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Method for the Accurate Preparation of Cell-Spiking Standards

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Preparation of calibration standards for cell enumeration is critical in characterizing the performance of any method or apparatus intended for recovering rare cells. Diluting a cell suspension serially is prone to statistical sampling errors as the cell suspension becomes more dilute, whereas transferring and injecting cells individually into a diluent with a micromanipulator is time-consuming. We developed a simple and robust method using a surface-modified glass capillary to siphon and eject cells. One-dimensional confinement of cells offered by the capillary made cell enumeration by visual counting simple and rapid, and cell ejection from the capillary was near 100% when the appropriate surface coating and cell solution was used. The residence time of cells in the capillary, however, could affect the percentage of cells that was ejected from the capillary. To characterize the performance of this method, we enumerated the ejected cell using both visual counting under a microscope and automated detection using a chip-based flow cytometer.

Accurately prepared standards for cell enumeration are highly important for characterizing the performance of methods or apparatus involved in the recovery of rare cells, which may include circulating tumor cells in body fluids,[1−8] fetal cells in maternal blood,[9−14] or stem cells.[15−18] Typically, a concentrated cell suspension is pipetted into a diluent, and to reach the concentration level of rare cells, the mixture is then subjected to a succession of serial dilutions. The cell concentration is calibrated by sampling a small volume at some point of the dilution cascade, and the cells within are counted either manually using a hemacytometer[19] or automatically using a flow cytometer.[17,20,21] This serial-dilution approach is convenient but relies heavily on the assumption that sampling is statistically representative: each time a small aliquot is taken, it is assumed that the concentration of the cells within is exactly the same as in the remaining solution. While this assumption is reasonably accurate for concentrated cells, it is prone to error if either the operator is inconsistent with maintaining the uniformity of cells in solution (e.g., via a vortexing or pipetting protocol) or the cells are highly diluted so that sampling is no longer statistically representative.

With the availability of micromanipulators,[22,23] it is technologically feasible to pick up cells individually and deposit them into a diluent. The use of micromanipulator, however, is time- and cost-intensive: the operator typically needs to use a micropipette that is mounted on a high-graduation micromanipulator to siphon up cells individually while following the process under the microscope, transfer the capillary tip gingerly into the diluent, and then eject the cells while counting the number of cells expelled. This approach is reasonable for injecting a small number of cells, but for more than a few repetitions, this process becomes laborious.

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This paper describes a simple yet accurate method to prepare standards for rare-cell enumeration and spiking using a large-diameter glass capillary. The surface of the glass capillary was modified to improve manual siphoning and ejection of cells. This procedure is highly accurate as the glass capillary can be readily inspected under a microscope before and after the ejection of cells. Unlike counting cells dispersed on a microscope slide or in solution, the inner diameter of the capillary is matched with the field of view of the microscope; this one-dimensional confinement greatly facilitates visual determination of cell counts because it eliminates the need to keep track of the inspected areas or the search in three dimensions for new cells that are either resting on the microscope slide or still dispersed in solution. With this procedure, a rare-cell standard can be prepared in as little as a few minutes. We characterized the effect of cell adhesion in the capillary and surface-modification strategies and validated the cell enumeration manually, as well as with an automated microfluidic flow cytometer.

EXPERIMENTAL SECTION

Materials. Glass capillary tubes (0.4 mm I.D. × 75 mm) were obtained from Drummond Scientific (Broomall, PA). Disposable positive-displacement pipet tips with pistons (Microman CP-25) were from Gilson Medical Electronics (Middleton, WI). For surface modification of glass capillary walls, tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane ("TDF-silane") was obtained from United Chemical Technology (Bristol, PA), and 2-[Methoxy(polyethyleneoxy)propyl] trimethoxy silane ("PEG-silane") was obtained from Gelest (Morrisville, PA). For fluorescent-labeling of cancer cells, Phycoerythrin-antihuman CD326 ("PE-anti-EpCAM"; 0.2 mg mL^{-1}) from Biolegend (San Diego, CA) and Pan-Keratin (C11) Mouse mAb (Alexa Fluor 647 conjugate) ("Alexa 647-anti-CK"; 0.01 mg mL^{-1}) from Cell Signaling Technology (Danvers, MA) were used. All aqueous solutions were phosphate-buffered salt solution (1 × PBS) from Fisher Scientific (Pittsburgh, PA) unless indicated otherwise.

