



Supporting Information

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Facile and Rapid One-Step Mass Preparation of Quantum Dot Barcodes

Sébastien Fournier-Bidoz, Travis L. Jennings, Jesse M. Klostranec, Winnie Fung, Alex Rhee,
W.C.W. Chan*

Institute of Biomaterials and Biomedical Engineering

Terrence Donnelly Center for Cellular and Biomolecular Research

University of Toronto

160 College Street, 4th Floor

Toronto, Ontario M5S 3G9, Canada

E-mail: warren.chan@utoronto.ca

Supplementary Section

Section 1: Supplementary Methods

Flow-Focusing technology. The flow-focusing process developed by Ganan-Calvo *et al.*^[1, 2] allows the generation of steady capillary microjets, which after undergoing capillary instability and break up, lead to the production of monodispersed micrometric droplets. The microjet is driven by the aerodynamic suction of highly accelerated co-flowing liquid streams. As seen in the insets of Figure 1a, a hole in the nozzle chamber wall is located just opposite a micro-needle's end feeding the polymer and QD solution. The water flow is forced outside the chamber through the hole while acting as a sheath fluid for the polymer/QD flow. Once the co-flowing jets pass through the orifice, the pressure gradient which is the main accelerating force in the axial direction, vanishes and the jet evolves under the influence of the viscous shear stresses exerted by the water flow on the polymer/QD/organic solvent flow and the capillary stress. Since the shear stresses are here an accelerating force, they play a stabilizing role while surface tension is the destabilizing cause in capillary jets. Perturbations grow downstream until the jet eventually breaks up into microdroplets. The break up process is governed by the Weber number (We), usually defined as the aerodynamic-to-capillary force ratio and the liquid-to-liquid velocity ratio. The droplets obtained from the break up of capillary microjets are monodisperse only if the breaking time is sufficiently small to avoid non-symmetric perturbations. In other words the Weber number must be maintained below a certain value ($We < 40$) to guaranty monodisperse microbeads. On the other hand, values of We of the order of unity or larger ($We > 1$) are required to generate the capillary microjet. The jet diameter and the resulting droplet size can be controlled by varying polymer concentration and/or flow rates. Increasing the focusing flow while keeping constant the focused flow leads to smaller particles. Similarly, decreasing the focused flow while keeping the focusing flow constant leads to smaller particles. Particles with average diameters of 3.4 μm were obtained using 0.5 mL/h for the focused flow and 5 mL/min

for the focusing flow (polymer concentration = 4 wt%). On the other hand, particles with 7.2 μm average diameters were obtained when using 2 mL/h for the focused flow and 3 mL/min for the focusing flow (polymer concentration = 4 wt%). When using a 10 wt% polymer solution (2 mL/h for focused flow and 3 mL/min for focusing flow), 9.0 μm particles were obtained.

TEM imaging. A solid sample of dried CCFF barcodes was embedded in a Quetol/Spurr resin mixture and polymerized overnight at 65°C. 100nm cross-sections on 200 mesh copper grids were cut on an RMC MT6000 ultramicrotome and further imaged at 100 kV on an FEI Tecnai 20 TEM, with a Gatan Dualview camera.

DNA conjugation. Microbead barcodes were concentrated down via centrifugation into a total volume of 200-300 μL , which was then pipetted gently on top of 10 mL 50:50 v/v water:glycerol mixture in 15 mL Falcon tubes for fractionation. Vials were centrifuged at 1000 rpm for 5 minutes and the top 7 mL was decanted and discarded to remove the small microbeads ($\sim 1 \mu\text{m}$) from the larger microbeads ($\sim 5 \mu\text{m}$). The fractionated microbeads were then cleaned from the glycerol solution by suspending the pellet in 1.5 mL nanopure 18M Ω H₂O, sonicating for 2 minutes, centrifuging at 10k rpm for 5 minutes, and repeating this process five times. Fractionated, cleaned microbeads were finally suspended in 500 μL and counted on a hemacytometer to obtain concentration information. Capture strand DNA was bound to the microbead surface using N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide coupling (EDC, Sigma-Aldrich). Briefly, 4.5 million beads were placed into a 1.5 mL vial and suspended to a volume of 200 μL in MES pH 4.5 buffer. Separately, a stock solution of EDC was prepared immediately before use by dissolving 0.04 g EDC into 500 μL MES pH 4.5 buffer. All strands of DNA were purchased HPLC-purified from Integrated DNA Technologies and used without further purification. A stock solution for each primary amine-modified capture strand DNA was prepared at a concentration of 1 pmol/ μL in MES pH 4.5. Finally, 50 μL of EDC solution and 50 μL of capture strand DNA solution were added to each microbead sample separately, and mixed on a shaker for 3 hours. When the reaction had come to completion, excess DNA and EDC were

removed by adding 200 μL 0.1% Tween-20 solution, heating to 95°C for 2 minutes to denature surface-adsorbed DNA, then immediately centrifuged at 10k rpm for 3 minutes and the supernatant decanted. This wash process was repeated one more time and the cleaned, DNA-bound microbeads were resuspended in 400 μL 20 mM PBS pH 7.4 containing 0.1% Tween-20.

Protein conjugation. Approximately 2.5×10^6 barcoded microbeads were suspended in 100 μL of 0.1M MES buffer (pH 5.5), centrifuged at 8,000 RPM and aspirated twice. The probes were then redispersed in 100 μL of MES buffer with ~ 1 mg of EDC and incubated on a vortex for 15 minutes. Microbeads were then centrifuged at 9,000 RPM and aspirated before being resuspended in 50 μL of PBS buffer with corresponding antigen (at a concentration of ~ 10 mg/mL) and 50 μL of carbonate bicarbonate buffer (pH 9.4). These samples were incubated on a vortex for ~ 1 hr, then centrifuged at 6,500 RPM, aspirated and resuspended in 50 mM glycine quenching buffer with 0.1% Tween. Next, the samples were incubated on a vortex again for 30 minutes, then centrifuged at 5,500 RPM, aspirated and resuspended in 100 μL TRIS wash buffer (10 mM Trizma base, pH 8.0, 0.05% Tween). Following centrifugation and aspiration, the microbeads were dispersed in 3% milk/PBS buffer (3% = 3g milk powder/100 mL PBS) and incubated on a vortex for ~ 1 hr. After two washes with wash buffer, the samples were stored dry until further use.

