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A Rapid Method To Verify Single-Cell Deposition Setup For Cell Sorters

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Single-Cell Deposition

AUTHORS NAMES AND AFFILIATIONS:

Olivia Roos Rodrigues¹, Simon Monard²

¹MRC Centre for Regenerative Medicine, University of Edinburgh, EH16 4UU,
Edinburgh, UK

²The Cytometry Laboratory, Walter and Eliza Hall Institute, Parkville, 3052
Victoria, Australia

CONTACT INFORMATION OF CORRESPONDING AUTHOR:

Simon Monard

Address: The Walter and Eliza Hall Institute, 1G Royal Parade, Parkville,
Victoria 3052, Australia

Tel.: +61 3 9345 2541 Fax: +61 3 9347 0852

E-mail address: monard@wehi.edu.au

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REVIEWERS:**1-Derek Davies**

The Francis Crick Institute, Lincoln's Inn Fields Laboratory, London, United Kingdom.

Email: derek.davies@crick.ac.uk.

2- Andy Riddell

Cambridge Stem Cell Institute, Cambridge, UK

Email: ar289@cam.ac.uk

BRIEF STATEMENT

The submitted report describes an easy and reliable method of validation for single-cell plate sorting that can be adopted in any standard flow cytometry facility. It consists of a method that reassures both the researcher and sorter operator that the cell sorter is adjusted optimally for the type of plate being used. The method can be used prior to cells sorting.

CONFLICT OF INTEREST DISCLOSURE:

The authors have no conflict of interest to declare.

ABSTRACT

Studying single cells reveals biology that cannot be explored using bulk techniques. Cell sorters provide the opportunity of separating single cells either for cell culture or for downstream molecular applications such as qPCR to study specific gene expression and single cell mRNA sequencing. Some of these molecular studies can be expensive so the investigator will often want reassurance that the cell sorter can reliably deposit a single droplet into each well of a 96-well or 384-well plate. Such plates may contain very small volumes of fluid as reducing the volume of fluid used can reduce the cost of the assay. To miss some of the wells could leave the data set incomplete requiring costly repetition. To verify this by microscopy is at best very time consuming and at worst impossible.

Here, an inexpensive colorimetric method is described for verifying whether a well, in either a 96- or 384-well plate, did receive a single sorted droplet from a cell sorter into the fluid at the bottom of the well. The droplet consists of particles suspended in an enzymatic solution, horseradish peroxidase (HRP), which is deposited into microtiter plate wells containing a substrate, 3,3',5,5'-tetramethylbenzidine (TMB). This method requires no special equipment or expertise and is rapid enough to be performed directly prior to the single-cell sorting experiment.

KEY TERMS:

Single-cell sorting; flow cytometry; horseradish peroxidase; colorimetric method of detection; polystyrene microspheres

INTRODUCTION

Single-cell sorting into plates or tubes has become an essential resource in biological research. After sorting, cells can be cultured, each well giving rise to a clonal culture or they can be studied in downstream applications on a molecular level using for example polymerase chain reaction (PCR). High-throughput single cell transcriptomics offers an unbiased molecular approach for understanding the extent, basis and function of gene expression variation between seemingly identical cells (1). Single-cell gene expression, single-cell mRNA sequencing (2) and single-cell DNA sequencing are amongst many single-cell genomic techniques that are being currently used by researchers to study and identify differences between individual cells in a homogeneous population (3, 4).

Several techniques in cell sorting such as index, positional and reflective plate sorting have been developed in response to increasing demands in single-cell analysis (5). These techniques sort cells into multi-well plates and keep track of cell of origin and the sorted well location. Improvements in the sampling of population diversity (6) have further increased the power and potential of single-cell sorting. All single-cell sorting would greatly benefit from an efficient and reproducible method of validating the accuracy of single-cell deposition.

