical benefits of PMP removal are illustrated by comparing a well containing PMPs to a well without PMPs (Fig. 3B). Visually, the PMPs cause the well to become nearly opaque, making bright field imaging of the target cells difficult. To expand this analysis, the solutions were analyzed by spectrophotometry both before and after PMP removal within the sieve chamber. Spectrophotometry revealed that removal of the PMPs decreases fluorescent interference by seven-fold, seen in both red (572 nm) and green (492 nm) channels (Fig. 3C).

#### Sieve chamber staining

To expand the capabilities of the VerIFAST we integrated intracellular staining into the process. Cells isolated with the VerIFAST were assayed for intracellular proteins on-device without any transfer steps. Elimination of the transfer step is

key to conserving cells, as cells are inherently lost during the transfer. Similar to the PMP removal process, the same microporous membrane was used to add and replace fluid without perturbing the cellular sample on the front side of the membrane (Fig. 4B). This is performed in a two-step fluid handling process (Fig. 4B) and is operated entirely by pipette for all fluid transfer steps, a simple and flexible alternative to syringe pumps. The first step is to aspirate the liquid from the rear well (left). The difference of hydrostatic pressures forces the liquid through the membrane, from the front well to the rear well, thereby enabling complete removal of the liquid. Once the liquid is removed, new liquid is added into the rear well. Again, the difference in hydrostatic pressure will force the liquid through the membrane, but this time to fill the front well. As this form of washing is not reliant upon diffusion, fluid transfers can be performed rapidly within the device. Using a single well is beneficial for multi-step methods, but can include problems of carryover and incomplete fluid removal, which can be ameliorated through the use of extra wash steps without sample loss. The sieve chamber can be used for a variety of processes (*e.g.* fixation, permeabilization, washing, fluorescent staining) without requiring removal of the cell sample from the device.

The use of a membrane for fluid replacements simplifies complex, multi-step and multi-fluid processes into a single well. Further, it enables both rapid fluid replacements and efficient use of space. For example, a thirteen step intracellular staining process for LNCaPs, including stains for intracellular proteins (pan cytokeratins and the proliferation marker Ki-67), can be completed in a single well of the VerIFAST (Fig. 4C, Process detailed in supplementary information). Importantly, all of the cells are contained within the well and are never directly contacted, thereby minimizing cell loss. To characterize the effectiveness of the membrane in terms of reducing cell loss, cells were counted after each wash for five washes ( $n = 3$ , Fig. 4E). The data shows that cell loss due to the membrane is statistically negligible through five wash steps,

making the membrane and the VerIFAST a simplified platform to enable isolation and staining of precious samples.

## Conclusions

We present a device that integrates cellular isolation with downstream analytical methods for extracellular and intracellular staining in an integrated, flexible platform well suited for rare or precious samples. By incorporating a front-end settling chamber, we add a passive pre-processing step resulting in a six-fold reduction in background through a single traverse with high efficiency regardless of background cell number. An in-line staining chamber is used to perform extracellular staining quickly and without wash steps, resulting in a viable population of labeled cells for downstream assays. Finally, a membrane-separated well is used to perform more sensitive analytical methods on an unperturbed sample, enabling the observation of cytoplasmic or nuclear proteins within an isolated cell population without significant cell loss. Cellular isolation is a powerful tool that is used for a litany of diagnostic and research techniques. An integrated method that can expand the abilities of isolation methods to include ubiquitous downstream techniques of immunohistochemistry and flow cytometry will enable and enhance the analysis of circulating tumor cells, in which rare samples need to be interrogated without sample loss.

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