Cell Culture. SKBr-3 and MCF-7, both human breast tumor-derived cell lines, were from the American Type Culture Collection (ATCC). Cells were maintained in the recommended culture media (McCoy's 5A or EMEM) containing 2 mM L^{-1} glutamine, 10% fetal bovine serum, and 50 μg mL^{-1} penicillin/streptomycin at 37 °C with 5% CO_2 in a humidified environment. HEK293, NG-108−15, CHO-K1, HeLa, CV-1, and CH-27 cell lines were cultured similarly in their recommended culture media supplemented with glutamine, fetal bovine serum, and penicillin/streptomycin. All cells were seeded at 0.5−1 × 10^4 cells cm^{-2} in a T-75 flask and allowed to grow for 3−5 days until cells reached ~80% confluency. To prepare cell suspensions, adherent cancer cells were treated with 5 mL 0.25% (w/v) trypsin and ethylenediaminetetraacetic (EDTA, sodium salt, concentration 2.5 g L^{-1}) for 5 min, diluted with media, and washed (centrifuged at 300 × g for 5 min then resuspended in 5 mL of corresponding diluent). CH-27 cells only required washing. Cells were mixed by vortex or micropipette to obtain uniform suspension and were then adjusted to the desired cell concentration.

Cell Staining. Cells were fixed in 2% paraformaldehyde solution for 15 min at room temperature with intermittent vortexing to maintain the cell suspension, washed twice in 1 × PBS, and then permeabilized with 0.5% Triton-X 100 for 15 min and washed again. Cells were incubated in PE-anti-EpCAM at 1:5 (antibody:cell suspension) volume ratio and Alexa 647-anti-CK at 1:10 ratio for 2 h at room temperature. After incubation, the mixture was washed three times to remove the free antibody and then adjusted to the desired cell concentration. Subsequently, the fluorescent cell suspension was maintained at 4 °C throughout the automated cell counting using the microfluidic flow cytometer.

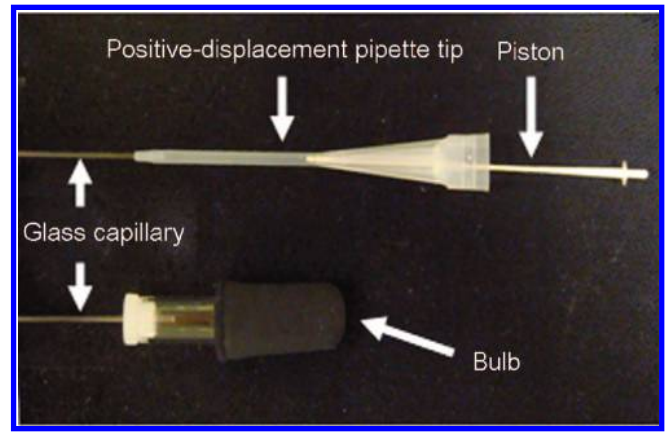

Figure 1. Photograph of the capillary-based cell-enumeration (lower) and cell-spiking (upper) apparatus. Cell suspension (4 μL) was siphoned into a glass capillary using a rubber bulb and counted manually under microscope while in the capillary. To eject cells, the glass capillary was inserted into a disposable positive-displacement pipet tip, and the capillary content was expelled by pushing in the piston at a constant speed.

Surface Modification of Glass Capillaries. Glass capillaries were placed in a vacuum desiccator jar along with a few drops of TDF-Silane or PEG-Silane in an open vial and maintained in vacuum overnight. The effectiveness of TDF-silane surface treatment was checked by inserting a treated capillary into DI water: the capillary should exhibit no capillary force to draw in liquid.

Manual Cell Enumeration and Preparation of Spiked Standards. Cell suspension was siphoned into a glass capillary and ejected using the apparatus shown in Figure 1. For cell enumeration, the cell suspension was siphoned into a surface-modified glass capillary using a rubber bulb. The rubber bulb was then slipped off the capillary, leaving no external pressure that could cause expulsion of liquid from the capillary. The glass capillary was placed under a Zeiss Axiovert 100 inverted microscope (equipped with a 150 W mercury arc lamp and optical filters) and the cells within the capillary were counted manually. To eject the cells, a disposable positive-displacement pipet tip was slipped onto the capillary initially without the piston, and then, as the piston was inserted into the capillary tip, a column of air was sealed between the piston and the cell suspension. The capillary content was carefully dispensed as a single droplet by pushing in the piston at a constant speed. Excessive speed could cause uncontrollable spraying of liquid. Unless otherwise noted, cells were ejected exactly after 3 min residence time in the glass capillary. All data points were repeated at least five times. Cells were not fixed or labeled in manual counting experiments.

