Video Article

Counting Proteins in Single Cells with Addressable Droplet Microarrays

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Abstract

Often cellular behavior and cellular responses are analyzed at the population level where the responses of many cells are pooled together as an average result masking the rich single cell behavior within a complex population. Single cell protein detection and quantification technologies have made a remarkable impact in recent years. Here we describe a practical and flexible single cell analysis platform based on addressable droplet microarrays. This study describes how the absolute copy numbers of target proteins may be measured with single cell resolution. The tumor suppressor p53 is the most commonly mutated gene in human cancer, with more than 50% of total cancer cases exhibiting a non-healthy p53 expression pattern. The protocol describes steps to create 10 nL droplets within which single human cancer cells are isolated and the copy number of p53 protein is measured with single molecule resolution to precisely determine the variability in expression. The method may be applied to any cell type including primary material to determine the absolute copy number of any target proteins of interest.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56110/>

Introduction

The goal of this method is to determine the variation in abundance of a target protein in a cell population with single cell resolution. Single cell analysis provides a number of benefits that are not available with traditional ensemble biochemical methods.^{1,2,3,4,5} Firstly, working at the single cell level can capture the rich heterogeneity of a cell population that would otherwise be lost by the averaging that occurs with traditional ensemble biochemical techniques. The majority of work-horse biochemical methods work with the bulk, requiring, as they often do, millions of cells to produce a result. Of course, the consequences of assessing entire cell populations depends on a number of factors, for example, the heterogeneity in protein expression where some important features of the distribution of protein abundance may be missed. From a practical perspective, the sensitivity required of single cell techniques make them capable of working with amounts of biological material that is insufficient for even the more sensitive bulk techniques to function. A key example of this is the study of rare cell types such as circulating tumor cells (CTCs) where even for patients with a poor prognostic outlook less than 10 CTCs might be present in a single 7.5 mL blood draw.⁶ Here we present the methodology required to perform single cell protein measurements using a reduced volume antibody-based assay employing oil-capped droplets printed on an antibody microarray.

Microfluidic droplet platforms are high throughput, able to generate thousands of droplets per second, and capable of isolating, and even culturing, single cells in individual droplets to perform a wide array of biochemical assays. Droplet-based techniques are well suited for single cell analysis,^{7,8,9} with notable recent examples including DropSeq¹⁰ and inDrop¹¹, which have been greatly aided by the power of amplification techniques. The limited amount of material and no methods of amplification for proteins make single cell proteomics especially challenging.

Droplets may be analyzed by a number of methods and fluorescence microscopy has been widely used. Single molecule techniques such as total internal reflection fluorescence (TIRF) microscopy allows fluorescent molecules to be visualized with unparalleled signal-to-noise ratio.¹² Due to the exponential decay of the evanescent field, only fluorophores in high proximity to the surface (order of 100nm) are excited making TIRF a good strategy in detecting small amounts of a target molecule in a complex mixture. The inherent optical sectioning strength of TIRF also helps to avoid wash steps and limits assay time and complexity. However, TIRF requires planar surfaces and examples of TIRF microscopy applied to droplets in flow involve the formation of a planar surface of which to image.¹³ To this end, single cell proteomic techniques often design microfluidic chips around surface-immobilized capture agents in a microarray format.^{4,14}

The droplets, themselves, may be formed in arrays on planar surfaces, so-called droplet microarrays.^{15,16,17} Spatially organizing droplets into arrays allows them to be conveniently indexed, easily monitored over time, individually addressed and, if required, retrieved. Droplet microarrays can achieve a high density of micro-reactors with thousands of elements per chip which are either free-standing or supported by microwell structures.^{18,19,20} They may be formed by sequential deposition by liquid handling robots, inkjet spotters, contact microarrayers^{21,22,23,24,25,26} or they can self-assemble on surfaces such as superhydrophilic spots patterned on a superhydrophobic surface.^{27,28,29}

With these considerations in mind, Addressable Droplet Microarrays (ADMs) were designed to combine the versatility, spatial addressability and reduced volumes of droplet microarrays with the sensitivity of single molecule TIRF microscopy to quantitatively measure protein abundance.⁵ ADMs enable single cell analysis forming a droplet microarray containing single cells over an antibody microarray, which is then capped with oil to prevent evaporation. The volumes of the droplets are discrete to prevent sample loss, which would otherwise be achieved by on-chip valving in continuous flow microfluidics.³⁰ The absolute amount of target protein from a single cell is extremely small; however, the reduced volume of the droplets allows for relatively high local concentration in order that they are detected using a sandwich antibody assay - antibody is immobilized in a distinct region, or spot, on a surface which captures protein which in turn binds to a fluorescently labelled detection antibody present in the droplet volume. As a label-free approach (i.e. protein targets do not need to be labelled directly), ADMs are generally applicable to analyzing cells from primary sources, such as processed blood, fine needle aspirates and dissociated tumor biopsies, as well as cells from culture and their lysates.