To determine the effectiveness of single-cell sorting is technically challenging. If cells are sorted into flat bottom wells and sufficient time is allowed for the cell to settle on the bottom it may be possible to use microscopy to determine whether each well does in fact contain a cell. Such a procedure is time

consuming as unstained cells can be difficult to visualize. For the purpose of demonstrating the instrument is sorting a single particle per well, fluorescent microspheres can be sorted into wells of a flat bottom plate and the plate viewed with a fluorescence-inverted microscope. The fluorescence of each microsphere can be seen even with lower magnification but the microspheres must be allowed to settle on the bottom of the wells. If cells are sorted into other types of plates with conical or round bottom wells, microscopy is not an option as all particles will not be in the same optical plane. Furthermore, some types of plates such as the white 384-well amplification plates (Nunc™ Rochester NY, USA) are not transparent so some other form of verification will be necessary. To demonstrate that the cell sorter is depositing a single sorted droplet into the bottom of a microtiter plate well is also challenging. It is possible to sort drops onto the lids of microtiter plates and assume a single droplet will fall in that position but generally the sorted droplets are not moving directly vertically and this will require some consideration. Recently, a method was described to address this problem. The investigators used discs of litmus paper placed in the bottom of 96- and 384-well plates to see the exact position of the sorted droplets (7). The approach, however, required multiple sorted droplets and careful inspection of the discs with a magnifying glass.

Here, we describe a rapid method that can demonstrate a single sorted droplet has been deposited into the fluid in the well. The system can easily distinguish whether a well received more than one droplet and the readout is a clearly visible color change in the well. Although depositing a single droplet into a well does not automatically mean a single cell or particle is contained

within that droplet, other tests can be used to determine if each sorted droplet contains the particle of interest. The drop delay can be verified using several methods: A known number of cells or microspheres can be sorted into several puddles onto a microscope slide. The drop delay is changed for each puddle by one whole drop. The number of particles in each puddle are counted using phase contrast microscopy. Adjustments are made until almost all the particles are all found in the same puddle and that drop delay is used. The drop delay can also be validated by using the RMAX method described by Riddell and colleagues (8) where the number of target cells in the waste stream are determined.

Fluidigm (South San Francisco, CA) have developed several approaches to single-cell genomics based on micro- and nanofluidic technology. The Fluidigm Biomark™ instrument allows multiplexing of samples and oligonucleotide probes and single-cell sorting often precedes the amplification step. Using Fluidigm Biomark™ and single-cell sequencing technologies can be costly thus, the investigator may want proof that single droplets are being sorted into each well and determine the likely occupancy rates for wells in a particular type of plate.

In 1971, Weemen et al. proposed the use of horseradish peroxidase (HRP) as an alternative to radioactivity in an immunoassay (9). Several years later 3,3',5,5'-tetramethylbenzidine (TMB) was synthesized and presented as a safer substrate for detecting the peroxidase activity in blood (10). TMB became the substrate of choice for enzyme-linked immunosorbent assays (ELISA).

The method described below uses the enzyme HRP and TMB substrate to detect single droplets sorted into microtiter plate wells. Neither of these reagents are expensive or hazardous and no special equipment is needed although a plate reader of the type used in ELISA detection is helpful. In our assay, unlike in ELISA, the background is very low as wells that have not been sorted into contain no HRP so false positives are not a problem giving the assay exquisite sensitivity.

HRP uses hydrogen peroxide (H_2O_2) to oxidize both organic and inorganic compounds (10). HRP catalyzes electron transfer reactions, it can reduce H_2O_2 to O_2 and H_2O by using TMB as the donor molecule. TMB in its reduced substrate form is colorless, when it is oxidized it turns blue. The progress of the reaction can be monitored by the appearance of the blue coloration or the absorbance at 650 nm can be measured on a spectrophotometer or plate reader. Color intensity and development time will vary depending on the assay conditions. For endpoint assays, the reaction may be stopped with an acid eg. H_2SO_4 to form a yellow product. This can also be quantified on a plate reader but measuring absorbance at 450 nm. In our experiments, we found that terminating the reaction by addition of a stop solution was not required as the development of the blue stain in small volumes of TMB was very rapid, usually evident by the time the plate is removed from the cell sorter. A plate reader is not necessary but allows a quantitative measure of the color development.

