DROPBLOT DESIGN INTEGRATES DROPLET MICROFLUIDICS WITH SINGLE-CELL ELECTROPHORESIS FOR TARGETED PROTEOMICS

Yang Liu[1*] *and Amy E. Herr*[1]
[1]University of California, Berkeley, California, USA

ABSTRACT

Single-cell analyses are revolutionizing biomedicine and biology, with genomics (DNA) and transcriptomics (RNA) tools leading the way. At the protein-level, single-cell analyses are limited to mass spectrometry and immunoassays. Neither assay provides comprehensive coverage of proteome for single cells, missing key protein forms (called isoforms). Here, we introduce a hybrid droplet-electrophoresis device, termed "DropBlot", to detect proteins from patient-derived tissue biospecimens relevant to clinical medicine and pathology. The DropBlot takes advantage of water-in-oil (W/O) droplets to encapsulate single cells derived from chemically fixed tissues, thus providing a picoliter-volume reaction chamber in which said cells are lysed and subjected to harsh lysis conditions (100ºC, 2 hours), as needed for fixed cells. We report an all-in-one microdevice to facilitate cell-laden droplet loading with >98% microwell occupancy. Droplets remain intact under the electric field and protein isoforms are shown to electromigrate out of the droplet and into a microfluidic separation channel where protein sizing takes place via the action of electrophoresis in a photoactive polyacrylamide (PA) gel.

KEYWORDS

Proteoform, Droplet, Microfluidics, Fixed Cells, Single-Cell Immunoblotting.

INTRODUCTION

Microanalytical tools underpin many of the single-cell genomic (DNA) and transcriptomic (RNA) advances of recent years.[1] Similarly, microfluidic tools are playing a significant role in understanding single-cell level protein expression and function;[2, 3] as is critical for disease diagnostics[4]. Proteoforms are different phenotypes (forms) of protein molecules translated by the same gene[5], and play a vital role in cell states and cellular heterogeneity.

While a suite of single-cell protein analysis tools have been developed, many rely on antibody probes that have low specificity and cannot differentiate the similar proteoform molecules.[6] A workhorse method to analyze the lysate of individual cells is capillary electrophoresis (CE), which conducts protein electrophoresis in fused silica capillaries. However, the low-throughput and analyte loss due to absorption bottleneck its application in the measurement of single-cell heterogeneity.[7, 8] Mass spectrometry is another powerful tool to analyze proteoforms without antibody probes. While promising in single-cell analysis, mass spectrometry can presently only detect high abundance species and small-to-intermediate sized proteins.[9, 10]

As a complement to existing tools that have difficulty detecting proteoforms, our group has pioneered single-cell immunoblotting microanalytical tools design and clinical translation[11, 12]. The immunoblotting tools utilize an 'open microfluidic' design concept, with no enclosed channels. An open microwell (no 'lid') is used to isolate and manipulate a single cell prior to lysis and protein electrophoresis. Nevertheless, the 'open' microwell structures suffer from diffusion-based protein target losses even before analysis. These target losses limit the duration of lysis (5-60 sec), which makes the device unable to analyze **F**ormalin-**F**ixed and **P**araffin-**E**mbedded (FFPE) specimens that are widely used in clinical pathology labs and diagnostics[13, 14] and require harsh conditions to reverse protein crosslinks and extract target proteins[15]. To address these cell preparation challenges, we present a hybrid droplet-electrophoresis device (DropBlot) designed to support harsh-cell lysis conditions and, thus, applicability to the ~1 billion patient-derived FFPE tissue samples preserved in clinical repositories.

RESULTS AND DISCUSSION

Overview of DropBlot

DropBlot (Figure 1a) incorporates a droplet generation device with a single-cell immunoblotting chamber, on which droplet trapping, cell lysis, on-chip protein separation, antigen probing, and nucleic acid recovery are performed. To our knowledge, this is the first such hybrid design. In this device (Figure 1b), cell suspensions and lysis buffer are encapsulated into water-in-oil (W/O) droplets. Droplets are then loaded onto PA-gel stippled with microwells. A suspension of cell-laden droplets is then loaded via gravity-based sedimentation onto the microwell setup. Cells are then chemically lysed while encapsulated in the droplets, with each droplet centered in a microwell. The droplet, enclosed by mineral oil, prevents protein lysate loss due to the diffusion and sustains stability under harsh lysis conditions (high temperature, long-time incubation). After the harsh lysis step completes, protein analysis is initiated by