Measuring the variation in protein abundance across a cell population is important in determining the heterogeneity in response, for example, to a drug and will help in providing insight into cellular functions and pathways, assessing subpopulations and their behavior as well as identify rare events that would otherwise be masked by bulk methods. This protocol describes how to produce and use addressable droplet microarrays to quantitatively determine the abundance of the transcription factor p53 in human cancer cells and may be used to investigate the role of p53 in response to chemotherapeutic drugs. The target protein is determined by the choice of capture and detection antibodies and may be modified to include more or different targets. Instructions are provided to build a simple apparatus incorporating a concentric nozzle from general lab consumables to manually array 10 nL droplets capped with oil. The full experimental process is described whereby each droplet is then loaded with a single cell, which is then lysed and the expression of protein determined with single molecule resolution using TIRF microscopy.

Protocol

1. Preparation

1. Make chips and print antibody microarrays

1. Attach an adhesive silicone/acrylic isolator to a coverslip functionalized to support an antibody microarray. This is referred to as the chip.
NOTE: Various surface chemistries have been tested for their suitability with addressable droplets.⁵ Surface chemistries may need to be optimized for alternative capture agents. ADM isolators are available commercially or may be produced by laser cutting acrylic (CAD file for isolator used in this work is provided as a download; see the **Supplemental Code File**).
2. Switch on the microarrayer and set the humidity to 75%.
NOTE: Relative humidity reduces evaporation of printing solution from the microarray pin and reduces intra- and inter-spot variation.
3. Clean the microarray pin in pin cleaning solution for 5 min by ultrasonication. Rinse the pin with ultra-pure water using a wash bottle and dry using nitrogen.
NOTE: Suspend the pin as to only immerse the pin tip. A microscope slide with an appropriately drilled set of holes will help if a pin holder cannot be obtained.
4. Make 5 mL of print buffer comprising of 3 × saline-sodium citrate (SSC) buffer, 1.5 M betaine supplemented with 0.01% sodium dodecyl sulfate (SDS). Store at 4 °C indefinitely.
5. To prepare a printing solution, thaw anti-p53 antibody (p53/Mdm2 ELISA kit; see the Table of Materials) aliquots stored at -80 °C. Mix it 1:1 with print buffer to a final concentration of 0.5 mg/mL. Load 5-10 µL of printing solution in a 384 well-plate using a micropipette and place in the microarrayer.
6. Load chips assembled in step 1.1.1 into the microarrayer. Program the microarrayer to print spots at coordinates defined by the center of each well of the isolator.
NOTE: Microarrayers employ a range of, predominantly, proprietary software and so the reader is encouraged to consult the relevant literature. The microarray required for the ADM isolator featured in the accompanying CAD file in step 1.1.1 comprises of rectangular elements; the relevant distances are provided.
7. Store the chips in airtight containers and wrap with foil. Store at 4 °C up to 6 weeks.
NOTE: The foil is to prevent any photo-damage. Storage at 4 °C limits the degradation rate of biomolecules and any deleterious reactions that may act to reduce microarray activity. An airtight container allows chips to equilibrate to room temperature before use without condensation forming on the chip surface. Contact microarray printing exploits surface tension and adhesion between the print solution and the print substrate to produce spots. Pre-printing or blotting is normally required to remove excess solution from the microarray pin to yield uniformly sized spots. Do not discard the sacrificial pre-print coverslip since it can be used to assess batch quality.

2. Prepare syringes, tubing and concentric nozzle for dispensing addressable droplets

1. Disassemble 100 µL (for the aqueous droplet) and 1 mL (for the capping oil) glass Hamilton syringes and rinse parts with distilled H₂O.
2. Feed a 100 mm length of 150 µm ID/360 µm OD fused silica tubing through a 40 mm length of 1.0 mm ID/1/16" OD PFA tubing until it protrudes by 2 mm. This will form the concentric nozzle.
3. Apply a thin layer of cyanoacrylate glue to the end of a 10 µL pipette tip and insert into the 40 mm piece of 1.0 mm ID/1/16" OD PFA tubing. If required, reposition the fused silica tubing to maintain a 2 mm protrusion at the nozzle before the adhesive sets.
4. Insert the other end of the fused silica capillary into the end of a 200 mm length of 0.014" ID/0.062" OD PTFE tubing and connect this to a 100 µL Hamilton syringe filled with 4% bovine serum albumen (BSA) in phosphate-buffered saline (PBS) (PBSA).
NOTE: Proteins and other biochemical species may non-specifically bind to surfaces and can be lost or denatured. BSA is used to 'block' surfaces to minimize non-specific binding by sacrificially binding to those surfaces.
5. Insert a 400 mm length of 1.0 mm ID/2.0 mm OD FEP tubing into a 200 µL pipette tip until it forms a seal. Apply a thin layer of cyanoacrylate glue to another 200 µL pipette tip and push this into the first tip to fix the tubing in place. Connect the open end of the 1.0 mm ID/2.0 mm OD FEP tubing to a 1 mL Hamilton syringe filled with mineral oil.