DNA multiplexed experiments. For multiplexed hybridization detection, 380k QD-barcodes from each separate sample were placed into each of 6 vials to make identical bead mixtures in a total volume of 450 μL . Hybridization of AlexaFluor® dye-labeled detection strands to QD-barcodes was tested by adding 5 pmol quantities of the detection strand to three-replicates using the following scenarios:

	Detection 1	Detection 2	Detection 3	Detection 4	Detection 5	Detection 6
Mixture 1:	No	Yes	No	Yes	Yes	No
Mixture 2:	Yes	Yes	No	No	No	Yes

Each mixture was warmed to 45°C for 3 minutes, cooled to room temperature, and then placed at 4°C for 10 minutes immediately prior to analysis on a Beckman-Coulter flow cytometer. All possible control experiments for non-specific binding as well as cross-reactivity were performed as described in the supplementary information. The following DNA strands (all listed 5'3') were used:

Capture-1 NH2-AAA AAA AAA ACG TCC TTT GTC TAC GTC CCG

Detection-1 AF647-CGG GAC GTA GAC AAA GGA CGT TTT TTT TTT

Capture-2 NH2-GAG ACC ATC AAT GAG GAA GCT GCA GAA TGG GAT

Detection-2 AF647-ATC CCA TTC TGC AGC TTC CTC ATT GAT GGT CTC

Capture-3 NH2-CAT AGT GGT CTG CGG AAC CGG TGA GT

Detection-3 AF647-ACT CAC CGG TTC CGC AGA CCA CTA TG

Capture-4 NH2-CTT TAT AAG GAT CAA TGT CCA TGC

Detection-4 AF647-GCA TGG ACA TTG ATC CTT ATA AAG

Capture-5 NH2-TCA GAA GGC AAA AAA GAG AGT AAC T

Detection-5 AF647-AGT TAC TCT CTT TTT TGC CTT CTG A

Capture-6 NH2-AGT TGG AGG ACA TCA AGC AGC CAT GCA AAT

Detection-6 AF647-ATT TGC ATG GCT GCT TGA TGT CCT CCA ACT

Protein multiplexed experiments. Samples were resuspended in 100 µL of target antibody solution, composed of phosphate buffer saline (PBS) buffer and antibodies at a concentration of

~ 0.001 mg/mL. It should be noted that the total number of antibodies incubated per sample were the same. If six types of antibodies were incubated, then the total number of antibodies for that sample equaled the same as for a sample that incubated with only one type of antibody. The incubation on a vortex lasted ~ 1 hr followed by two washes with wash buffer. The samples were then incubated on a vortex for ~ 1 hr with 100 μ L of PBS with ~ 0.0005 mg/mL of cy5 conjugated goat anti-mouse IgG antibodies. After this incubation, samples were washed twice using wash buffer before being resuspended in 500 μ L of PBS buffer and analyzed using flow cytometry. Antibody and antigen used and their suppliers are described in the supplementary data.

SWELL barcoding process. This is a 2-step process involving the synthesis of microbeads and followed by the encapsulation of QDs inside them. Polystyrene microbeads were synthesized according to a modified published procedure.^[3] However, there are several other ways to synthesize uniform sized microbeads and they have been described in a review by Horak.^[4] Swelling step was carried out according to a published procedure.^[5]

POLYM barcoding process. This is a 2-step process involving the displacement of the tri-n-octylphosphine (TOPO) stabilization ligand around the ZnS-capped CdSe QDs with oleic acid. These QDs were precipitated out of chloroform by methanol and then centrifuged. 70 mg were redispersed into 10 mL of oleic acid and the solution was stirred at 150°C for 1h. Then, they were precipitated out of chloroform by methanol to remove the excess of oleic acid and the modified QDs were redispersed in 7 g of styrene for further use. The second step was performed according to published procedures.^[6]

Buffer study. Microbeads containing a single color barcode (605 nm emission QD) were produced using the SWELL, POLYM and CCFE approaches as described above. The following 10 mM buffer solutions at pH 6.7 were used: PBS, TRIS, HEPES, Citrate, MES, NaHCO₃ and 18 M Ω water as reference. Each sample contained 600 μ L of buffer solution and 600 μ L of

beads (~ 6 millions) suspended in 18 M Ω water. Samples were left rotating overnight and fluorescence spectra of the suspensions were recorded using a triangular quartz cuvette into a Fluorimeter (Fluoromax-3 from Horiba). This experiment was conducted three times to obtain an average and a standard deviation.

pH study. Microbeads containing a single color barcode (605 nm emission QD) were produced using the SWELL, POLYM and CCFF approaches as described above. pH solutions were prepared by adding drops of 0.1 M HCl or 0.1 M NaOH into 15 mL of 10 mM PBS buffer at pH 6.7 until desired pH level was reached. Each sample contained 1.0 mL of pH solution ranging from pH=0 to pH=14 and 500 μ L (~ 5 millions) of barcoded microbeads suspended in 18 M Ω water. Samples were left rotating overnight and fluorescence spectra of the suspensions were recorded using a triangular quartz cuvette into a Fluorimeter (Fluoromax-3 from Horiba). This experiment was conducted three times to obtain an average and a standard deviation.

Heat study. Microbeads containing a single color barcode (605 nm emission QD) were produced using the SWELL, POLYM and CCFF approaches as described above. Each sample was prepared using 1.2 mL of 18 M Ω water and 300 μ L of beads (~ 3 millions) suspended in water. Samples were then heated at 95 $^{\circ}$ C for 15 minutes in a preheated aluminum heat block. After the sample returned to room temperature, fluorescence spectra of the suspensions were recorded using a triangular quartz cuvette into a Fluorimeter (Fluoromax-3 from Horiba). This experiment was conducted three times to obtain an average and a standard deviation.

Leakage study. Microbeads containing a single color barcode (605 nm emission QD) were produced using the SWELL, POLYM and CCFF approaches as described above. 1.5 mL of microbead barcodes (~ 15 millions) were suspended in 18 M Ω water. Samples were recovered after initial fluorescence spectra measurement using a triangular quartz cuvette into a Fluorimeter (Fluoromax-3 from Horiba) and subjected to 5 minutes of sonication followed by 2 minutes of centrifugation at 10,000 x g. Supernatant was extracted and emission spectrum was taken.

Remaining beads were redispersed in 18 MΩ water to the same volume at which sample was recovered, vortexed, and spectrum was taken. The cycle was repeated three times. For all studies, three separate experiments were conducted.

Ag & Ab suppliers

Protein	Antigen	Antibody
Glucagon	Sigma, Synthetic, #G2654	Sigma, Mouse IgG1, Clone K79bB10, #G2654
Albumin	Sigma, 96-99%, Human, #A1653	ABR, Mouse IgG1, Clone Alb1, #MA1-26210
Transferrin	Sigma, >=98%, Human, #T3309	ABR, Mouse IgG1, Clone 2A2, #MA1-20108
Hemoglobin	Sigma, Human, #H7379	ABR, Mouse IgG1, Clone Haem1, #MA1-26212
Insulin	Sigma, recombinant from yeast, #I2643	Sigma, Mouse IgG1, Clone K36AC10, #I2018
Streptavidin	Sigma, <i>S. avidinii</i> , #85878	ABR, Mouse IgG1, Clone S10D4, #MA1-20010

Calculation of concentration factor from QDs in solution to QDs in microbeads. For this calculation, the following parameters have been selected. The microbead size is 5 μm, the polystyrene density equals 1.05 g/mL, and the polymer concentration in chloroform is 4 wt%. The diameter of a bead (d_b) is directly linked to d_d using the following equation:

$$d_b = d_d \times (4 \text{ wt}\% / 1.05 \text{ g/mL})^{1/3}$$

Therefore, a 5 μm diameter bead derives from a 15 μm droplet which has a 27 times larger volume and consequently, the QDs are 27 times more concentrated in the final solid microbead matrix compared to their state while contained in the initial chloroform droplet.