Chip-Based Cytometry. An automated chip-based cytometer was used to validate the cell counting and spiking procedure. We constructed the cytometer (Figure 2A) by sending the 488 nm output of a solid-state diode pumped laser (Coherent Sapphire, Santa Clara, CA) and the 633-nm output of a HeNe laser (Coherent, Santa Clara, CA) into a Nikon TE2000 inverted microscope. Prior to entering the 20 × objective, the two laser beams were shaped using cylindrical optics to form a collimated

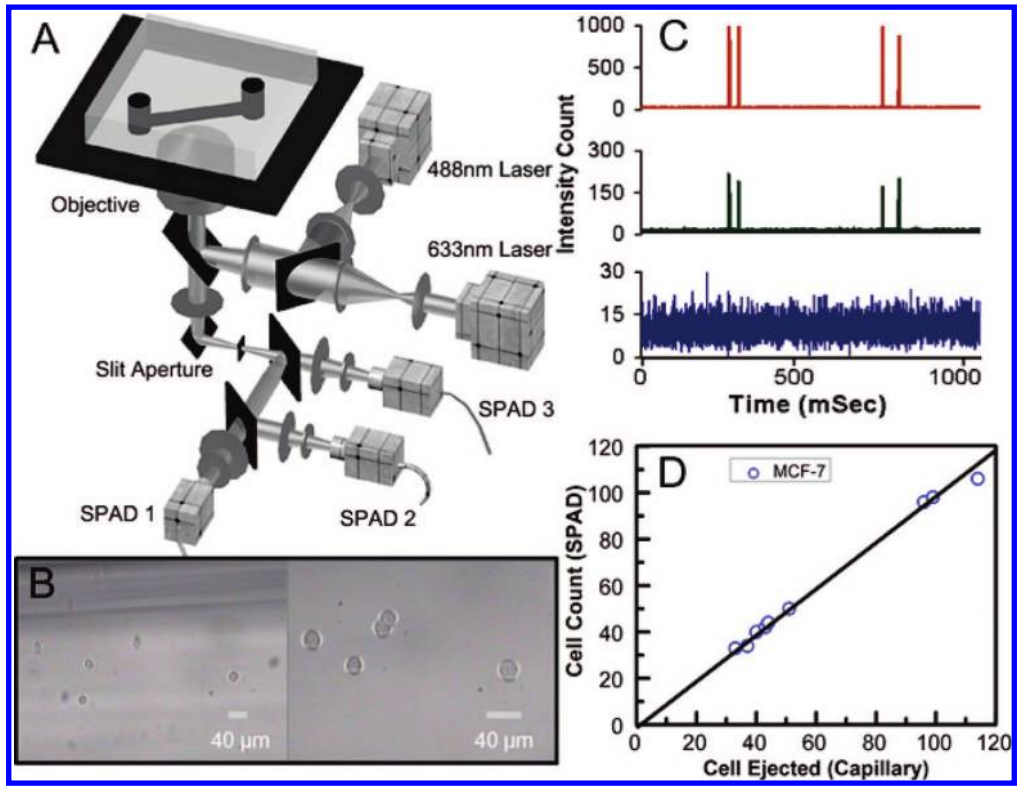

Figure 2. (A) Schematic of the automated chip-based cytometer; SPAD: single-photon avalanche diode. (B) Cells (MCF-7 in PBS) in a glass capillary under a 10 × (left image) and 20 × (right image) objective. The inner diameter of the capillary matches the field of view of the 10 × objective. (C) Typical signal traces from the three SPADs as fluorescently labeled cancer cells pass through the detection region of the cytometer. Top trace: 645−700 nm fluorescence, for detecting Alexa 647-anti-CK; middle trace: 560−610 nm fluorescence, for detecting PE-anti-EpCAM; bottom trace: 500−540 nm as negative control. (D) A plot showing the detection efficiency of the chip-based cytometer, which is defined as the ratio between the cells detected by the cytometer and the cells ejected out of the glass capillary and into the chip.