6. Insert the 200 μL pipette tip assembly into the 10 μL pipette tip of the concentric nozzle. Place the syringes in separate syringe pumps as they will need to be operated independently.
7. Fill the 100 μL syringe with 4% PBSA blocking solution. Reattach and flush the 'aqueous' tubing with the blocking solution. Repeat twice for a total of 3 flushes.
8. Fill the 100 μL syringe with 0.125 $\mu\text{g mL}^{-1}$ detection antibody (anti-p53 antibody (DO-1) labelled with Alexa 488) in 4% PBSA and re-attach tubing. Replace blocking solution in tubing by dispensing 25 μL detection antibody solution.
9. Flush the 'oil' tubing with mineral oil until all tubing and the nozzle fills with oil. Refill the 1 mL syringe with mineral oil and re-attach tubing. Secure the tubing and nozzle assembly to an XYZ manipulator.

3. Prepare microinjector and micromanipulator

NOTE: Steps below make specific reference to components of the microinjector and micromanipulator apparatus specified in the Table of Materials, but are generally applicable to any such apparatus.

1. Assemble the microinjector by attaching the pressure tubing and the capillary holder.
2. Slowly rotate the piston dial until the mineral oil completely fills the line.
3. Mount the capillary holder into the translation head mount.
4. Fix the capillary in the capillary holder with the grip head.
5. Pipette a solution of 4% PBSA into one of the wells of the isolator.
6. Translate using the micromanipulator and immerse the tip of the capillary into the solution.
7. Slowly fill the capillary with 4% PBSA and leave to block for 10 min.
8. Eject the microcapillary and swivel the translation module so that the assembly is clear of the chip.

2. Form Addressable Droplets and Load with Single Cells

1. Form addressable droplets

1. Secure the chip on the microscope stage.
NOTE: The microscope is an inverted fluorescence automated microscope capable of single molecule TIRF fitted with an encoded XY stage and an electron multiplying charge-coupled device camera (EM-CCD).
2. Record the microscope stage coordinates of each spot in the array using the automated microscope control software.
3. Set the XYZ manipulator on the microscope stage. Place the nozzle at an angle of 50-60°. Ensure that the nozzle has sufficient length and clearance to reach the chip. Set the 'aqueous' and 'oil' syringe pumps to dispense 10 nL at 100 $\mu\text{L}/\text{min}$ and 5 μL at 100 $\mu\text{L}/\text{min}$, respectively.
NOTE: During preparation, the aqueous solution at the head of the nozzle may dry.
4. Dispense aqueous solution until a bead of fluid is visible at the head of the nozzle. Dab with a dust free wipe to remove it.
NOTE: Depending on the number of conditions under investigation, reserve a number of wells to serve as reservoirs for cells. For a single cell line/type a single reservoir will suffice.
5. Using the automated microscope control software, set the microscope stage coordinates to an antibody spot in the array and focus on the coverslip surface.
6. Careful not to disturb the spot, using the XYZ manipulator, align the glass capillary tip of the concentric nozzle to the side of the antibody spot and dispense 10 nL of aqueous solution. Without moving the stages, dispense 5 μL of oil to cap the aqueous solution. Slowly raise the nozzle clear of the droplet and move to the next well.
7. Repeat step 2.1.6 for all antibody spots in the array.
8. After 30 min, image all spots in the array using the automated microscope control software to determine the background of single molecules bound to each antibody spot prior to loading cells.

2. Load addressable droplets with cells

1. Remove the cell culture flasks from the incubator and detach cells using trypsin/EDTA solution.
NOTE: In this study, the BE human colon carcinoma cell line was used and cultured using Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) in a CO_2 incubator.
2. If required, fluorescently stain cells.
 1. Resuspend cells in a solution of 0.125 $\mu\text{g}/\text{mL}$ detection antibody (anti-p53 antibody (DO-1) labelled with Alexa 488) in 10% FBS in L-15 media. To ensure a single cell suspension, filter the cell solution through a 40 μm pitch cell strainer.
3. Count cells using a hemocytometer and dilute or concentrate the cell solution to a concentration of $25\text{--}400 \times 10^3$ cells/mL. This ensures that cells sediment with sufficient spacing to comfortably manipulate the microcapillary.
4. Load single cells into addressable droplets from the cell reservoir using the micromanipulator and microcapillary.
NOTE: Non-adherent cells may be loaded in bulk into the micropipette and dispensed one by one into addressable droplets. Despite surface blocking treatment, adherent cell lines will tend to non-specifically stick to the glass microcapillary inner wall and be lost.
5. Using a 10 $\times$ objective to observe, use the microinjector to aspirate a single cell into the microcapillary from the cell reservoir.
6. Store the micromanipulator stage coordinates if using an electronic manipulator stage or manually note the z-position.
7. Retract the micropipette by translating it upwards to clear the 1 mm height of the chip. Perform this manually with a joystick or automatically using the 'Eject' feature of an electronic manipulator stage.
8. Set the stage coordinates to that of an addressable droplet the using automated microscope control software. 'Inject' the micropipette by returning it to the stored (or noted) z-position. Perform this manually with a joystick or automatically if using an electronic manipulator stage.
NOTE: The microcapillary will pierce the capping oil and be located within the aqueous portion of the addressable droplet.
9. Dispense the cell in the addressable droplet using the microinjector. The volume of a 10 nL addressable droplet will increase by less than 1%.
10. Repeat 2.2.5-2.2.9 for the remaining addressable droplets leaving some free for the experimental control where addressable droplets do not contain a cell.