Calculation of wt% from # beads/mL. The diameter of a bead (d_b) is expressed in microns.

$$\text{wt}\% = 1.05 \times \frac{4}{3} \times 3.14 \times (d_b \times 10^{-4} / 2)^3 \times \# \text{beads/mL} \times 100$$

Section 2: Supplementary tables and figures

	Ag						Ab						AF IgG	Notes
	Glucagon	Albumin	Transferrin	Streptavidin	Insulin	Hemoglobin	Glucagon	Albumin	Transferrin	Streptavidin	Insulin	Hemoglobin		
1	+	+	+	+	+	+	+	+	+	+	+	+	+	6 Ag&Ab
2	+	+	+	+	+	+	-	+	+	+	+	+	+	1 Ab
3	+	+	+	+	+	+	+	-	+	+	+	+	+	
4	+	+	+	+	+	+	+	+	-	+	+	+	+	
5	+	+	+	+	+	+	+	+	+	-	+	+	+	
6	+	+	+	+	+	+	+	+	+	+	-	+	+	
7	+	+	+	+	+	+	+	+	+	+	+	-	+	
8	+	+	+	+	+	+	-	-	-	-	-	-	+	No Ab
9	+	-	-	-	-	-	-	+	+	+	+	+	+	Cross-Reactivity
10	-	+	-	-	-	-	+	-	+	+	+	+	+	
11	-	-	+	-	-	-	+	+	-	+	+	+	+	
12	-	-	-	+	-	-	+	+	+	-	+	+	+	
13	-	-	-	-	+	-	+	+	+	+	-	+	+	
14	-	-	-	-	-	+	+	+	+	+	+	-	+	
15	-	-	-	-	-	-	+	+	+	+	+	+	+	No Ag
16	+	+	+	+	+	+	+	+	+	+	+	+	+	No IgG
17	+	+	+	+	+	+	+	+	+	-	-	-	+	Figures
18	+	+	+	+	+	+	-	-	-	+	+	+	+	

Table S1. Chart depicting all microbead and antibody-antigen systems. These experiments test for the degree of non-specific binding and cross-reactivity of the barcode in protein assays.

	Capture Strand microbeads						Detection Strand DNA						Notes
	1	2	3	4	5	6	1	2	3	4	5	6	
1	+	+	+	+	+	+	+	+	+	+	+	+	Detection all
2	+	+	+	+	+	+	-	+	+	+	+	+	Minus one detection strand
3	+	+	+	+	+	+	+	-	+	+	+	+	
4	+	+	+	+	+	+	+	+	-	+	+	+	
5	+	+	+	+	+	+	+	+	+	-	+	+	
6	+	+	+	+	+	+	+	+	+	+	-	+	
7	+	+	+	+	+	+	+	+	+	+	+	-	No detection strand
8	+	+	+	+	+	+	-	-	-	-	-	-	
9	+	-	-	-	-	-	-	+	+	+	+	+	Cross-Reactivity
10	-	+	-	-	-	-	+	-	+	+	+	+	
11	-	-	+	-	-	-	+	+	-	+	+	+	
12	-	-	-	+	-	-	+	+	+	-	+	+	
13	-	-	-	-	+	-	+	+	+	+	-	+	
14	-	-	-	-	-	+	+	+	+	+	+	-	No bound capture strands
15	-	-	-	-	-	-	+	+	+	+	+	+	
16	+	+	+	+	+	+	-	+	-	+	+	-	Figures
17	+	+	+	+	+	+	+	+	-	-	-	+	

Table S2. Chart depicting all scenarios of microbead, capture strand, and detection strand systems. These experiments test for the degree of non-specific binding and cross-reactivity of the barcode of all DNA sequences.

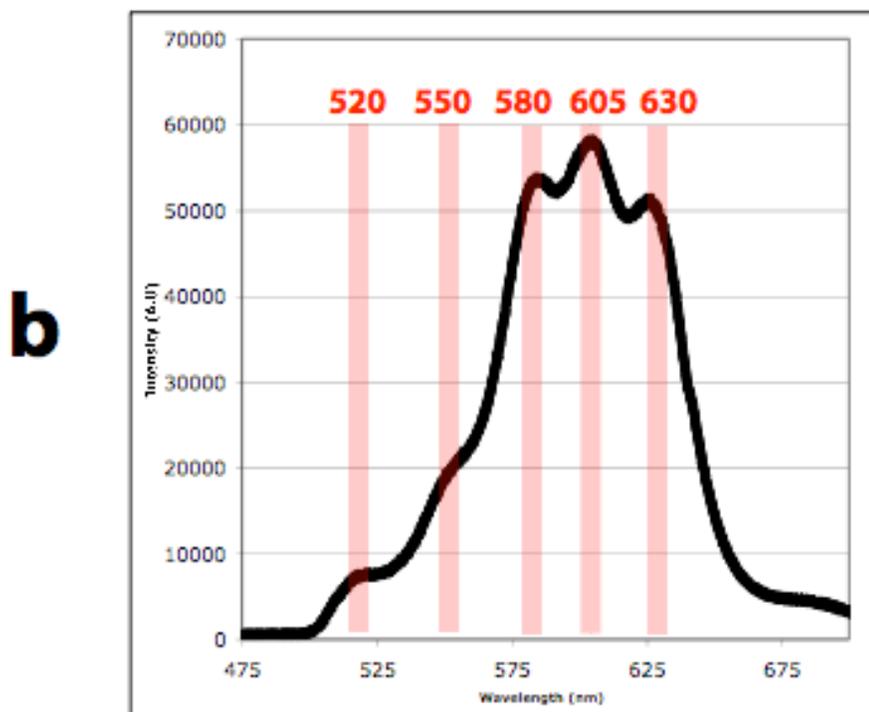
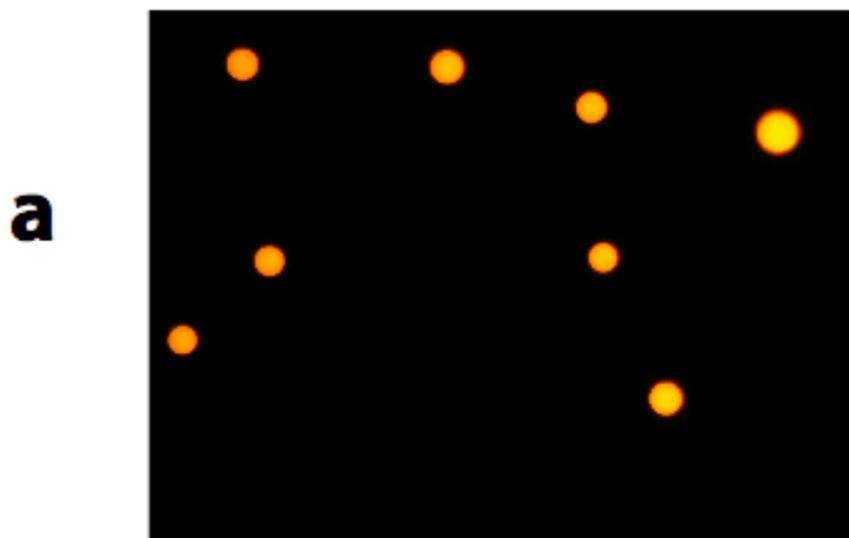


Figure S1. Presentation of a 5-color CCF produced barcode. a) Fluorescent image of CCF barcodes generated using 5 QD colors simultaneously. **b)** Corresponding fluorescence spectrum.