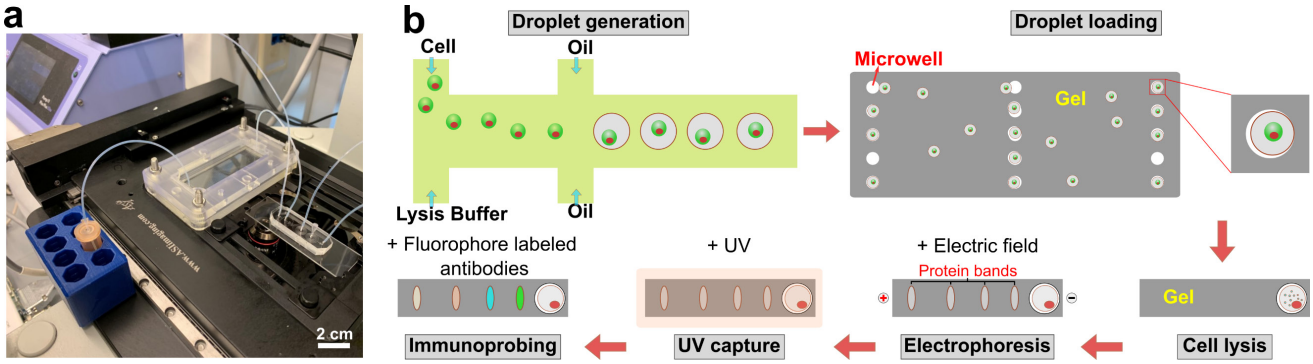

Figure 1: DropBlot: a hybrid droplet and single-cell protein electrophoresis bioMEMS device to understand the proteome of even rugged cell specimens. (a) A photo of the device assembles with droplet generation stage and all-in-one electrophoresis chamber. (b) Workflow for single-cell electrophoresis using the integrated system.