NOTE: A simpler alternative to loading single cells into addressable droplets using a microcapillary and micromanipulator is to replace the solution in step 1.2.8 with a solution of detection antibody in 4% PBSA containing cells at a concentration on the order of 10^5 cells/mL, equivalent to 1 cell/10 nL. Single cell occupancy will be Poissonian and the cell concentration will need to be optimized.

3. Lyse cells and image array

1. Image cells in droplets using brightfield microscopy, including any fluorescence imaging.
NOTE: Imaging takes approximately 3 min to image ~100 droplets using an automated microscope. Perform imaging with a 60 NA = 1.49 oil-immersion objective. No filters are required for brightfield imaging whereas standard filter sets are used for fluorescence imaging.
2. Image all spots in the array using single molecule TIRF microscopy.
NOTE: Settings are used as in step 2.3.1 with a specialized TIRF filter set. These images will be subsequently analyzed in section 3 to determine the background of single molecules bound to each antibody spot prior to lysis. Single molecule imaging using total internal reflection fluorescence (TIRF) microscopy takes approximately 5 min to image ~100 spots.
3. Focus on a cell in an addressable droplet. Achieve complete optical lysis by focusing a single 6 ns laser pulse (wavelength 1064 nm, pulse energies $14.1 \pm 0.3 \mu\text{J}$ per pulse) close to the location of the cell.
NOTE: The laser pulse sets up an expanding cavitation bubble that shears the cell and liberates cellular constituents into the droplet volume.^{4,31} The mechanical processes due to laser-induced lysis do not disturb the oil-water interface at low pulse energies. Optical lysis typically takes approximately 20-30 min to lyse 100 cells. As discussed in the Discussion section, there are a number of alternative methods to lyse single cells if optical lysis setup is not possible.
4. Repeat for all cell-containing addressable droplets leaving 5 free for the experimental control where addressable droplets contain an un-lysed cell.
5. Note the time at which each cell is lysed.
NOTE: These times will be used to correct individual binding curves for each spot in step 3.1.9. Often it is sufficient to note the times when the first and last cells are lysed and estimate the rest assuming an adequately consistent time between lysis events.
6. Acquire single molecule images using TIRF microscopy of all spots every 10 min for the first 30 min then every 20 min for a further 60 min. If only interested in the amount of protein bound at equilibrium, image all spots after incubating the chip for 90 min at room temperature.
NOTE: The time to reach equilibrium will depend on the droplet volume and the affinities of the antibodies used in the assay. TIRF microscopy is performed using a laser excitation source at $\lambda = 488 \text{ nm}$ and 1.5 mW power as measured at the back aperture using an optical power meter. Single molecule images are acquired by setting the acquisition settings on the EM-CCD camera to 900 ms acquisition time, 16-bit digitization at 1 MHz readout rate and an EM gain factor of 10. The isolator may be re-used by carefully removing the coverslip.

3. Data Analysis

1. Single molecule counting (non-congested/digital regime)

1. Using Fiji (or Matlab), for any non-congested antibody spot, load the image acquired before lysis (background, BKD).
NOTE: The operations in the following steps are straightforwardly performed using Fiji image analysis software. A user guide may be found at the following link <https://imagej.nih.gov/ij/docs/guide/146.html>.
2. In Fiji, select the BKD image. Under the "Image" menu select "Duplicate" to duplicate BKD. Select the duplicate image and under the "Process > Filters", select "Gaussian Blur" and specify a 50 pixel radius.
3. Flatten the BKD image so that there is an effective uniform intensity distribution of the excitation light source by dividing the BKD image by the blurred BKD image, producing BKD_FLAT.
 1. Under "Process" select "Image calculator...", specifying the operation "Divide", the images required, and selecting the boxes "Create new window" and "32-bit (float) result."
4. Subtract each pixel in the image by 1 ("Process > Math", select "Subtract..." and specify a value of 1). The average pixel intensity should be 0.
NOTE: Field flattening and flattened background images may be checked easily since the sum of all pixels should be 0 and any remaining offset may be more straightforwardly compensated for.
5. Select a 50 pixel $\times$ 50 pixel area in any of the 4 corners of the BKD_FLAT image. Measure the pixel intensity standard deviation (σ) by selecting "Analyze" and "Measure".
NOTE: The summary statistics of the selection will be presented in a Results window. This determines *off-spot* background where there is unlikely to be a high density of single molecules. The area to be measured may be manually selected using the default menu selection tools or by running the macro code "makeRectangle(x, y, width, height)"; this creates a rectangular selection, where the x and y arguments are the coordinates (in pixels) of the top left corner.
6. Set the image threshold to 3σ and create a binary image SM_MASK.
 1. Under "Image > Adjust", select "Threshold..." and set the lower threshold level to 3σ .
NOTE: Pixels whose value are below the threshold are set to zero and pixels exceeding the threshold are set to 1. The threshold will determine the confidence with which thresholded pixels belong to a single molecule.
7. In the segmented image SM_MASK, set pixel intensity values to zero of any objects that do not have a size of $4-9 \text{ pixel}^2$ and a circularity of $0.5 - 1$ by selecting "Analyze" followed by "Analyze Particles" and specifying the size and circularity.
NOTE: The pixel size may need to be optimized for other fluorophores and microscope set ups. Due to the type of camera noise single pixels may be above this threshold and not be discarded despite not being single molecules. The pixel size criterion will correctly discard such pixels. Single molecules are generally circular in shape. A circularity value of 1 indicates a perfect circle whereas a value approaching 0 indicates an increasingly elongated shape. The remaining objects are single molecules and may be counted. A mask may be set to demarcate the area of the spot to discriminate *on-spot* and *off-spot* counts per frame. This is easier for frames acquired at later times when there is a sufficient signal on-spot which can then be applied to earlier frames.