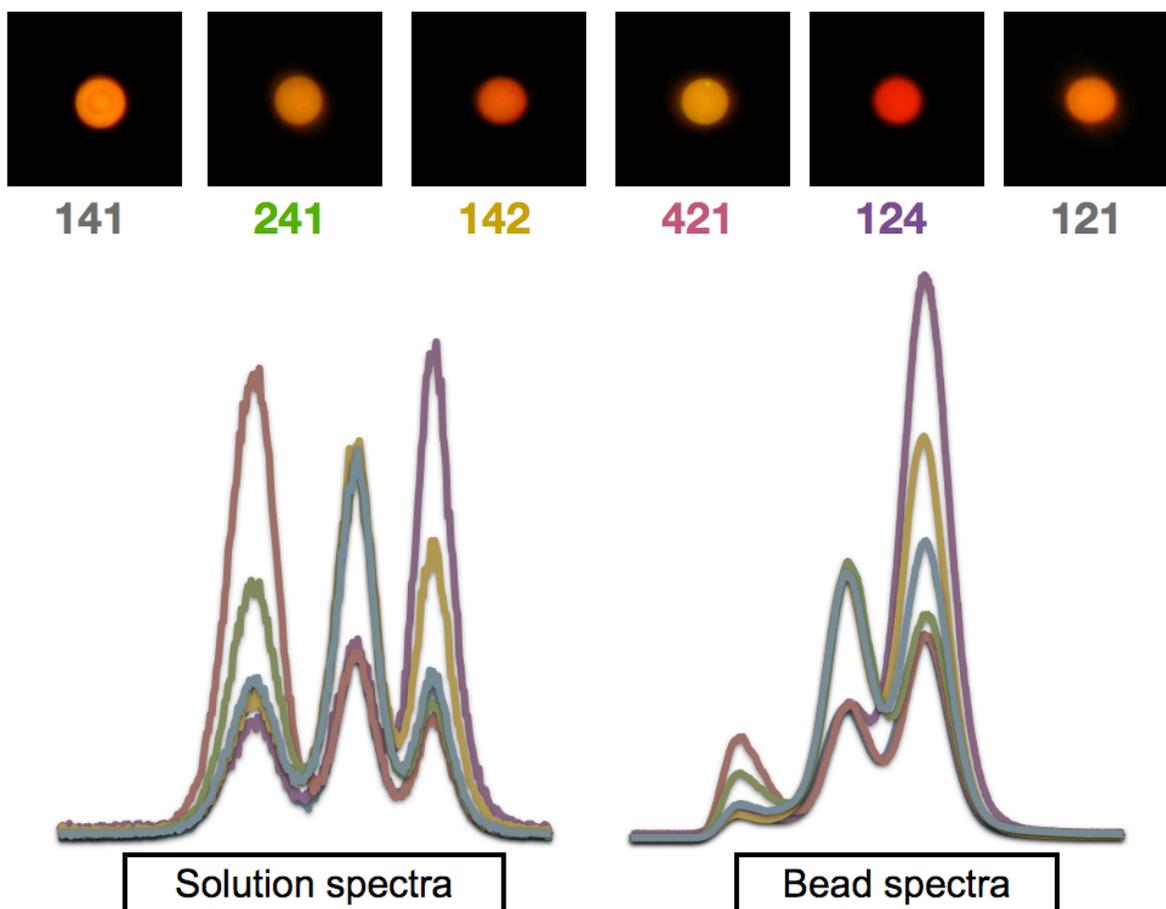


Figure S2. Photophysical effects observed during the production of barcodes. As an example, the barcode series 283 is represented here. 6 different shades of orange are created with a unique spectrum associated to each. When moving from solution spectra to bead spectra, significant intensity changes are taking place due to photophysical effects such as FRET and photon reabsorption. The resulting spectra still give rise to unique emission profiles for each barcode.

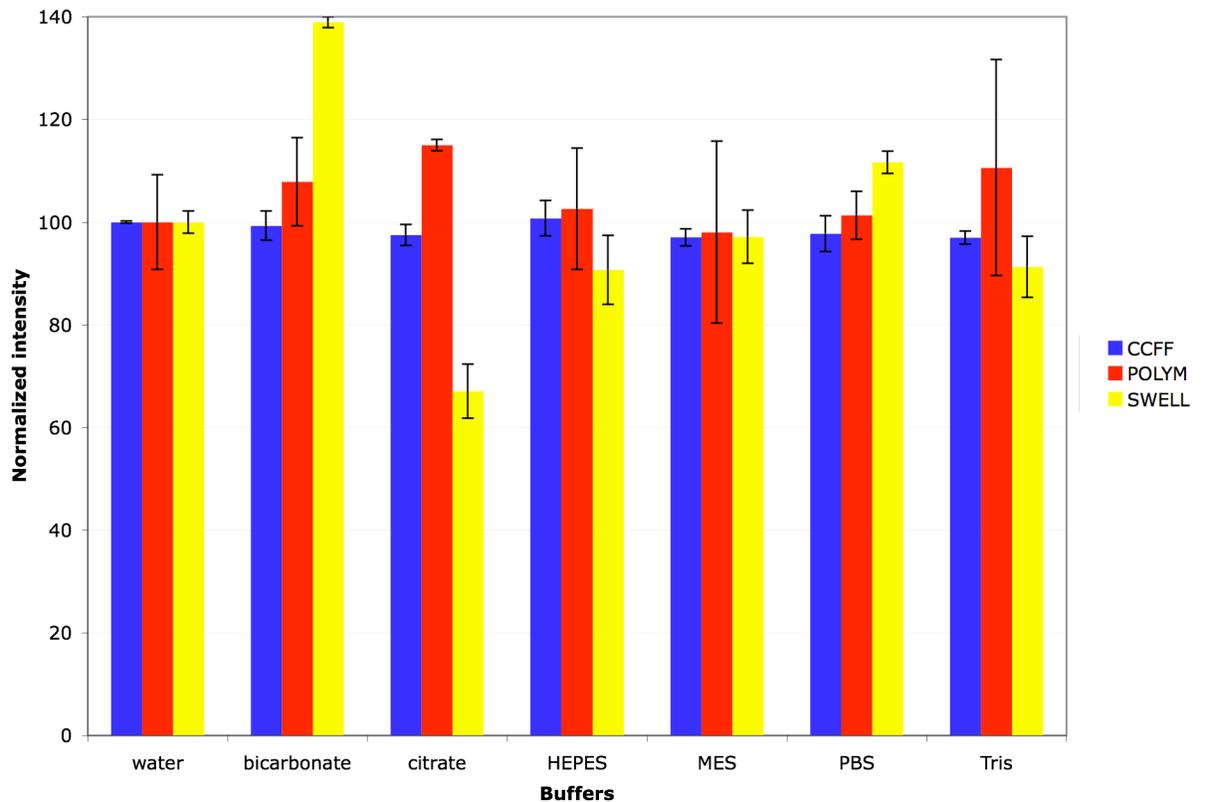


Figure S3. Effect of buffers on the emission intensity of barcodes. The effects of 6 commonly used buffers including bicarbonate, citrate, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MES (2-(N-morpholino)ethanesulfonic acid), PBS (phosphate buffer saline) and Tris (trishydroxymethylaminomethane) were compared. To prevent any other effect related to pH, each buffer was adjusted to the same pH=6.7. The SWELL barcodes are the most affected by buffer ions. Citrate, HEPES and Tris buffers decrease the fluorescence of the microbeads by up to 33%. On the other hand, bicarbonate and PBS buffers have positive effects and tend to increase the fluorescence of the microbeads while MES has no significant effect. Interestingly, the POLYM barcodes do not statistically suffer from any loss of fluorescence and in the case of citrate ions, the fluorescence is even enhanced by 15%. Lastly, the CCFF approach appears to be the most robust in terms of stability as there is near-to-no buffer effect observed in the fluorescence signal variation (within 3% of the original fluorescence intensity).