978-1-940470-04-7/HH2022/$25©2022TRF
DOI 10.31438/trf.hh2022.29

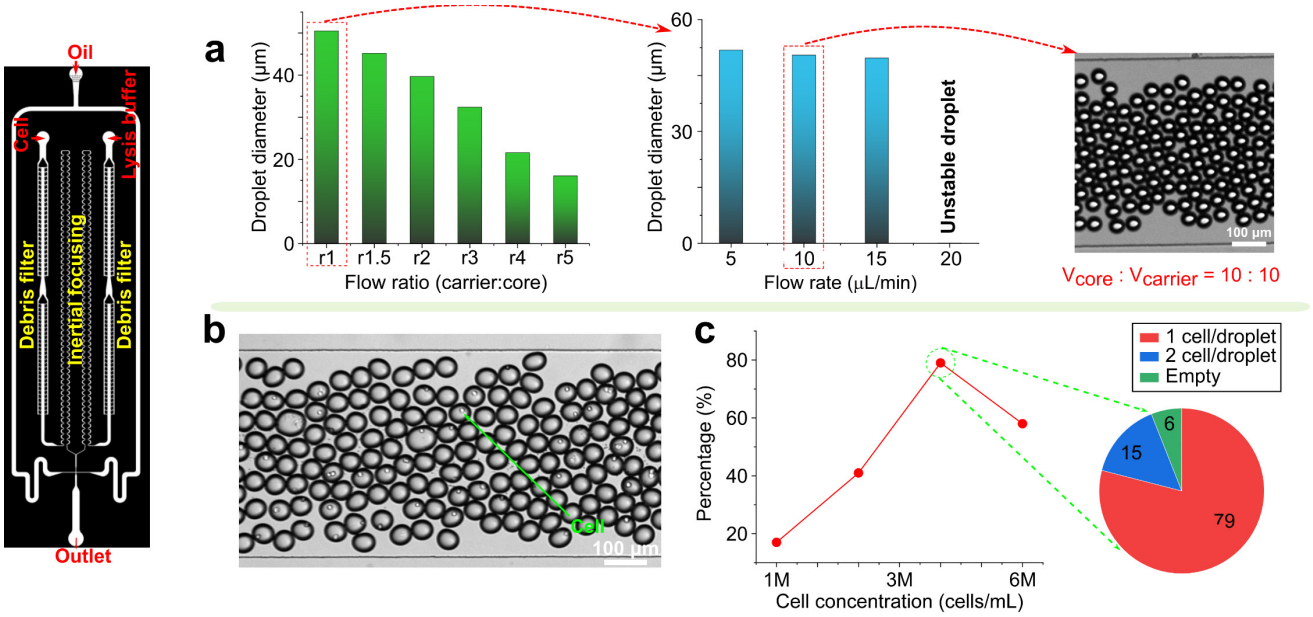

Figure 2: Optimization of droplet generation for cell encapsulation. (a) Study of droplet size with different parameters: flow ratio between carrier and core medium (left); flow rate of core medium when the flow ratio was 1 (middle). Stable droplets of 50 μm in diameter were generated (right). (b) Bright-field images of cell encapsulation when the initial concentration was 3M cells/mL. (c) The percentage of droplets containing one cell with a different initial cell concentration.

applying an electric field to the PA gel and droplet system, causing protein electromigration out of the W/O droplet and into the PA-gel molecular sieving matrix. Once protein electrophoresis initiates, protein targets separate based on differences in electrophoretic mobility (proportional to molecular mass or 'size'). Resolved proteins are covalently bonded to the gel by UV-initiated capture and then labeled with fluorescent antibodies. Using microscopy, we observe that the droplets remain intact after in-gel immunoblotting.

Droplet Generation and Cell Encapsulation

The first requirement for single-cell encapsulation is the sustained creation of homogeneous, high concentration cell suspensions and lysis buffer with or without barcode beads. For this purpose, we designed a droplet-generation device containing 3 major components: (1) filters to remove debris from lysis buffer and cell suspension; (2) sigmoidal microchannels with alternating curvatures to inertially focus particles into a narrow stream. (3) Droplet generation region to produce W/O droplets. The inertial-focusing region was designed based on our previous work[16] and has been reported to efficiently align cells at low flow rates (10 – 100 μL/min).

Using a syringe, we injected mineral oil containing 2% Span 80 surfactant into the OIL inlet, while cell suspension and lysis buffer were separately injected into corresponding inlets. The dependence of droplet size was investigated using flow ratios ($V_{carrier}$: V_{core} = 1-2), producing droplets with diameters ranging from 41 to 51 μm (Figure 2a). To increase the cell capture efficiency, different concentrations of cells were tested with the 5 million per mL cell concentration yielding the highest percentage (79%) of droplets loaded with just one cell (Figure 2 b-c). There were 6% of droplets loaded with zero cell and 15% of droplets loaded with two cells.

Droplet Stability Study and In-Droplet Cell Lysis

Cells were chemically lysed in droplets with RIPA-like buffer containing Sodium Dodecyl Sulfate (SDS), Sodium Deoxycholate, Triton X-100, and Tris-Glycine. This new system is robust to various lysis temperatures (23-100ºC) and durations (1–180 min) and is designed to retrieve antigens from fixed or FFPE samples. To investigate in-droplet cell lysis, we screened cells in a range of SDS lysis buffer concentrations, which have strong hydrophilic properties and the hydrophilic-lipophilic balance (HLB) value of SDS is 40. We observe an emulsion pattern that is dependent on SDS concentration. We further observe stable droplet generation with ≤0.5% SDS in the core medium (Figure 3a). Furthermore, the

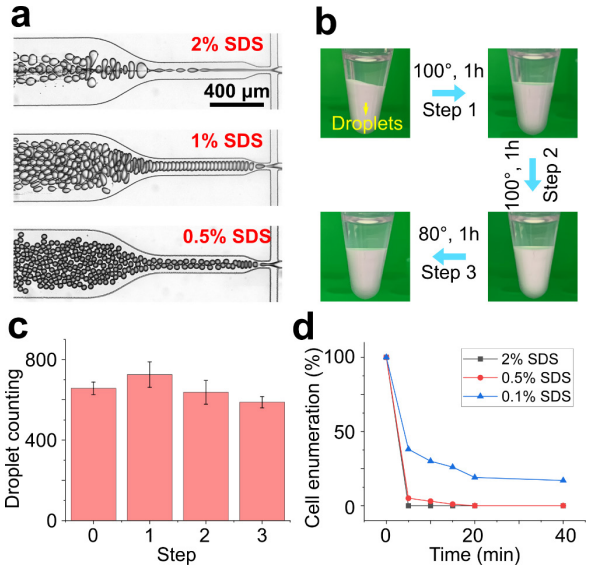

Figure 3: Study of droplet stability with different lysis buffer and incubation conditions. (a) Droplet generations with 0.5 – 2% SDS in the core medium (Vcore : Vcarrier = 10:15). (b) Stability of droplets containing 0.5% SDS under a series of incubations. (c) Droplet enumeration after incubations (step1: 100ºC for 1h; step 2: 100ºC for 1h; step 3: 80ºC for 1h). Droplets were incubated and imaged on a glass slide with a hydrophobic surface. (d) Cell (MCF7) lysis with different concentrations of SDS at 95 ºC.