The method relies on the fact that particles in conventional droplet-based sorters are contained in a core stream. When droplets are formed a particle to be sorted will be within the core of fluid within a larger droplet of sheath fluid.

This core consists of the fluid in which the cells were suspended. If the particles to be sorted are suspended in buffer containing HRP, the sorted droplet will contain some of this enzyme. Prior to sorting, microtiter plates containing TMB are prepared. A single droplet sorted into a well in such a plate will contain enough HRP to turn the well blue. If a plate reader is used to detect and quantify the color change, the method can be used to determine the number of drops sorted in each well. For these purposes, the particles themselves can be anything that will trigger the cell sorter. In this study, both uncoupled carboxylate polystyrene microspheres and a lymphoblastoid cell line were used. We show that the droplet core volume changes with sample pressure and that as expected the core of the droplet is reduced with a smaller nozzle size. Droplet formation and deflection is not affected by the different types of particles analysed in this study. The nozzle sizes used for these experiments were both 70 μm and 100 μm . Here, we describe an easy, rapid and reproducible colorimetric method that allows verification of single droplet deposition into microtiter plate wells. This procedure can be performed immediately before sorting wells adding TMB to culture media and adding HRP to a cells suspension to be sorted is not recommended.

MATERIALS AND METHODS

Microspheres and HRP

The microspheres used in this experiment were 4.5 μm and 10 μm Polybead® carboxylate microspheres (Polysciences Inc, PA, USA). The microspheres were not coupled to any protein. Generally 2 μl of 4.5 μm

microspheres and 5 μ l of 10 μ m microspheres were added to 0.5 ml of PBS. Horseradish peroxidase (HRP) (Alpha Diagnostic International, TX, USA and ThermoFisher Scientific, MA, USA) was used at a final concentration of 50 μ g/ml or 250 μ g/ml.

Absorbance Measurement

Two plate readers were used: BMG Omega™ Series plate reader (Offenburg, Germany) and a Molecular Devices V-max™ Kinetic microplate reader (CA, USA). On both instruments absorbance was read at 650 nm.

Plate Imaging

Plates were imaged in several ways. Some were simply photographed with the digital cameras in a Blackberry Curve™ smart phone, (Waterloo, Ontario, Canada) others were photographed using a transilluminator and a Nikon SLR digital camera with macro lens mounted on a bellows. This produced the best quality image with even illumination.

Cell Culture

The lymphoblastoid human B-cell line U266 was grown and maintained in RPMI media with 10% FCS (Invitrogen, CA, USA). Cells were used directly from culture at a concentration of approximately one million cells/ml.

Cell Sorter settings:

FACS Jazz

BD FACSJazz™ cell sorter (BD Biosciences, San Jose, CA, USA), equipped

with blue argon (488 nm) and red diode (633 nm) lasers with a 100 μ m nozzle was setup for single-cell sorting with single cell 1.0 drop mode. Drop delay was calculated manually using Accudrop™ beads (BD Biosciences, San Jose, CA, USA). The sheath pressure used was 20 psi and the sample offset was set at 0.3psi and was kept constant for the duration of the experiment. The drop drive frequency was 30KHz and the drop drive amplitude was 15V. The instrument was triggered on forward scatter and the sort gate was set on forward scatter and side scatter of microspheres. Sheath fluid was preservative-free, DNase, RNase, protease-free, phosphate buffered saline (Fisher Bioreagents, Fair Lawn, NJ, USA).

Moflo MLS

A three laser Moflo MLS (Beckman Coulter, FL, USA) equipped with a blue argon laser (488 nm), a red diode (640 nm) laser and a yellow-green (561 nm) solid state laser was setup for single-cell sorting using single cell sort mode and single drop deflection. Only the 488 nm laser was used. The drop delay was calculated in the usual way by sorting microspheres onto microscope slides and viewing by microscopy. When sorting with a 100 μ m nozzle the sheath pressure was set at 20 psi and drop drive frequency at 31KHz. When sorting with a 70 μ m nozzle sheath pressure was 60 psi and the frequency 98KHz. The instrument was adjusted such that when the sheath and sample pressure read the same pressure no sample flowed to the interrogation point. When the sample pressure was increased to 0.1 psi above the sheath pressure, sample would begin to flow. Measurement of sample and sheath pressure was only accurate to one decimal place. Sheath fluid was Dulbecco's phosphate buffered saline (Invitrogen, CA, USA).