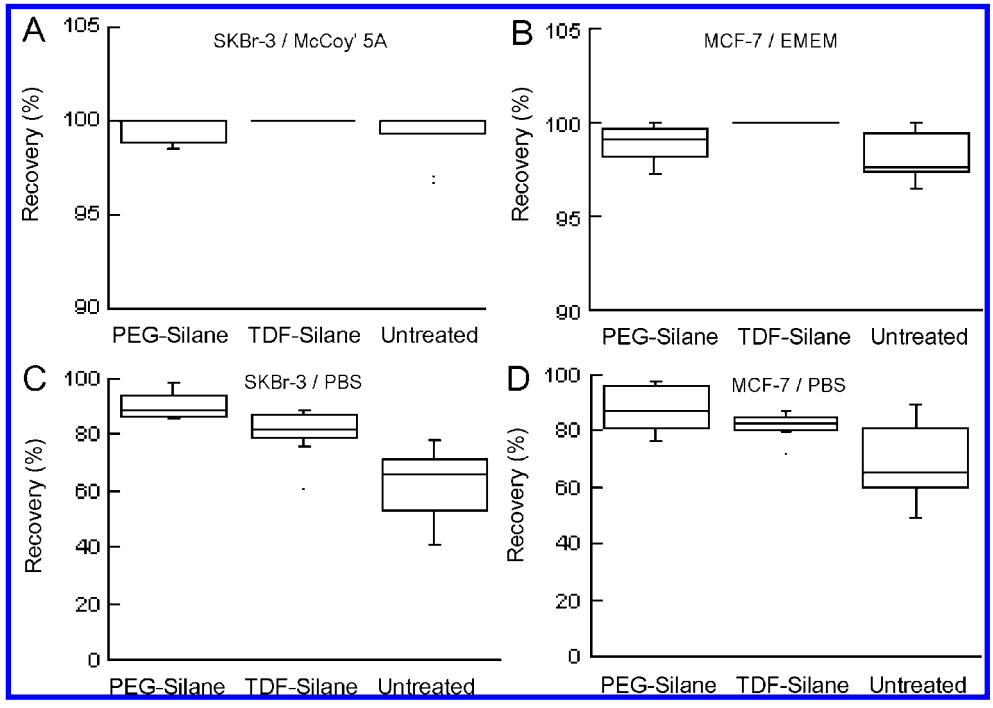

Figure 3. Effect of cell solution and the surface modification of the glass capillary on the recovery of cells: (A) SKBr-3 in culture media; (B) MCF-7 in culture media; (C) SKBr-3 in PBS; (D) MCF-7 in PBS. To distinguish the effects between cell adsorption versus retaining liquid, we used a piece of Kimwipe to draw out all liquid inside the capillaries that were either untreated or treated with PEG-silane (TDF-silane-treated capillaries did not require the use of Kimwipe to eject all liquid), so the observed differences in recovery can be properly attributed to cell adsorption on the capillary walls. Recovery refers to the percentage of cells ejected from the glass capillary as compared to the original number of cells siphoned into the capillary. The residence time of cells in the capillary was exactly 3 min.

Table 1. Accuracy and Efficiency of Transferring Cells Using a TDF-Silane-Treated Glass Capillary in Culture Media

	cells counted in the capillary				cells deposited onto the coverslip				
run	single	2-cell clumps	3-cell clumps	>3 cell clumps	single	2-cell clumps	3-cell clumps	>3-cell clumps	transfer efficiency
1	15	2	3	0	15	2	3	0	100%
2	10	2	1	0	10	2	1	0	100%
3	10	4	2	1	10	4	2	1	100%
4	14	2	0	0	14	2	0	0	100%
5	10	5	0	0	10	5	0	0	100%
6	2	1	0	0	2	1	0	0	100%
7	1	0	0	0	1	0	0	0	100%