8. For the same non-congested antibody spot, load and repeat steps 3.1.1 - 3.1.7 for all image frames captured as a time-resolved series acquired post lysis. Use the lysis times noted in step 2.3.5. to correct any binding curves.
- 2. Single molecule counting (congested/analogous regime)**
1. In order to calculate the average intensity of a single molecule, before proceeding, repeat steps 3.1.1 - 3.1.8.
 2. Multiply the images BKD_FLAT and SM_MASK to produce an image whereby non-zero pixel values are associated with single molecules.
 1. To perform this, under the "Process" menu, select "Image calculator...", specify the operation "Multiply" and the images required, and select the boxes "Create new window" and "32-bit (float) result."
 3. Sum all pixel intensity values by selecting "Analyze" and then "Measure"; the summary statistics of the selection will be presented in a Results window. Divide by the number of counted single molecules as per step 3.1.8 to calculate the average intensity per single molecule.
 4. For any congested antibody spot, load the image acquired post-lysis and flatten with a blurred background image as per steps 3.1.2 and 3.1.3.
 5. Subtract each pixel in the flattened image by 1 ("Process > Math", select "Subtract..." and specify a value of 1)
 6. Select a 50 pixel × 50 pixel area in any of the 4 corners of the image and measure the pixel intensity standard deviation (σ). See step 3.1.5 for details.
 7. Create a binary image mask by setting the image threshold to 3σ and set pixel intensity values to zero of any objects with a size less than 4 pixel². To perform this, under the "Analyze" menu, select "Analyze Particles" and specify the size and circularity.
 8. Multiply the flattened congested antibody spot image by the binary image mask.
 1. To perform this, under the "Process" menu, select "Image calculator...", specify the operation "Multiply" and the images required, and select the boxes "Create new window" and "32-bit (float) result."
 9. Sum of the remaining pixel intensities by selecting "Analyze" followed by "Measure"; the summary statistics of the selection will be presented in a Results window.
 10. Divide the sum of pixel intensities by the average single molecule intensity to calculate the number of single molecules bound to the congested spot.
- 3. Calibration curve for absolute quantification**
1. Repeat sections 1 and 2 to form 10 nL addressable droplets with the detection antibody in 4% PBSA solution spiked with a known concentration recombinant protein.
 2. Perform a concentration series of 10^2 - 10^7 recombinant proteins per droplet by varying the known concentration of recombinant protein added to the solution. It is recommended to work in terms of number of proteins per droplet to make calibrating single cell data straightforward.
 3. Use the concentration series data in step 3.3.2 to calibrate any single molecule counts per spot to protein abundance per droplet and by extension protein abundance per single cell.

Representative Results

The absolute basal protein copy number of p53 was determined with single cell resolution in a human colon cancer cell line, BE cells. We demonstrate how p53 expression can vary over several orders of magnitude and show a weakly positive correlation between cell size and protein copy number within the resting BE cell population.

Addressable Droplet Microarrays are formed when aqueous droplets are dispensed at antibody spot locations and capped with oil. Here, the droplets support a sensitive p53 protein assay. Coverslips are arrayed with capture antibody spots using a contact microarrayer and a pin. The anti-p53 capture antibody is taken from a commercial ELISA test kit. The printing conditions were such that capture spots were approximately 100 μ m in diameter with a maximum capture capacity of $6.2 \pm 0.5 \times 10^5$ proteins per spot.³²

The use of an isolator bonded to the coverslip allows a higher density of droplets to be formed since it limits the spread of oil to nearby antibody capture spots. In each well of the isolator droplets are formed by dispensing an aqueous solution, which is then capped with oil to prevent evaporation (**Figure 1A**). A minimal amount of oil may be dispensed to cap the droplet; however, it is useful to dispense an amount which fills each isolator well exactly such that the oil surface is flat for imaging. Isolators are either commercially sourced or made by laser cutting acrylic sheets. Silicone isolators are available pre-cut wells in a 4 × 6 array ($n = 24$) but may be modified to a 4 × 11 array ($n = 44$) using a biopsy or multi-hole leather punch. The laser cut isolator in **Figure 1B** shows an array of 100 hexagonal wells each with a maximal diameter of 2.89 mm, a center-to-center separation of 3.9mm and a height of 0.5mm. The droplets may be stored and remain stable for extended periods of time (observed up to 3 months).