Cell Sorting Setup

The cell sorters were carefully adjusted so that the sorted droplets were as close as possible to the centre of each well. This adjustment was done by having the trajectory of the sorted drops as near vertical as possible and covering the plate with adhesive aluminium foil. The foil is smoothed down firmly allowing the profile of the well to be seen. Sorting 50 droplets onto the foil cover produces a small but visible puddle. Polystyrene or plastic film covers were not used as they can carry an electrostatic charge that may cause the puddle to move. It may be necessary to shim the plate or make modifications to the plate holder to ensure droplets fall into the centre of all of the wells of the plate as the movement of the plate may not be exactly parallel to the well positions of each row. This is particularly important for sorting into 384-well plates. Another approach used on the Moflo cell sorter was to make a paper template of the position of the bottom of the wells. The template had a cross in the center of each well. The template was secured to the plate holder. The instrument was adjusted such that the sorted droplets were sorted exactly onto the centers of the crosses. The plate was then placed on top of the template such that the center of each well was exactly over the crosses on the plate template being secured with Bluetac™.

Multiple droplet 96-well plate sorting

96-well plates Falcon™ (Corning Tewksbury, MA, USA) were prepared with 100 µl TMB High Sensitivity Substrate Solution (Biolegend, San Diego, CA, USA) in each well. The microspheres were suspended in either 50 or 250

$\mu\text{g/ml}$ HRP in PBS. Immediately before acquisition the microspheres were re-suspended and sorted based on forward scatter vs. side scatter.

When sorting, 4 microspheres per well were sorted into Row A, 3 microspheres per well in Row B, 2 microspheres per well in Row C, 1 microsphere per well in Row D, no microspheres per well in Row E and 1 microsphere per well for the rest of the plate Rows F-H.

Immediately after sorting the plate was removed and left in the dark for 30 minutes. The plates were photographed and read on a BMG Omega Series plate reader measuring absorbance at 650 nm.

Single droplet 384-well plate sorting

White 384-well amplification plates (Nunc™ Rochester NY, USA) were prepared by adding 10 μl of TMB to each well. 4.5 μm microspheres were suspended in PBS containing 50 $\mu\text{g/ml}$ HRP.

BD FACSJazz™ cell sorter was carefully adjusted such that all sorted droplets were as close as possible to the centre of each well. Single microspheres were sorted into plates using several different patterns.

After sorting the plates were incubated in the dark for 15 minutes after which they were photographed.

Single droplet 96-well plate sorting

A sample was prepared containing 0.5 ml of U266 cells in culture medium, 2 μl of 4.5 μm microspheres, 5 μl 10 μm microspheres and 25 μl of 5 mg/ml stock of HRP. This gave a final concentration of HRP of 250 $\mu\text{g/ml}$. 5 μl of

TMB was added to each well of 96-well vinyl Microtiter® plates (Thermo Scientific NY, USA).

The Moflo was adjusted, as described previously, so that the sample would just begin to flow at 0.1 psi above sheath pressure. Then the sample pressure was set to 0.2 psi above sheath pressure and the sample flow rate allowed to stabilize. The forward and side scatter gain was adjusted so that all three particle sizes could be observed simultaneously and a sort gate was made for each of the particle types.

One droplet containing one 4.5 µm microsphere was sorted into column 1, one droplet containing one 10 µm microsphere was sorted into column 5 and one droplet containing one cell was sorted into column 9. Sample pressure was increased to 0.4 psi above sheath pressure, flow rate was allowed to stabilize then one droplet containing one 4.5 µm microsphere was sorted into column 2, one droplet containing one 10 µm microsphere was sorted into column 6 and one droplet containing one cell was sorted into column 10. This was repeated for both 0.6 psi and 0.8 psi sample pressures in subsequent columns.