elliptical beam with an aspect ratio of 10 to 1.[24] Using a combination of half-waveplate and polarizing beam splitter, the intensity of each beam could be adjusted, while mirrors independently steered the light to create a spatially co-localized excitation region. Collected back through the objective and after passing through a polychroic mirror (Semrock, Rochester, NY), fluorescence was imaged onto a rectangular slit aperture (Melles Griot, Carlsbad, CA and Edmund Optics, Barrington, NJ) located in the image plane. The fluorescence was split into three wavelength bands by two dichroic mirrors before passing through the bandpass filters (Chroma, Rockingham, VT) and refocused onto the three single-photon avalanche diodes (SPADs) (SPCM-AQR-14, Perkin-Elmer, Fremont, CA). One SPAD collected fluorescence in the wavelength range of 560−610 nm to capture the peak emission from phycoerythrin; a second SPAD collected the fluorescence in the range of 645−700 nm from Alexa 647, and a third SPAD collected the range of 500−540 nm and was used as a negative control to eliminate false-positives from antibody aggregates or contaminants. The SPAD outputs were recorded by a PCI-6602 counter/timer board (National Instruments, Austin, TX) and analyzed with LabView, MATLAB, and Origin.

The microfluidic chips were fabricated in PDMS according to procedures described previously.[25,26] The chip design consists of a single microchannel of 200 μm (W) × 50 μm (H).

To count cells in flow, MCF-7 cells were first fixed and labeled with Alexa 647-anti-CK and PE-anti-EpCAM as described earlier and adjusted to appropriate concentration with culture media. The cells were then siphoned into a TDF-silane-modified glass capillary and counted manually at least in triplicates under microscope in both bright-field and fluorescence mode. The content of the capillary was ejected from the capillary and into the fluidic reservoir of the chip, which has been primed with Isoton III hematological diluent (Coulter Diagnostic, Hialeah, FL). The nominal volumetric flow rate on the chip was 15 μL/min, and the average transit time of cells through the detector region was 200 μs.

Cell Viability. TDF-silane-modifed capillaries were rinsed repeatedly and immersed overnight in 70% ethanol and then autoclaved. The capillaries were filled with SKBr-3 suspension (~10^7 cells/mL) using a rubber bulb. The capillaries were divided into three groups of eight capillaries, with each group in its own Petri dish, covered with McCoy's 5A, and maintained at 37 °C with 5% CO_2 in a humidified environment for a specific residence time. Excess McCoy's 5A prevented the evaporation of solution within the capillaries, but the media did not enter the capillaries because of the hydrophobic capillary walls. The entire content of the capillaries was subsequently expelled (with repeated rinsing to remove adherent cells) onto a 24-well plate, each group occupying one well; a control sample was prepared by using a Rainin L-100 pipet to aliquot 30 μL of SKBr-3 suspension and deposited immediately onto the same 24-well plate. The 24-well plate was then maintained overnight at 37 °C with 5% CO_2 in a humidified environment.

Cell viability was determined with Invitrogen's LIVE/DEAD Viability/ Cytotoxicity assay kit. This assay is based on detecting intracellular esterase activity of live cells with calcein AM and plasma membrane integrity of dead cells with ethidium homodimer-1 (EthD-1). The cultured cells were washed twice in 1 × PBS, and then incubated at room temperature for 30 min in a solution of 2 mM calcein AM and 4 mM EthD-1 prepared in 1 × PBS. The cells were washed once again with 1 × PBS and inspected under the microscope.

RESULTS AND DISCUSSION

Manual Cell Counting. Table 1lists the number of SKBr-3 cells siphoned into a glass capillary and deposited subsequently onto a coverslip, as counted manually under the microscope. The glass capillary was modified with TDF-silane to render the surface hydrophobic and prevent retention of any liquid by capillary force, as well as wetting the outside of the capillary. The cell suspensions were deliberately mixed under lesser agitation to retain ~20% of the cells in the form of clumps. In all runs, cells were expelled at 100% transfer efficiency. We noted no loss of the cells inside or outside of the glass capillary and that the piston, which was separated from the cell suspension by a column of air, did not cause any damages to cells as one might expect if it were in direct contact. All multicell clumps were fully accounted for; the clumps did not dissociate under the shear stress of ejection. As compared with other manual counting methods, this capillary-based method can accurately account for 1 cell in a 4 μL volume, whereas a conventional manual hemacytometer has a minimum threshold 62 cells in 10 μL, and a microchannel-based hemacytometer has a threshold of 1−2 cells in 1 μL.[21] However, neither conventional nor microchannel-based hemacytometer is designed to transfer cells for preparing spiked samples; these hemacytometers are intended to analyze a small aliquot, and at such dilute concentrations the sampling of aliquot can be a significant source of error.