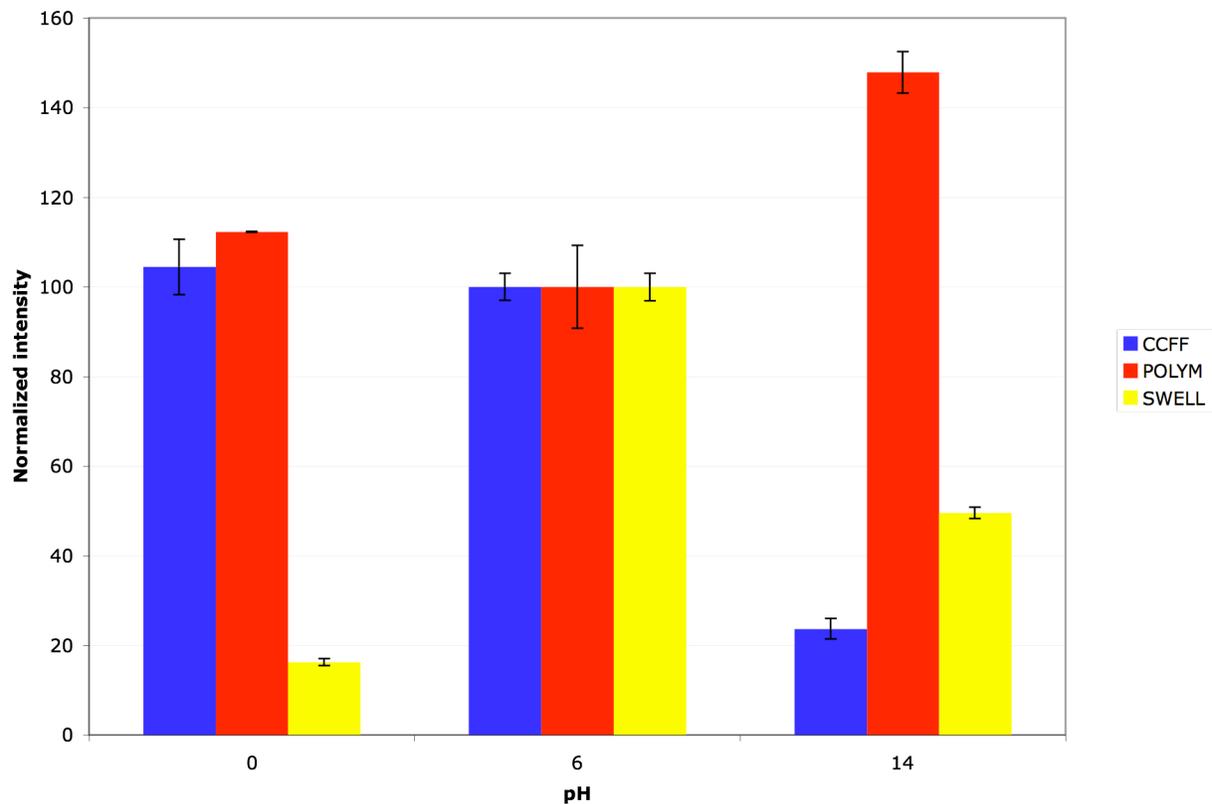


Figure S4. Effect of pH on the emission intensity of barcodes. When comparing the 3 methods, both POLYM and CCFF are very robust over the entire acidic pH range whereas SWELL fluorescence is quenched by more than 80 %. In highly basic solutions, the intensity obtained for CCFF does not reflect the emission of the barcodes as the anhydride-based polymer forming the microbeads hydrolyses and dissolves because of the absence of cross-linker inside the beads. POLYM fluorescence increases by 50 % whereas SWELL decreases by 50 %.

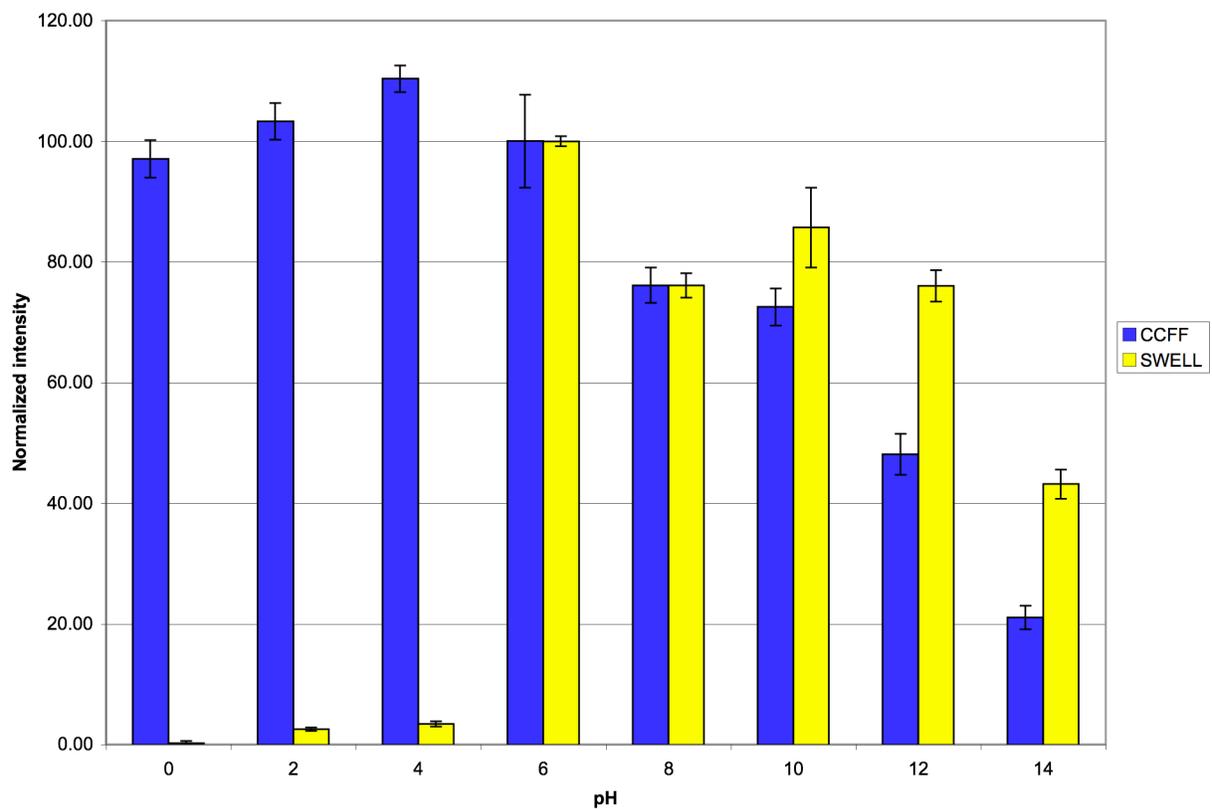


Figure S5. Detailed pH study for CCF and SWELL barcodes. When comparing both methods, SWELL barcodes can be considered stable in the range of pH from 6 to 12 whereas any acidic solution quenches their fluorescence completely. CCF barcodes tend to be stable in the pH range of 0 to 10 at which point, the copolymer starts dissolving.