5 minutes after the completion of the sorting, the plates were photographed and read on the plate reader Molecular Devices V-max™ Kinetic plate reader.

Single droplet 96-well PCR plate sorting

Eppendorf® Twin Tec 96-well PCR plates (Hamburg, Germany) were prepared by adding 5 µl TMB to rows A and B, 2 µl TMB to rows C and D, 1 µl TMB to rows E through H. The plates were kept on ice and were covered

by adherent cellophane to prevent evaporation. Extra care was taken to adjust the trajectory of the sorted droplets as the target is very small.

A sample was prepared containing 2 μl of 4.5 μm microspheres suspended in 0.5 ml PBS containing 250 $\mu\text{g/ml}$ HRP. Single droplets were sorted into all but the last two rows. Immediately after sorting, cellophane covers were applied to the plates and the plates photographed within 5 minutes.

RESULTS

Multiple droplet 96-well plate sorting

The colorimetric transition from clear liquid to blue can be detected from as little as one sorted droplet containing 50 $\mu\text{g/ml}$ in 100 μl of TMB (Fig. 1A). However, the change is more rapid and obvious if 250 $\mu\text{g/ml}$ of HRP is used (Fig. 1B). This colorimetric change can be easily quantified by using a plate reader such as those used for ELISA. Measuring the absorbance at 650 nm gives a highly reproducible, quantitative result (Fig. 1C). Sorting into 96-well plates has been repeated twenty times and inter-assay results were qualitatively comparable.

384-well plate sorting

The color development is faster when sorting single droplets into wells within 384-well plates containing 10 μl of TMB (Fig. 2). The color develops within a few minutes with the first wells already showing a blue coloration by the time the plate is removed from the cell sorter. Sorting into 384-well plates was also repeated more than ten times and inter-assay results were qualitatively comparable.

Single droplet 96-well plate sorting with increasing sample pressure.

Fig. 3. shows that sorting 4.5 μm microspheres, 10 μm microspheres and cells produced very similar results. The droplet formation and deflection appears to be unhampered by the presence of cells. Both sizes of microspheres and the U266 cells were sorted sequentially at each sample differential so any differences observed were not due to the difficulty of duplicating identical sample pressures. The plate images (Fig. 3A and 3B) and the graphs quantifying TMB absorbance of respective sorted wells (Fig. 3C and 3D) show as expected, that increasing sample differential progressively increases the sample core volume and thus the absorbance values. TMB absorbance mean values represented in Fig. 3C and 3D have been calculated after subtracting respective background absorbance values measured in the last row of wells which received no sorted droplets. The absorbance values read with the 70 μm nozzle sort are reduced in comparison to the 100 μm nozzle by about half using a similar sample differential. For this experiment only 5 μl of TMB was added to the bottom of vinyl ELISA type plates. The color appears faint as the fluid is very thinly spread on the bottom of the well. Several droplets missed their target wells, these wells are circled in black and were not used for the calculated mean values. The 100 μm nozzle plate showed 4 missed target wells while the 70 μm nozzle plate revealed 1 missed target wells. A dot plot of forward and side scatter is shown with sort gates demonstrating all particles on the same plot (Fig. 3E).

Single droplet 96-well PCR plate sorting

Sorting into PCR amplification plates produced a blue coloration almost immediately. The color appears most intense when 5 μ l of TMB is used moderately intense with 2 μ l TMB and less intense with 1 μ l TMB. Nevertheless, the signal can clearly be seen when using only 1 μ l of TMB (Fig. 4).

DISCUSSION

An easy and reproducible colorimetric technique is described that can be used for the validation of single droplet sorting using HRP solution and microtiter plates containing TMB. This method was developed simply to reassure the researcher and operator that the cell sorter is adjusted correctly and the deflected droplet will reach its target. It cannot demonstrate that the droplet contains a particle of interest only that the droplet arrived at its destination. Initially, it is demonstrated that a single sorted droplet from a sample of microspheres suspended in HRP solution can be detected in a microtiter plate well containing 100 μ l of TMB. It is then shown that the system can easily quantify whether a well received one, two or more sorted droplets.