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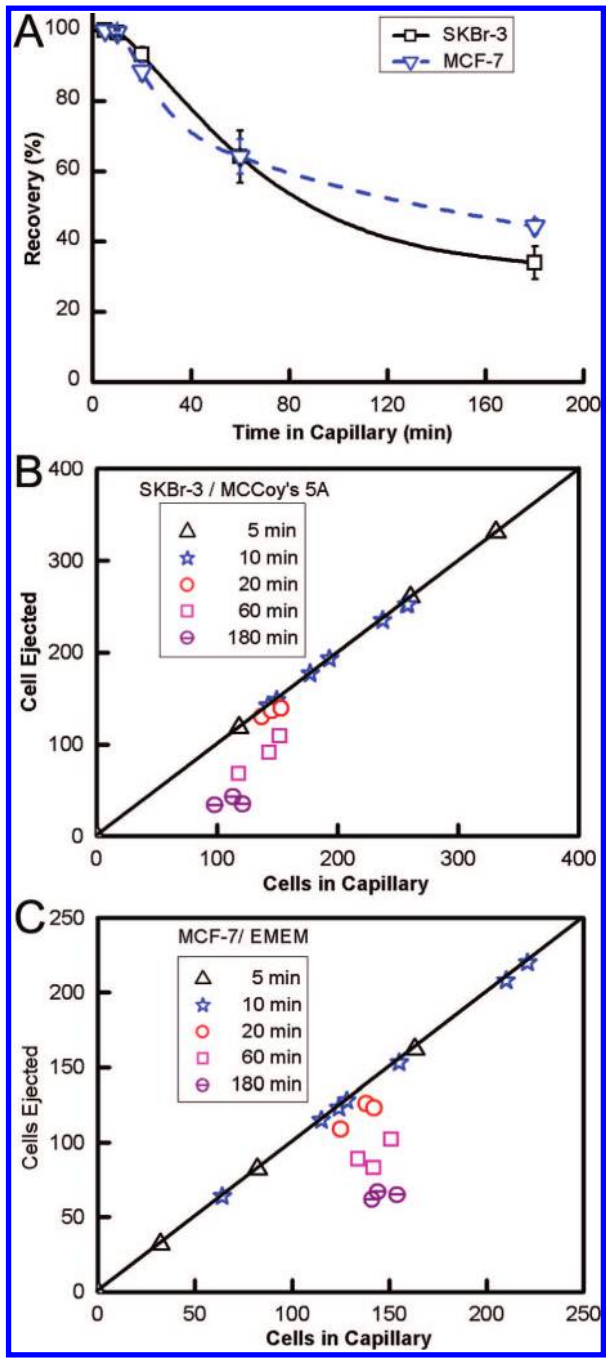

Figure 4. (A) Effect of residence time of cells (in culture media) in TDF-silanized glass capillary on the cell recovery (the percentage of cells ejected from the glass capillary as compared to the original number of cells siphoned into the capillary). The lines that connect the data points were drawn to guide the eye. (B) Cell ejection as a function of the number of cells, as well as the residence time in TDF-silanized glass capillary in the case of SKBr-3 in McCoy's 5A. Straight line indicates 100% ejection. (C) Cell ejection in the case of MCF-7 in EMEM.

For an untreated glass capillary, it was impossible to completely eject the cells as the hydrophilic surface would always retain liquid on the inner wall.

Verification of Spiked Sample with Automated Cytometer. To eliminate the possibility of human error in manual counting, we verified the cells ejected from a capillary using an automated cytometer shown in Figure 2A. MCF-7 cells were fluorescently labeled, siphoned into a TDF-silane-modified glass capillary,

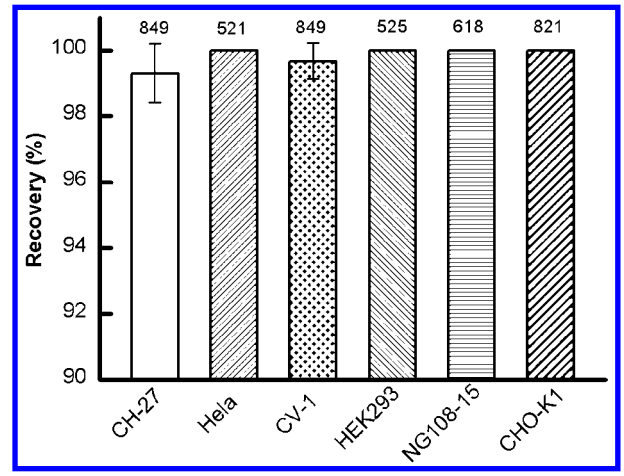

Figure 5. Recovery of various cell lines. The number on top of each bar indicates the total number of cells counted in determining the average recovery ($N = 6$ for each cell line).