50 µm W/O droplets loaded with 0.5% SDS maintained stability under harsh conditions: 100ºC for 2 hours and 80ºC for 1 hour, used for the lysis of the FFPE sample (Figure 3b-c). The lysis efficiency of cells was determined by the concentration of SDS presented in the lysis buffer. MCF7 (human breast cancer cell line) cells isolated in droplets were observed completely lysed within 15 min using 0.5% SDS at room temperature (Figure 3d). Lysis buffer with 2% SDS can lyse cells within 5 minutes while the buffer with 0.1% SDS cannot completely lyse cells and about 25% of cells remain intact after 40 minutes of incubation.

Validation of Droplet Sealing with Protein Ladder

The W/O droplets act as a closed reaction chamber in which we perform cell lysis. To measure the protein-permeability of these droplets, we loaded a cocktail of known protein standards mixed with lysis buffer (SDS: 0.5%) into droplets and measured the mean

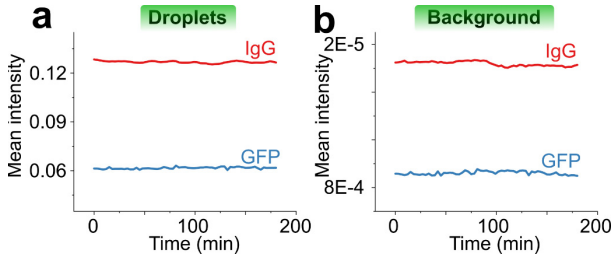

Figure 4: Droplet diffusion assay in W/O droplet. (a) Mean fluorescence intensity of ~300 droplets over time (0–180min). (b) Mean fluorescence intensity of background over time (0-180min).

fluorescence intensities of individual proteins (Immunoglobin (IgG*, 150 kDa) labeled with AF647 and Green Fluorescent Protein (GFP, 26kDa)). As is shown in Figure 4a, there was no obvious fluorescence intensity change during the 180-min assay (room temperature). Meanwhile, the intensity of the background did not increase over time (Figure 4b). We can conclude that W/O droplets with lysis buffer are stable and prevent protein loss from the cell-laden droplet during a 180-min experiment.

Protein Electrophoresis with DropBlot

Using a customized loading chamber, more than 98% of microwells on the gel were occupied with one lysate-laden droplet (Figure 5a), thus providing a convenient, well-controlled approach to sample loading into each microwell for subsequent electrophoretic analysis of each protein lysate. Here, we generated droplets with 50 µm in diameter and loaded the droplets onto the gel platform (with an array of 60-µm diameter microwells in the gel). An applied electric field strength of 40 V/cm was applied to the system for the electrophoresis step. Using fluorescence microscopy, protein (BSA, Bovine Serum Albumin) electromigrated out of the droplet and then out of the microwell and into the molecular sieving gel for electrophoresis (Figure 5b, t = 20s). The droplet remains intact after electrophoresis. The fluorescence intensity of each lane (Figure 5c) was analyzed with MATLAB to calculate the migration distance. While we demonstrate an electric field of 40 V/cm to resolve BSA, future experiments of different strengths would help to balance the electrolysis and Joule heating. We will develop a model to simulate the protein migration in the DropBlot and optimize parameters including droplet position, electric field strength, and electrophoresis time. Furthermore, we will investigate the conditions that are required to achieve maximum separation resolution for proteins isoforms, such as Her2.