Droplets are created manually using a home-built apparatus, which translates a concentric tubing nozzle (**Figure 1C**), which is connected to two syringe pumps (**Figure 1A**). The concentric tubing nozzle is comprised of a fused silica capillary, which dispenses the aqueous phase, fed through a short length of PEEK tubing, which dispenses the oil phase. Using a microscope, the nozzle is aligned to an antibody spot and the pumps would first dispense the aqueous solution immediately followed by the oil (**Figure 1E**, steps 1-4). Once all droplets in the ADM are formed, a micropipette and micromanipulator (see the Table of Materials) are used to load each droplet with a single cell (**Figure 1D**). The micropipette would capture a cell from a reservoir of cells, dispensed in one of the wells on the chip, be translated to a droplet well and then penetrate the oil cap to deliver the cell into the aqueous droplet (**Figure 1D**, steps 5-8).

All spots within the droplets are imaged prior to lysis and will be used to determine the degree of non-specific binding to the spots (**Figure 2A**). Single cells are fully lysed (including nucleus) using optical methods.³¹ Optical lysis relies on the shearing force of an expanding laser pulse-induced cavitation bubble to disrupt the cells. Care must be made that the oil-water interface not be disturbed by these processes by limiting the pulse energies and depositing cells away from the oil/water interface. Once cells are lysed, the array is imaged by TIRF microscopy using an automated microscope; frames are acquired every 10 min for 30 min and then every 20 min for a further 60 min. The conditions, such as antibody affinity, protein diffusion and droplet volume, are such that binding equilibrium is reached within the 90 min acquisition. A typical single cell pulldown is shown in **Figure 2A**.

The process of image analysis to determine the single molecule counts is schematically depicted in **Figure 2B**. Images of spots are either considered to be non-congested, where target protein concentration is such that single molecules are well separated and easily distinguished, or congested, where target protein concentration is higher and single molecule images overlap and are no longer individually distinguishable. Since the TIRF excitation profile is uneven, it is crucial that all acquired images be flat-field corrected. A Gaussian blur is applied to a non-congested frame, which acts as a smoothing filter to remove detail and noise and produce an image with the average profile of the TIRF excitation profile. This processed image may be used to 'flatten' the original image. For non-congested images, where, even at equilibrium, there are few single molecules, a frame may be processed to correct itself. This approach is not applicable to a congested spot, where single molecules densely occupy the spot and requires a background image to be acquired for flattening. Flattened frames are then thresholded for pixels with intensities at least 3 times the background standard deviation plus its mean value. Single molecules are then automatically detected by identifying 4-9 clustered pixels with circularity greater than 0.5 using the Particle Analysis features of Fiji. From the results, the average intensity of a single molecule may be calculated. It is used to determine the number of single molecules on a congested spot by dividing the antibody spot total intensity by the known average intensity of a single molecule. These steps may be automated using Fiji's script editor.

A concentration series is performed to determine the single molecule count at equilibrium in the droplets with known concentrations of recombinant p53 protein (**Figure 3A**). The conditions are such that the capture antibody spots capture a fraction of the total target protein, approximately 1% in the region of linearity (for 10^5 - 10^8 proteins per droplet $R^2 = 0.99$). The level of non-specific binding in the droplets is 187 ± 60 molecules. The results of the single cell pulldowns may then be converted to achieve distributions of absolute protein copy number per cell. **Figure 3B** shows the distribution of absolute p53 protein expression in single BE cells using ADMs. P53 protein expression in the BE cells, which may be assumed to be genetically identical or very similar, is a stochastic process. The distribution is Gamma-like - asymmetric and unimodal with a long tail. Consequently, the mean (1.82×10^6 proteins) can overestimate the modal protein abundance (bin 0 - 5.0×10^5 proteins), and the standard deviation (1.88×10^6 proteins) doesn't fully capture the features of the distribution. This distribution is an example of the importance of single cell measurements to capture the heterogeneity of protein expression in the cell population, which would otherwise be lost when averaging with bulk measurements. Additional parameters for each cell may be measured. Here, the cell volume is estimated by measuring each cell diameter prior to lysis in the droplet and assuming the cell is approximately spherical. **Figure 3C** shows a comparison of p53 absolute protein copy number in single BE cells as a function of cell size. There is a tendency for larger cells to have a higher total p53 copy number. Interestingly, it is possible from the distribution that there is coordination between p53 expression and cell size since there appears to be a minimum p53 copy number per cell; however, the mechanisms by which this arises requires further investigation.

Figure 1: Addressable Droplet Microarray Apparatus and Chip. (a) Droplets are dispensed manually using a 3-axis manipulator to translate a concentric tubing nozzle. The aqueous droplet is dispensed at specific locations in an antibody microarray followed by capping oil. (b) The isolator enables a higher density of addressable droplets on the substrate by limiting the spread of the oil. Isolators may be produced by laser cutting 0.5 mm acrylic sheets. To aid visualization of the droplets, blue food coloring dye is deposited using the apparatus in a). Scale bar 10 mm, inset scale bar 1 mm. (c) The concentric tubing nozzle allows addressable droplets to be formed, assembled as in step 1.2 of the protocol. (d) A microinjector and micromanipulator are used to isolate cells into individual addressable droplets, prepared as in step 1.3 of the protocol. (e) The major steps in the process of creating and loading addressable droplets is shown. An antibody spot is located using an encoded translation stage (1) where the tip of the capillary tubing is aligned (2) and the aqueous (3) then oil (4) components are dispensed. Single cells may be loaded using a glass microcapillary (5-8) which can repeatedly address the aqueous droplet (7) and deposit a cell (8). Scale bars 100 μ m. The red arrow in (5) and (8) highlights the isolated single cell and the inset of (8) shows the isolated single cell at high magnification (scale bar 10 μ m). Portions of this figure has been modified from reference¹², reproduced by permission of The Royal Society of Chemistry. [Please click here to view a larger version of this figure.](#)