The method works well for 384-well PCR plates containing 10 μ l of TMB. Color development is rapid with distinct blue color already developing by the time the plate is removed from the sorter.

The system works equally well whether the sorted particles are cells or microspheres. U266 lymphoblastoid cells are fairly large cells ranging from 10-20 μm diameter in size. Sorting these cells did not disrupt drop formation or deflection into wells. Both sizes of microspheres and the U266 cells were sorted without changing the sample differential and all three types of particles demonstrated similar results with progressive increases in droplet core volume as the sample differential increased. The larger volume of the cells compared to the microspheres seems to have minimal effect on the absorbance values.

Finally, it is demonstrated that the system can be used with very small volumes of TMB presenting a very small target. Droplets deposited into 1 μl of TMB cause a visible color change showing the robustness of the chemistry. The challenge of sorting into volumes of less than 2 μl is preventing the contents of the well from drying out and adjusting the sorter to deliver the droplet into such a small target. Faintness of the signal seen in the wells of the 96-well PCR plate when sorting a single droplet into 1 μl of TMB is probably caused by the TMB partially drying before the plate was photographed and partly the depth of fluid in the well is very small. It might be worth considering sorting into a dry plate and adding the fluid after sorting if that would be compatible with the downstream application. It is shown that that the blue color development is more pronounced with a 100 μm nozzle as it delivers a greater volume of HRP solution. Generally when single-cell sorting the event rates are low so larger nozzle sizes can be used. The advantage of smaller nozzles is that they allow higher drop drive frequencies and thus higher sample event rates. There may be occasions when a high

event rate is needed such as when sorting very rare cells and in this case increasing the HRP concentration might be prudent.

Although all the experiments done are using either 96-well or 384-well plates the system can just as easily be adapted to other formats such as single PCR tubes, strips of tubes, terasaki plates or 1536 well plates.

As already mentioned, the particles are used simply for triggering the instrument, an electronic test signal is available on the Moflo MLS instrument but was not used as we believe using particles would better mimic the behaviour of sorted droplets in a real cell sorting experiment. The behaviour of particles, especially cells in a jet of fluid, can be unpredictable due to non-random distribution of cells and formation of cell doublets or aggregates during sample preparation (11,12). The method described in this article could be used to investigate the effects of very large cells or aggregates on droplet formation.

In an attempt to demonstrate the sorted droplet contains the particle of interest, HRP was coupled directly to the surface of carboxylated microspheres. It was hoped that a single HRP coupled microsphere would be able to generate a visible color change in TMB. Unfortunately, a visible blue color change could not be generated with one microsphere. Several sizes of carboxylate microspheres and several coupling protocols were explored. Approximately 0.2 ng HRP is needed to achieve a rapid visible color change but binding this much HRP to a single bead might not be possible (see Supplementary Material). However, if this were achieved, a positive well would demonstrate that the droplet reached the well and the droplet did in fact contain the particle of interest. On the other hand, a well could show no

color change for two reasons, either the droplet never reached the well or, the droplet didn't contain the particle, thus a negative result could be ambiguous.

With this system using soluble HRP however, a negative well would show definitively that the droplet did not reach its target.

Some experiments using chemiluminescent reporters of HRP activity were performed which appears to have comparable or slightly better sensitivity but as a specific plate reader is a requirement for such an assay the method was not pursued.

Figures 3 and 4 show that several wells were missed. This rather than being a failure of the system is what the system was devised to demonstrate and suggests that's some further adjustment might be worthwhile. Single-cell sorting on the FACS Jazz™ was especially effective with droplets rarely missing their target.

The method described here is both sensitive and quantitative. By doing a standard curve of HRP concentration in a 96-well plate (see Supplementary Material), the absolute amount of HRP can be calculated in each droplet. As we know the concentration of HRP, the core volume of the droplet can be elucidated.