counted under microscope (Figure 2B), and then spiked into a diluent flowing through a straight microchannel. Figure 2C shows the signal traces from the three SPADs, with the top pane showing the fluorescence from Alexa 647-anti-CK, the middle pane showing that from PE-anti-EpCAM, and the bottom pane showing the negative control. Concurrent detection of Alexa 647-anti-CK and PE-anti-EpCAM but no signal in the third SPAD is required for positive identification of a spiked cancer cell. Simultaneous detection of signal in all three SPADs was regarded as false-positive, which might be caused by antibody aggregates or debris. Figure 2D shows consistently >97% agreement between the automated counting and manual counting, except for two data points (>91%). For these outliers, cell loss could have occurred on the chip at the inner wall of the fluidic reservoir, which was too deep to be inspected thoroughly using a microscope.

Diluent and Surface Modification of Glass Capillary. Figure 3 shows four boxplots profiling the recovery (the percentage of cells ejected from the glass capillary as compared to the original number of cells siphoned into the capillary) as a function of surface modification of the glass capillary and the compounding effect of diluents (culture media or PBS) for live SKBr-3 and MCF-7 cells. Two observations can be made: first, cell recovery in culture media was always higher than that in PBS regardless of surface modification; and second, surface-modified glass capillary always resulted in higher recovery than unmodified capillary. Since culture media is invariably supplemented with bovine serum, the bovine serum albumin (BSA) within is well-known as an excellent blocker for protein and cell adsorption.

Surface treatment with PEG-silane provides a different surface-protection mechanism from that with TDF-silane: PEG-silane introduces polyethylene-like surface functional groups that are resistant to biofouling,[27] but the surface is nowhere near as hydrophobic as the fluorinated surface from TDF-silane. Thus, the choice of silanes presents a tradeoff between resistance to protein adsorption and resistance to wetting. To distinguish the effects between cell adsorption versus retaining liquid, we used a piece of Kimwipe to draw out all liquid inside the capillaries that were either untreated or coated with PEG-silane, so the observed differences in recovery can be properly attributed to cell adsorption on the capillary walls. Note for capillaries treated with TDF-silane, all liquid in the capillary was

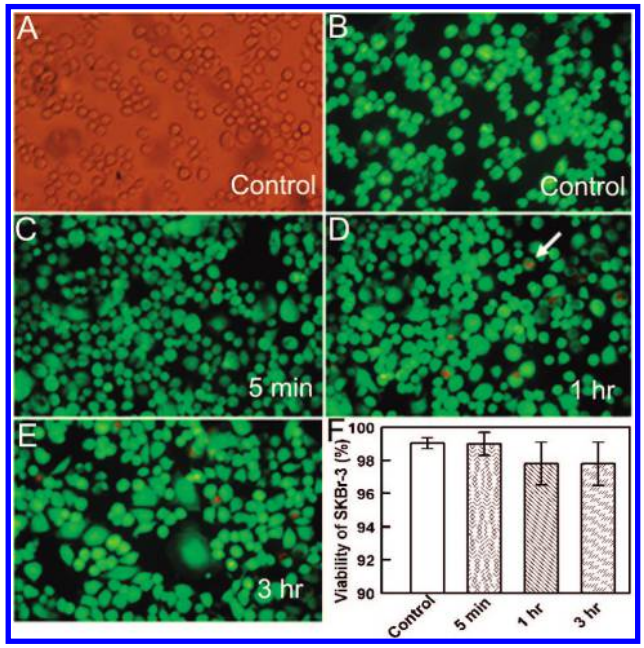

Figure 6. Viability of SKBr-3 cells following transfer. Live cells are identified by calcein AM (green), whereas dead cells (indicated with arrow) by ethidium homodimer-1 (red). (A) (bright-field) Control sample prepared by transferring 30 μL of SKBr-3 suspension ($\sim 10^7$ cells/mL) using a Rainin L-100 pipet and then cultured overnight. (B) Fluorescence image of the control sample. (C−E): Fluorescence image of SKBr-3 cells transferred using TDF-silane-modified capillaries and then cultured overnight: (C) 5 min residence time in the capillary; (D) 1 h residence time; (E) 3 h residence time. (F) Cell viability as a function of residence time in the capillary; results were averaged from at least 8 randomly sampled fields-of-view.