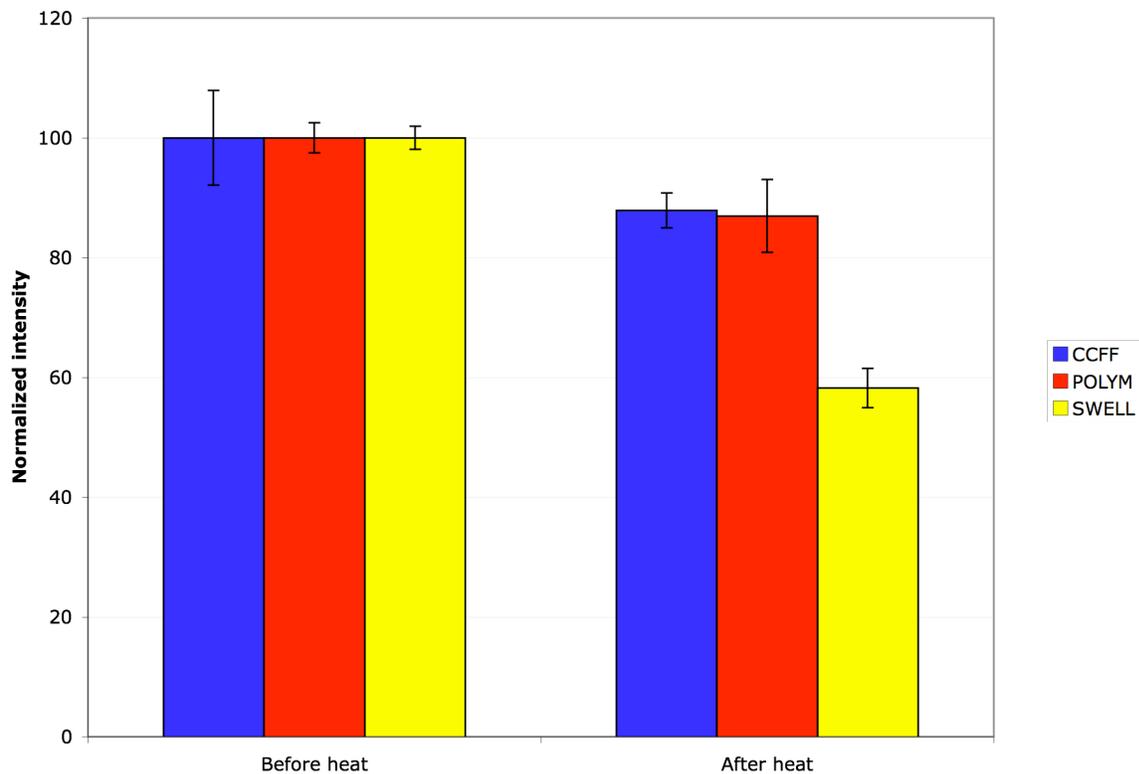


Figure S6. Effect of heat treatment on the emission intensity of barcodes. The barcodes fluorescence after exposure to a temperature of 95°C for 15 minutes was compared. The SWELL barcodes shows a fluorescence decrease of about 42% whereas POLYM and CCFF only lead to a decrease of about 12%.

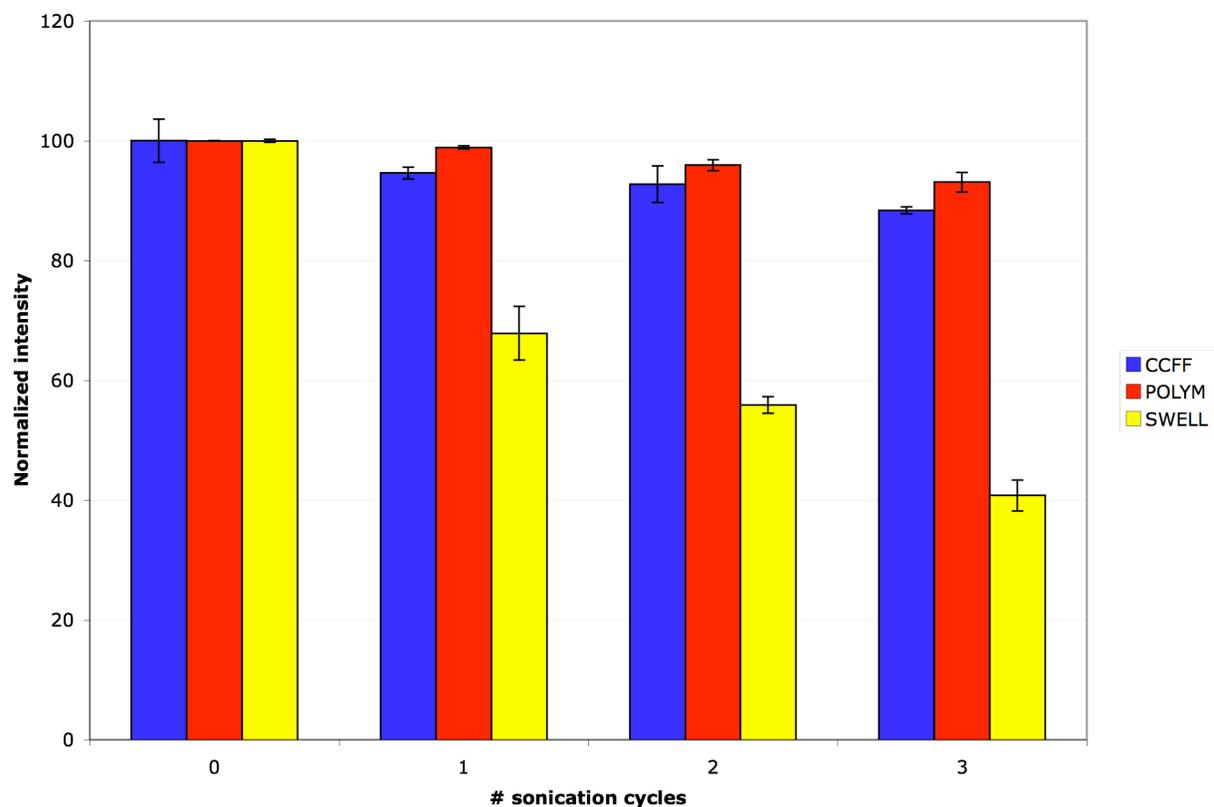
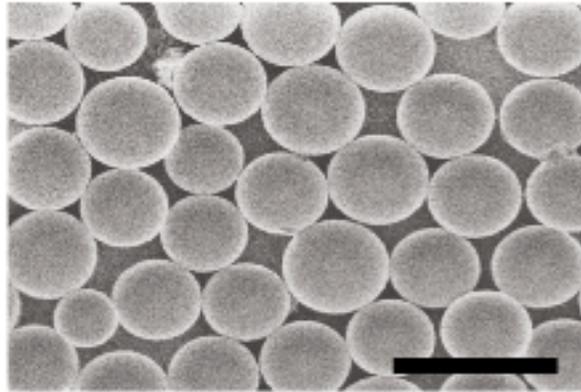
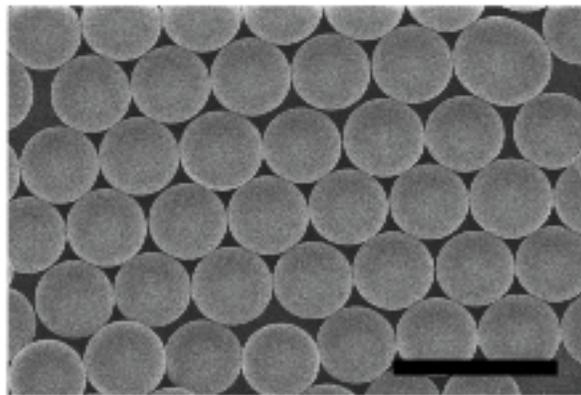