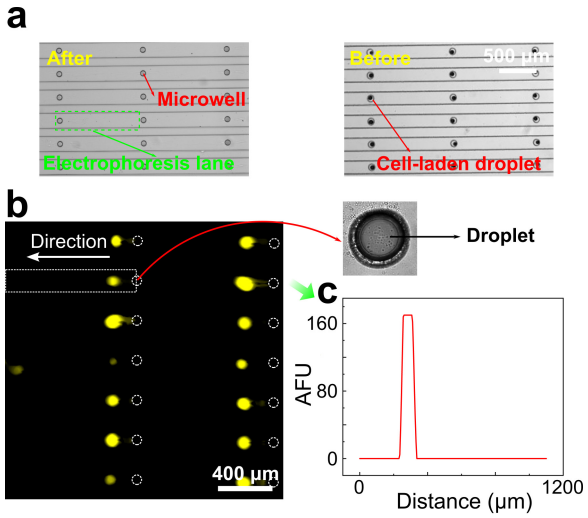

Figure 5: Protein electrophoresis with the DropBlot device. (a) Droplets loading onto polyacrylamide gel with microwells. Top: before loading. Bottom: after loading. (b) Fluorescence imaging of BSA (left) and bright-field image of the droplet at the 20s lapsed separation time. Electric field: 40V/cm. (c) Background subtracted fluorescence intensities (AFU) of one separation lane

CONCLUSION

Here we report a novel hybrid droplet-electrophoresis device (DropBlot) to deconvolve protein isoforms of fixed/FFPE samples at a single-cell level. The preliminary data confirmed the feasibility of this setup, wherein cells loaded in the droplet can be completely lysed under harsh conditions and extracted proteins can migrate out of the droplet and be resolved on the PA gel. Nucleic acids will be extracted from droplets after electrophoresis and are then processed to analyze DNA or mRNA. DropBlot will be tested with various fresh cancer cell lines and cells dissected from FFPE tissue. We anticipate that DropBlot will broaden the applications of single-cell immunoblotting in pathological diagnosis using FFPE cancer specimens.

METHODS

Fabrication of Droplet Generation Chips. Single emulsion generation chips were fabricated using standard soft lithographic techniques. SU-8 3050 (Kayaku Advanced Materials) was used to fabricate masters with a height of 60µm. PDMS was prepared with Sylgard 184 Silicone Elastomer Kit (Ellsworth Adhesives) and mixed with a ratio of 10:1. After curing (70ºC, 3 hours), the PDMS device was further baked for 48 hours at 80ºC to recover the hydrophobicity.

Polyacrylamide Gel fabrication. Wafer microfabrication and silanization are described in our previous work.[12] To prepare an 8%T polyacrylamide gel, the gel precursor solution was mixed with 30% (wt/wt) Acrylamide/bis-acrylamide (Sigma-Aldrich), N-(3-((3-benzoylphenyl) formamido)propyl) methacrylamide (BPMA, Pharm Agra Labs), 10X tris-glycine buffer (Sigma-Aldrich), and ddH_2O. Gels were chemically polymerized for 20 min with 0.08% (w/v) ammonium persulfate (APS, Sigma-Aldrich) and 0.08% (v/v) TEMED (Sigma-Aldrich). After polymerization, gels were collected with a razor blade and released from the wafer and stored in the RIPA-like buffer.

Cell Culture. Human breast cancer cell line (MCF7-GFP, ATCC) was used in this study. Cells were cultured in DMEM

medium (Thermofisher) supplemented with 10% (v/v) fetal bovine serum (Benchmark), 1% (v/v) penicillin/streptomycin solution (Thermofisher), and 0.1 mM non-essential amino acid solution (Thermofisher). The cells were cultured in the incubator (37ºC, 5% CO_2) and were released through incubation with 0.05% trypsin-EDTA solution (Thermofisher). The concentration of harvested cells was measured with a hemocytometer (Hausser Scientific) and resuspended with PBS to a specific concentration.

Fluidics Assembly and Generation of W/O droplet. Harvested cells or purified proteins were resuspended in PBS and injected into the cell inlet. Cell suspension and lysis buffer were mixed prior to the emulsion region. 2% (v/v) Span 80 surfactant (Sigma Aldrich) was spiked into mineral oil (Sigma Aldrich) and used as the carrier solution. Flow rates of the substrate and carrier solution were actively controlled by a syringe pump (Chemyx). The flow rates of each solution were adjusted to generate droplets of different sizes. Droplets could be collected in a 1.5mL Eppendorf tube or be directly loaded onto PA-gel with customized PDMS channels.

Protein Diffusion Assay. Proteins of different molecular weights were diluted to 5 μM with PBS. In this assay, we used Alexa-Fluor 647 labeled donkey anti-rabbit secondary antibody (IgG, Thermofisher) and rTurboGFP (Evogen). Proteins were encapsulated into 50 μm droplets and observed under an inverted microscope (Olympus) for 180 min.