Figure 2: Image analysis steps. Each spot in the array is periodically imaged by TIRF microscopy until equilibrium (t_{eq}) is reached (90 min for p53 assay). The equilibrium time depends on t in solution as well as the affinities of the antibodies' pair for the targeted protein. a) Example single molecule TIRF microscopy images of the background as well as an example single cell pulldown (scale bars 20 μ m). Inset image shows a magnified portion of the background image with some single molecules highlighted by red arrows (scale bar 5 μ m). b) Outline of image analysis detailed in step 3 of the protocol. Briefly, there are two broad regimes where the spots may be considered non-congested, single molecules are sparse, and congested, where the density of single molecules is such that there is significant overlap and may no longer be singly identifiable. A crucial step in the image analysis is field flattening to remove the effect of the non-uniform intensity profile of the excitation laser. In the digital counting regime, single molecules are identified directly, based on their intensity and shape parameters. In the analogue counting regime, the number of single molecules is calculated by measuring the total spot intensity at equilibrium and dividing by the average intensity of a single molecule, which is known from the digital counting regime. Steps may be straightforwardly automated in ImageJ to analyze the data. The bottom panel sequence of images shows accumulation of target protein on the antibody capture spot; initially, spots are not congested (t_1) whereas as target protein accumulates on the antibody spot, the single molecules can become increasingly congested (t_n) until equilibrium is reached (t_{eq}). Note that whether a spot remains non-congested or becomes congested depends on a number of factors, in particular the abundance of target protein in the analysis volume. [Please click here to view a larger version of this figure.](#)

Figure 3: Single cell data. Absolute quantification is achieved using a calibration curve. (a) Single molecule counts are made using known concentrations of recombinant protein. This is a calibration curve and is used to convert single molecule counted on each spot to number of target proteins in the analysis volume and hence single cell copy number. The horizontal red dashed line indicates the level of non-specific binding of 187 ± 60 molecules. The error bars are one standard deviation of the mean of 3 experimental runs. The dashed line represents 100% detection of protein. (b) A distribution of the single cell basal p53 protein expression in BE cancer cells is shown showing a long-tailed gamma-like distribution where p53 protein expression in some cells is significantly higher than the modal value. (c) Scatter plot of p53 protein copy number per cell as a function of cell volume showing how protein expression varies as a function of cell size. Portions of this figure have been modified from ¹² and reproduced with permission. [Please click here to view a larger version of this figure.](#)

Discussion

Addressable Droplet Microarrays are a sensitive and extensible method for quantitatively determining the absolute copy number of protein within a single cell.

Limiting the level of non-specific binding (NSB) is critical within the protocol to achieving as low a limit of detection as possible. Proteins and other biochemical species may non-specifically bind to a number of interfaces present within the droplets — the coverslip surface, the antibody spot and the oil/water interface. Proteins can be lost by partitioning into the interface or the oil itself. We have shown that 4% BSA present in the aqueous droplet is capable of limiting NSB of proteins using the antibodies specified in the protocol. Alternative protein targets and the antibodies used to detect them may require alternative blocking approaches, such non-ionic detergents or compatible surfactants. Fluorinated oils may also

be tested for their suitability in serving as the capping oil. In such cases, additional considerations such as the critical micelle concentration and the density of the capping oil must be made.

The unwashed printing buffer, which remains at each spot location after arraying aids in alignment. When initially depositing the droplets, care must be taken to not scratch or damage the antibody spot by the tip of the concentric tubing nozzle. In developing the method further, a fluorescent dye may be doped into the antibody printing buffer which becomes immobilized in addition to the capture antibody. This would allow for wash and blocking steps to be performed prior to droplet arraying; however, such a step would not necessarily be required if using automated methods of droplet arraying such as inkjet printing methods.

The surface of the commercially sourced coverslips upon which droplets are formed is coated with a hydrophilic polymer and the aqueous droplets produced upon them with a volume of 10 nL have a diameter of 751 ± 42 nm. There is a limit to how much the deposited droplet should wet the surface or spreads due to the weight of the capping oil since below a minimum thickness there is increased optical scatter from the TIRF excitation laser. The scatter increases the average level of background and can drown out signal when detecting single molecules. The antibody spot must also be located away from the droplet edge since the excitation laser light may partially or fully strike an area outside of the aqueous droplet. The critical angle condition will no longer be met for light striking the glass-oil interface as opposed to the glass-water interface.

To quantitatively determine the abundance of protein the number of single molecules bound to a spot must be determined through image analysis. To determine the total amount of protein in solution from the single molecule count a calibration curve is required and enables the technique to be absolutely quantitative.