Figure S7. Assessing QD-leakage from the microbeads. Identical barcodes prepared with the 3 different techniques were subjected to multiple sonication periods followed by centrifugation and supernatant removal and the intensity of the barcode was measured after each cycle. After 3 complete cycles, the intensity of both CCFF and POLYM barcodes is still retained (over 90% of their original value) whereas SWELL ones already lost 33% after the 1st cycle and are down to a 60% loss after the 3rd cycle.

CCFF



SWELL



POLYM

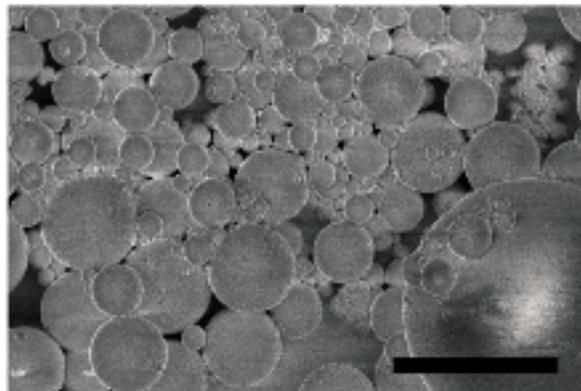


Figure S8. SEM micrographs of QD barcodes. SEM micrographs of microbead barcodes representative of each synthetic technique. Scale bar represents 10 microns.

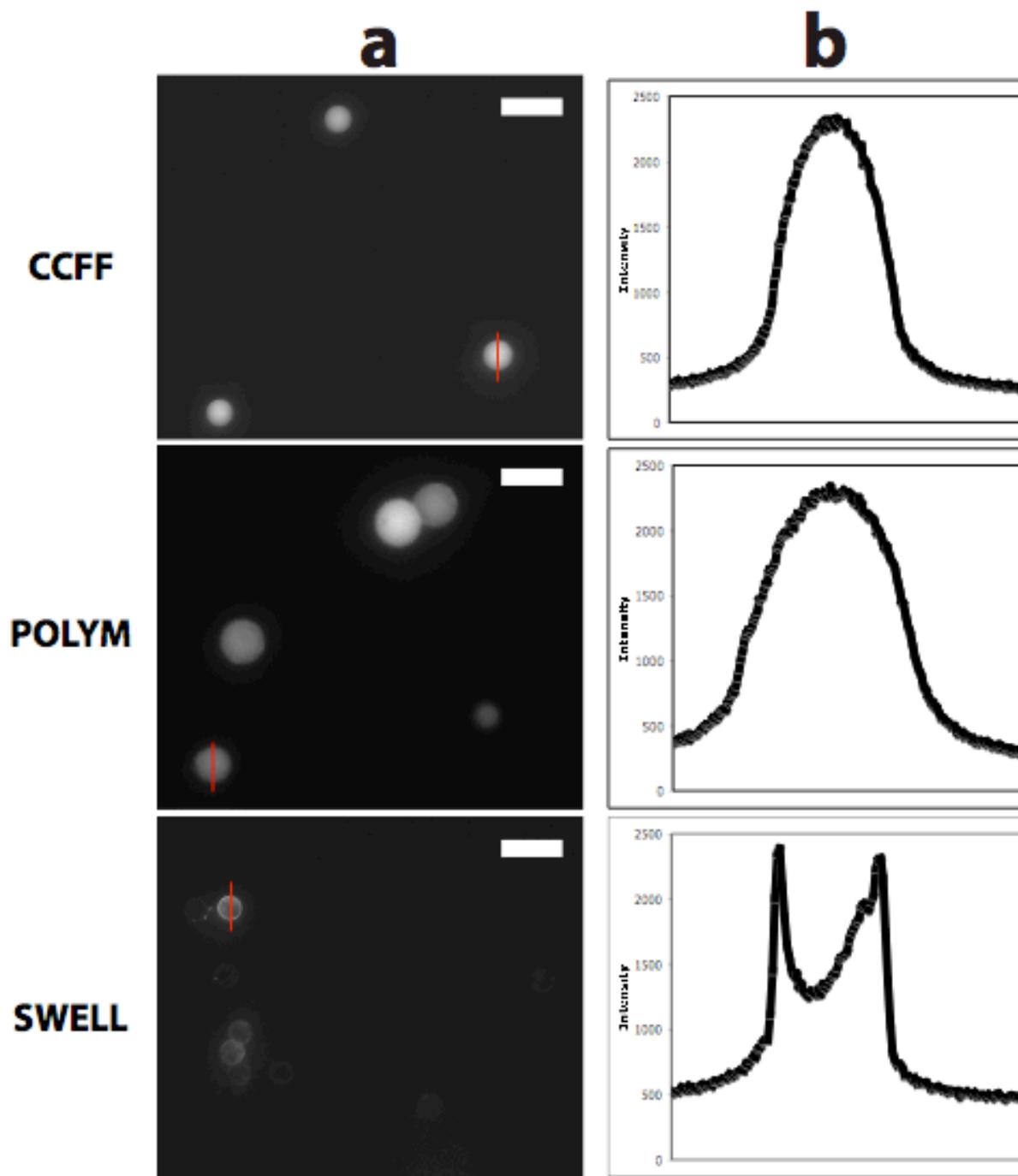


Figure S9. Fluorescence images and respective intensity profiles of barcodes. a) Fluorescence images of barcodes were taken using a 60x oil immersion objective (NA=1.3). b) Respective intensity profiles for each type of bead along the red line in a). It can be seen that CCFF and POLYM barcodes both have a homogeneous distribution of QDs inside the entire microbead volume whereas SWELL barcodes have their QDs mostly concentrated on the outer region of the microbead. Scale bar represents 10 microns.