Protein Electrophoresis using DropBlot. BSA(Bovine Serum Albumin)-loaded droplets were settled down on the PA-gel, which was placed in a customized electrophoresis chamber. 7.5 mL running buffer, containing 1X Tris-Glycine and 1% (v/v) SDS, was poured onto the chamber. We supplied a constant voltage of 240 V (the target voltage for an electric field of 40V/cm). Immediately After electrophoresis, the power was shut off and migrated proteins were photocaptured by 45 s UV exposure. The slides were then rinsed with deionized water and imaged with a microscope.

ACKNOWLEDGEMENTS

This work was funded by the National Institutes of Health (NIH) R01CA20301. We sincerely thank Herr Lab members Gabriela Lomeli and Ana Martinez for initial training on the operation of the single-cell western blot. We acknowledge all members of the Herr Lab at UC Berkeley. We are also grateful to R&D Machine Shop at UC Berkeley for the fabrication of the chamber.

CONTACT

*****Yang Liu, tel: + 1-706-254-6443; yl2021@berkeley.edu

REFERENCES

[1] E. Papalexi and R. Satija, *Nat Rev Immunol*, 2018, **18**, 35-45.
[2] A. J. Dickinson, P. M. Armistead and N. L. Allbritton, *Anal Chem*, 2013, **85**, 4797-4804.
[3] A. D. Hargis, J. P. Alarie and J. M. Ramsey, *Electrophoresis*, 2011, **32**, 3172-3179.
[4] S. Doll, F. Gnad and M. Mann, *PROTEOMICS–Clinical Applications*, 2019, **13**, 1800113.
[5] L. M. Smith and N. L. Kelleher, *Nature methods*, 2013, **10**, 186-187.
[6] Y. Liu, X. Chen, Y. Zhang and J. Liu, *Analyst*, 2019, **144**, 846-858.
[7] R. T. Kennedy, M. D. Oates, B. R. Cooper, B. Nickerson and J. W. Jorgenson, *Science*, 1989, **246**, 57-63.
[8] T. M. Olefirowicz and A. G. Ewing, *J Neurosci Methods*, 1990, **34**, 11-15.
[9] B. Budnik, E. Levy, G. Harmange and N. Slavov, *Genome Biol*, 2018, **19**, 161.
[10] Y. Zhu, G. Clair, W. B. Chrisler, Y. Shen, R. Zhao, A. K. Shukla, R. J. Moore, R. S. Misra, G. S. Pryhuber, R. D. Smith, C. Ansong and R. T. Kelly, *Angew Chem Int Ed Engl*, 2018, **57**, 12370-12374.
[11] S. M. Grist, A. P. Mourdoukoutas and A. E. Herr, *Nat Commun*, 2020, **11**, 6237.
[12] E. Rosas-Canyelles, A. J. Modzelewski, A. Geldert, L. He and A. E. Herr, *Nat Protoc*, 2021, **16**, 1062-1088.
[13] A. Smith, M. Galli, I. Piga, V. Denti, M. Stella, C. Chinello, N. Fusco, D. Leni, M. Manzoni, G. Roversi, M. Garancini, A. I. Pincelli, V. Cimino, G. Capitoli, F. Magni and F. Pagni, *J Proteomics*, 2019, **191**, 114-123.
[14] B. Heijs, S. Holst, I. H. Briaire-de Bruijn, G. W. van Pelt, A. H. de Ru, P. A. van Veelen, R. R. Drake, A. S. Mehta, W. E. Mesker, R. A. Tollenaar, J. V. Bovee, M. Wuhrer and L. A. McDonnell, *Anal Chem*, 2016, **88**, 7745-7753.
[15] A. Ly, A. Buck, B. Balluff, N. Sun, K. Gorzolka, A. Feuchtinger, K. P. Janssen, P. J. Kuppen, C. J. van de Velde, G. Weirich, F. Erlmeier, R. Langer, M. Aubele, H. Zitzelsberger, L. McDonnell, M. Aichler and A. Walch, *Nat Protoc*, 2016, **11**, 1428-1443.
[16] Y. Liu, W. Zhao, R. Cheng, A. Puig, J. Hodgson, M. Egan, C. N. Cooper Pope, P. G. Nikolinakos and L. Mao, *Lab Chip*, 2021, **21**, 2738-2750.