It should be noted that we found pronounced differences in the HRP reactivity from different suppliers which would mean that several concentrations should be tried to get optimal results. To use the method to validate accuracy of droplet placement does not require a plate reader or even a camera. The blue coloration develops quickly usually within a few minutes, which is obvious to the human eye.

Here, we demonstrate an easy and reliable validation method to aid with the setup of costly single-cell sorting experiments. The method is sensitive enough that a single sorted droplet can produce a rapid visible color change. This method is quantitative if a plate reader is used but otherwise requires no special equipment. It can be adapted to any type of sorting receptacle and any type of cell sorter.

LEGENDS TO FIGURES

FIGURE. 1.

96-well plates after sorting on the FACS Jazz™ cell sorter followed by a 30 minutes incubation in the dark. The blue coloration is produced by 3,3',5,5'-tetramethylbenzidine (TMB) upon catalysis by horseradish peroxidase (HRP).

Absorbance at 650 nm was read on a BMG Omega Series plate reader.

Row A received 4 droplets, row B 3 droplets, row C 2 droplets, row D one droplet, row E no droplets and rows F-H received 1 droplet per well.

(A) Plate prepared with 100 μ l of TMB in each well and HRP concentration of 50 μ g/ml.

(B) Plate prepared with 100 μ l TMB in each well and HRP concentration of 250 μ g/ml.

(C) TMB relative absorbance values of plate A. Relative absorbance values were calculated by dividing mean absorbance values of multiple droplet wells by the mean value obtained for single droplets. Values are represented as mean $\pm$ standard error.

FIGURE. 2.

384-well plates following single droplet sorting on the FACS Jazz™ cell sorter. The plate was prepared with 10 µl TMB in each well and HRP concentration of 50 µg/ml. Single droplets were deposited in alternate columns and the plate incubated for 15 minutes.

FIGURE 3.

96-well plates following sorting of single particles into 5 µl TMB in 7 replicate wells (rows A-G), row H having no sorted particles. Each particle size was sorted at increasing sample differentials of 0.2, 0.4, 0.6, 0.8 psi above sheath pressure. 4.5 µm microspheres (Columns 1-4 respectively), 10 µm microspheres (Columns 5-8) and single cells, U266 human B lymphoblastoid cell line (Columns 9-12). Both 70 µm A) and 100 µm B) nozzle sizes were used on a MoFlo MLS cell sorter. Plates were incubated for 5 minutes in the dark. Target wells missed are circled in black. TMB absorbance values were quantified for both 70 µm (C) and 100 µm (D) nozzle sizes. Absorbance values were measured on a Molecular Devices V-max™ Kinetic plate reader measuring absorbance at 650 nm and are represented as mean ± standard error. E) Representative forward scatter (FS) versus side scatter (SS) dot plot of sort gates for 4.5 µm, 10 µm microspheres and U266 cells.

FIGURE 4.

96-well PCR plate following single droplet sorting of 4.5 µm microspheres into rows A-F. Rows G and H had no droplets. The plate was prepared with 5 µl TMB in Rows A and B, 2 µl TMB in Rows C and D, 1 µl TMB in Rows E to H. The concentration of HRP was 250 µg/ml. Plates were incubated 5 minutes. Target wells missed are circled in black.

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Accepted Article

Figure 1a and b
584x669mm (72 x 72 DPI)

A

Figure 1c
649x342mm (72 x 72 DPI)

Accepted

Figure 2
616x410mm (72 x 72 DPI)

Accepte

Figure 3a and b
555x695mm (72 x 72 DPI)

Figure 3c and d
448x565mm (72 x 72 DPI)

A

Figure 3e
391x346mm (72 x 72 DPI)

Acce

Figure 4
578x381mm (72 x 72 DPI)

Accepte

Figure 5
617x387mm (72 x 72 DPI)

Accepte

Figure 6
619x332mm (72 x 72 DPI)

Accepted

SUPPLEMENTARY MATERIAL

Determination of standard curve for HRP

A serial dilution of HRP was performed and 5 μ l of each dilution was added to each well of a 96-well ELISA plate containing 50 μ l TMB. The highest concentration was 25 ng HRP/well. However concentrations above 1.56 ng/well are not shown as they caused saturation of the TMB color change with wells turning yellow preventing meaningful absorbance values at 650 nm. Plates were incubated for 15 minutes and read on the plate reader.