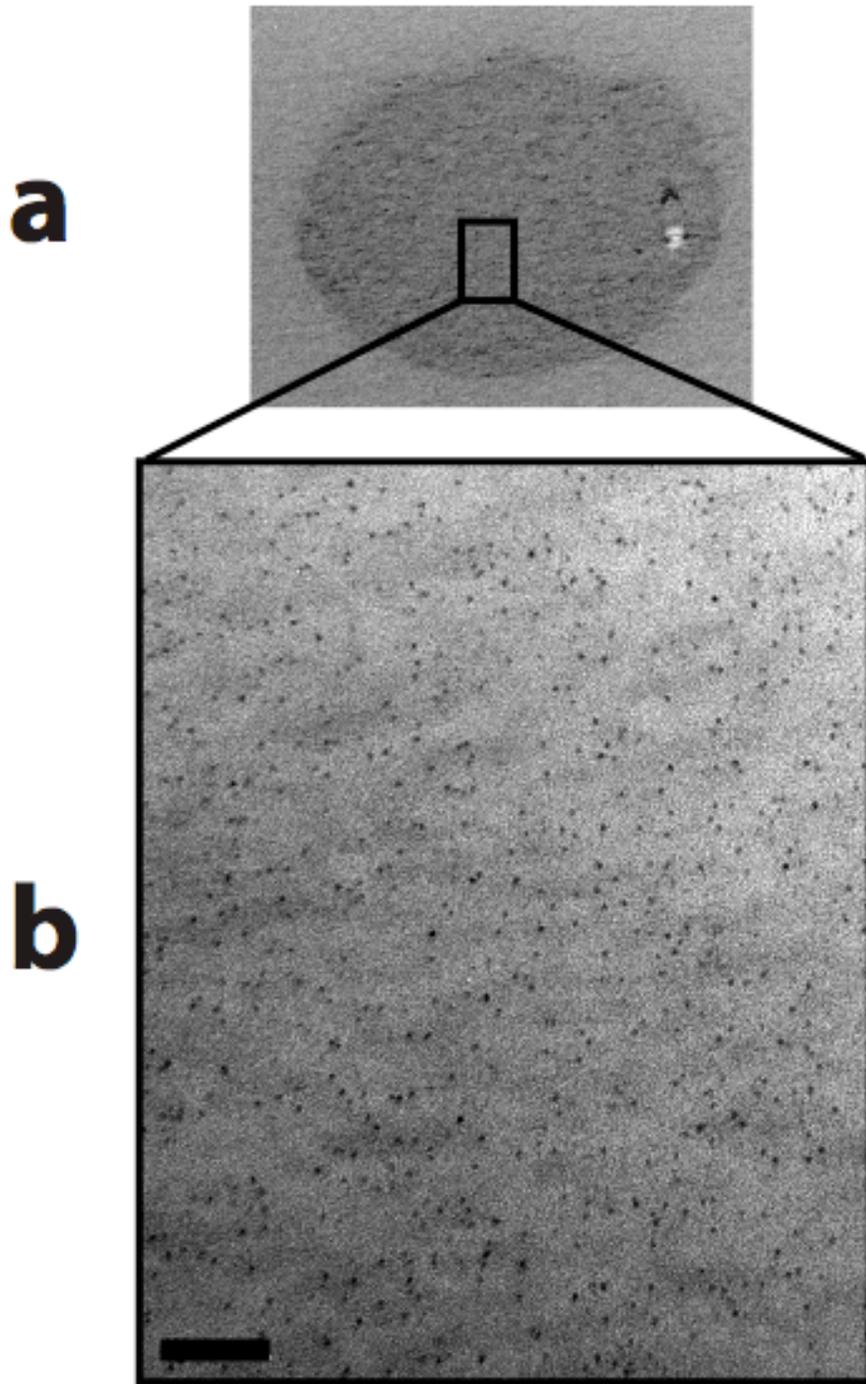


Figure S10. TEM cross-section of CCFF produced barcodes. a) TEM micrograph of a 100 nm thick cross-section of a typical 5 μm diameter CCFF microbead barcode containing both 630 and 580 nm QDs. **b)** 60x TEM micrograph of the same microbead revealing the presence of homogeneously dispersed QDs on the entire cross-section. Scale bar represents 100 nm.

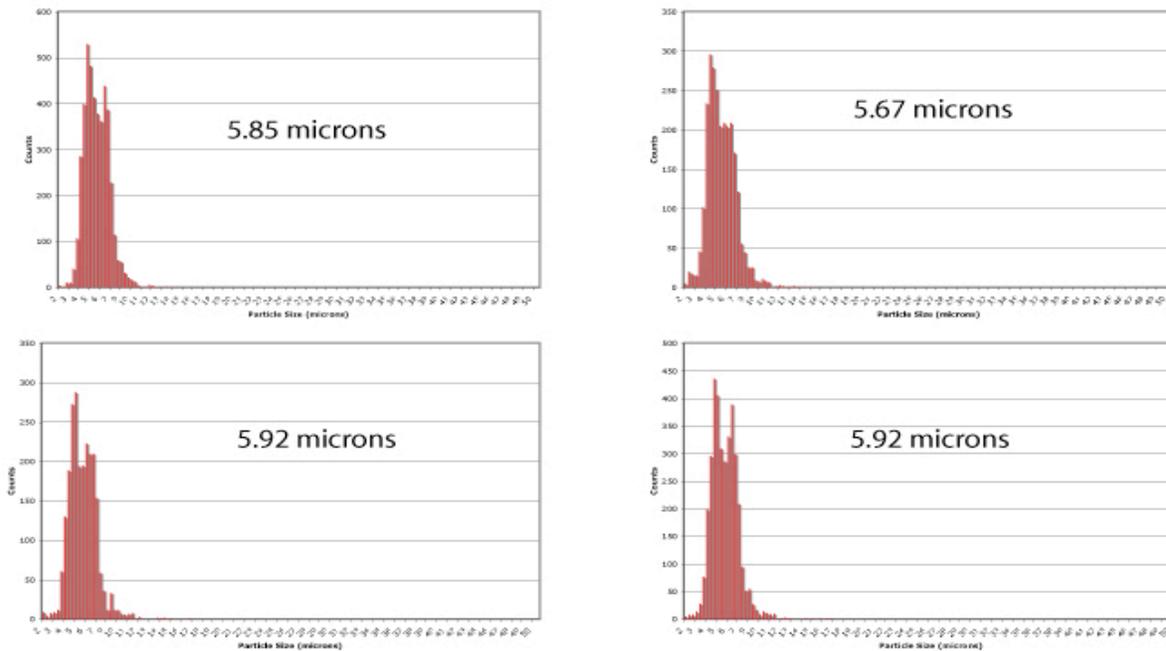


Figure S11. Batch-to-batch barcode distribution. Barcode distribution of 4 samples made using identical synthetic conditions obtained using a Vi-Cell XR instrument (Beckman Coulter). The batch-to-batch average is $5.84 \pm 0.12 \mu\text{m}$.

Section 3: Supplementary movie

Movie S1. Movie demonstrating the production of CCFF barcodes in real time under UV light. The white edges represent the walls of the container used during the demonstration. A graphic representation of the nozzle has been superimposed onto the movie allowing the viewer to see the nozzle that would be otherwise hard to see under the dark conditions of the experiment.

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