readily ejected without the aid of Kimwipe. When culture media was used as the diluent, surface-modification strategies resulted in very little difference in the recovery; however, when PBS was used as the diluent, we consistently observed that the recovery decreased in the following order: PEG-silane > TDF-silane > Untreated. The difference in surface adhesion between SKBr-3 and MCF-7 was not significant.

In summary, cell culture media should be used in cell-spiking experiments because it minimizes the adsorption of the cells onto the capillary walls, and when cell culture media is used, TDF-silane coating should be employed because it eliminates retention of the liquid within the capillary and thus ensures a complete ejection of the contained cells.

Residence Time in Glass Capillary. To further characterize the effect of cell adhesion, we mapped the cell recovery as a function of the cell residence time in the glass capillary. In this experiment, cells in culture media were siphoned into a TDF-silane-modified glass capillary and remained in the capillary for a specific duration. Cells in the capillary were counted in at least triplicates before and after ejection. Figure 4A shows the effect of residence time on the cell recovery: within 20 min the recovery remained around 90%, but more than 60 min the recovery drops to less than 70%. This behavior was observed in both SKBr-3 and MCF-7. This residence time is in reasonable agreement with Kwon et al.'s observation that cells seemed to be unable to recognize the glass surface within ~1 h.[28]

Figures 4B and 4C map the cells ejected over a range of cells siphoned into the capillary. We observed no loading effects, that is, cells did not become more difficult to eject under higher packing in the capillary. Within 5 min of residence time, the ejection was 100%; this is more than sufficient time for counting the capillary-confined cells on the microscope. And unlike using micromanipulators, siphoning up and ejecting a few hundred cells only required one swift operation, and the glass capillary did not need to be monitored under the microscope continuously.

Other Cell Lines. To demonstrate the versatility of this spiking process, Figure 5 shows the recovery of six additional cell lines using TDF-silane-modified capillaries. For each cell line the cell recovery was measured six times using a different capillary each time, and the results were averaged. Consistently >98% of the cells were ejected and accounted for using this approach. We detected no significant variation among these cell lines.

Cell Viability. To demonstrate that this method of transferring cells is gentle on the cells, we conducted a cell viability assay on the transferred cells. Figure 6 summarizes the findings. Figure 6A shows a bright-field image of a control prepared by transferring SKBr-3 cells using a Rainin L-100 pipet and then cultured overnight; Figure 6B shows the corresponding fluorescence image. Almost all cells in the control were viable. Panels C−E of Figure 6 show the fluorescence images of cells transferred using TDF-silane-modified capillaries with various residence times in the capillary and then cultured overnight. Figure 6F shows the percentage of viable SKBr-3 cells as determined by averaging at least eight fields-of-views: consistently >97% of the cells were deemed viable, but as the residence time in capillary increased, more dead cells were found.

CONCLUSIONS

This paper reports a simple method using a glass capillary to accurately prepare cell-spiking standards using consumables commonly available in a laboratory. To ensure complete ejection of the contents in the glass capillary, we recommend a surface treatment with silane and the use of culture media as the diluent whenever possible. Using cell culture media may not be appropriate under some circumstances as the complex matrix can complicate the chemical analysis. To avoid retaining liquid in the capillary, TDF-silane is preferred as the surface-modification reagent. TDF-silane coating is stable against ambient exposure for at least 1 month, ethanol immersion overnight, and autoclaving. For spiking with live cells, cell adhesion in the glass capillary can pose a problem if the residence time is significant. However, we expect that within 5−10 min residence time, which is more than sufficient to count several hundred cells in a capillary, surface adhesion of cells should be insignificant. This method for preparing cell-spiking standards is particularly useful for cell counts that range from a few to hundreds, a range that contains too few for methods that rely on statistical dilution and too many for techniques that require manual transfer and injection.

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