Coupling HRP to Microspheres

Beads were coupled to HRP using Polylink™ Protein Coupling kit for Carboxylate Microspheres (Polysciences Inc, PA, USA). 500 μ g of HRP was used for each coupling. After coupling beads were washed 3 times in Polylink™ Wash/Storage Buffer. For sorting experiments, 5 μ l HRP coupled beads were diluted in 500 μ l PBS.

Calculation of potential protein binding by carboxylate microspheres

Carboxylate microspheres are reported to be able to bind about 3 mg/m² of bovine serum albumin (BSA) which has a molecular weight of 65 KDa (Bangs Laboratories, Inc. TechNote 206 Rev. #006, 20 March 2015). Horseradish peroxidase is a 44 KDa protein so it should be possible under optimum conditions to bind at least 3 mg/m² to the surface of microspheres. A single 10 μ m microsphere has a surface area of about $3.14 \times 10^{-10} \text{ m}^2$ (Area = $4\pi r^2$ where radius=5 μ m) so as $3.14 \times 10^{-10} \text{ m}^2 \times 3 \text{ mg} = 9.42 \times 10^{-13} \text{ g}$. So a 10 μ m

microsphere should be able to bind approximately 9.5×10^{-13} g of HRP $\approx$ 0.001 ng = 1 pg.

Calculation of droplet volume.

Using the Moflo MLS with single drop deflection and single cell mode 200,000 droplets were sorted into a 12X75 mm test tubes. The volume of fluid in the tubes were measured and divided by 200,000.

For 100 μ m nozzle with a drop drive frequency of 31 KHz

: Volume 200,000 droplets was 540 μ l

so 1 droplet has a volume of 2.7 nl.

For 70 μ m nozzle with a drop drive frequency of 98 KHz

: Volume 200,000 droplets was 194 μ l

so 1 droplet has a volume of 0.97 nl.

DISCUSSION OF SUPPLEMENTARY MATERIAL

From Fig. 3 it can be observed that a good visible blue coloration corresponds to an absorbance value of about 0.3. From the standard curve of HRP against absorbance (Fig. 5) it is demonstrated that an absorbance of 0.3 corresponds to about 0.2 ng of HRP thus, 0.2 ng of HRP would need to be coupled to each microsphere to generate such a color change. This is approximately 200 times more HRP than the above calculation suggests is possible to bind to a 10 μ m microsphere. A fainter color change can be seen with a lower

absorbance but the authors are of the opinion that it is not possible to bind enough HRP to the surface of a microsphere for them to be useful as a single-cell deposition validation tool. The authors in the past have seen a blue signal develop from sorting as few as 5 microspheres. However, they now suspect this may have been contamination with soluble HRP as more recent couplings of HRP to a 10 μm microsphere have shown that about 150 microspheres would be needed to generate a clear visible color change. 50 microspheres generate good absorbance values on a plate reader (Fig. 6). This is in accordance with the calculation above and again suggests that binding enough HRP to beads would be problematic. Although the plates used in Fig. 3 contained 5 μl of TMB and the plates used for the standard curve (Fig. 5) used 50 μl TMB, the absorbance values for the same amount of HRP are comparable. With longer incubation times it may be possible to get a visible signal with less microspheres but to get a color change with a single particle unfortunately seems unachievable.

FIGURE 5.

Standard calibration curve showing TMB absorbance values obtained from a serial dilution of HRP solution. Concentrations of HRP range from 1.56 ng/well to 0.012 ng/well. The plate was prepared with 50 μl TMB. Plates were incubated 15 minutes.

FIGURE 6.

96-well plate following single droplet sorting of known numbers of 10 μm HRP coupled microspheres (50, 10, 5, 1 and 0). The plate was prepared with 50 μl TMB. Plates were incubated 15 minutes.

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Author/s:

Rodrigues, OR;Monard, S

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