To minimize photobleaching, spots are not imaged continuously and the TIRF excitation laser source power is minimized. The protocol as presented has been used successfully for photostable fluorophores such as the Alexa Fluor dyes. Alternative fluorophores may be used but photobleaching must be assessed and the imaging protocol adapted if necessary. The total number of single molecules per frame may be plotted against time to reproduce the binding curve although this is certainly not necessary if binding kinetics are not sought and imaging a single frame at equilibrium will suffice.

Although there is a requirement of two high affinity antibodies, this results in a highly sensitive and highly specific assay, crucial for measurements at the single cell level. The p53 antibodies used in this protocol are well optimized in detecting free p53 from single cells. The target protein is determined by the choice of antibodies and may be modified in a number of ways: 1, both the capture and detection antibodies may be replaced to bind alternative targets; 2, the detection antibody may be replaced to probe protein-protein interactions or post-translational modifications to the protein bound to the capture antibody, e.g. determine the phosphorylation status of captured p53; 3, multiplexed assays may be achieved by printing multiple capture antibody spots in close proximity — printing a 3×3 spot array is possible within 10 nL droplets. Of course, for any change of target protein a number of antibody screens, controls and optimizations must be undertaken.

Several methods for lysing single cells have been reported.³³ Optical lysis is a chemical-free approach to rapidly lyse individual cells without altering the contents of cells and maintaining the integrity of proteins and their complexes. Chemical lysis using detergents poses a problem to the integrity of the droplets when using mineral oil and can interfere with interactions between molecules.³⁴ However, for assays where chemical lysis is compatible it is an attractive approach since it is not reliant on equipment such as lasers or micropatterned electrodes.³⁵

The droplets show comparable performance with alternative single cell methods. In the current generation of droplets using the suggested p53 antibodies, the level of NSB is 187 ± 60 molecules per spot. The single molecule counts exhibit strong linearity ($R^2 = 0.99$) in the region spanning 10^5 – 10^8 recombinant p53 proteins per droplet. This suggests that while lower levels of protein may be detected in cells, there is a limit of quantitation of 10^5 p53 proteins; this corresponds to a single molecule count on the spots of 901 ± 83 . This is predominantly limited by the volume of the droplets and adsorption to the interface. The performance of the p53 assay is such that when performed in a 10 nL volume only $1.1 \pm 0.2\%$ of the total target protein is captured from solution; this increases to $85 \pm 9\%$ when reducing the analysis volume to 0.2 nL.³⁶ By reducing the volume of the droplets to below 1 nL and reducing adsorption, there is potential using the specified antibodies to p53 in improving the limit of detection to <50 proteins per cell. Therefore, addressable droplets have the potential to be used to measure very low abundance proteins from single cells.

The addressability of the droplets afforded by the easily penetrable oil cap permits the cell analysis volume to be sequentially challenged. The use of a micropipette enables the injection of a desired number of cells into each droplet in a plug of 10–20 pL, which does not significantly increase the volume. For the chip shown in **Figure 1B** with 100 wells, to form droplets and load them with single cells typically takes 75–90 min. Loading cells may also be achieved by addressing the droplet using the concentric tubing nozzle instead of the micropipette or using a solution containing cells when initially forming the droplets. The latter would be useful in limiting the number of steps in the protocol but, in both cases, the occupancy per droplet would follow a Poissonian distribution. This would limit the proportion of single cell data per chip; however, droplets with zero or multiple cells would serve as controls. An additional limitation in using the concentric nozzle to address droplets is the droplet volume would increase in 10 nL increments. With the suggested modifications above this could be improved. On-chip droplet microfluidics are capable of producing droplets at high frequency and have potential in being automatically combined with ADMs to produce and manipulate droplets and sequentially deposit them in a planar array. This would certainly overcome the limitations in production of ADMs while also enabling single molecule readout of droplets.

The ability to address each individual droplet has a range of possible uses. We have demonstrated the ability to sequentially load single cells. It also permits removal of the unbound cellular material post lysis and post analysis by pipette for analysis using other methods. Examples of subsequent analysis would include quantitative real-time PCR or mass-spectrometry.

One of the current bottlenecks for laboratories wanting to take a quantitative approach to single cell protein analysis is the high technical barrier and the need for specialized equipment. With ADMs, miniaturized, high sensitivity analyses may be achieved without the need for clean room facilities to fabricate microfluidic lab-on-a-chip devices. Experiments are not limited by the design of the chip and their volume and position may be altered without the need for fabricating new masters. Certainly, there is scope for improvement in simplifying the technique and lowering the

barrier to entry for single cell analysis to just a microarrayer, to print both antibody and droplet microarrays, and a fluorescence microplate reader, to image.

A number of clinical applications of single cell analysis are emerging, particularly in the treatment of cancer. Tumor heterogeneity is a major challenge to effective chemotherapy. In tumor development, mutations arise with increasing frequency and heterogeneity can result in morphological, genetic, and proteomic variability. Single cell analysis is able to resolve cellular heterogeneity within a tumor and provide a platform to help better understand and predict drug resistance and guide therapy.

Disclosures

The authors have nothing to disclose.

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