Bar-coded hydrogel microparticles for protein detection: synthesis, assay and scanning

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This protocol describes the core methodology for the fabrication of bar-coded hydrogel microparticles, the capture and labeling of protein targets and the rapid microfluidic scanning of particles for multiplexed detection. Multifunctional hydrogel particles made from poly(ethylene glycol) serve as a sensitive, nonfouling and bio-inert suspension array for the multiplexed measurement of proteins. Each particle type bears a distinctive graphical code consisting of unpolymerized holes in the wafer structure of the microparticle; this code serves to identify the antibody probe covalently incorporated throughout a separate probe region of the particle. The protocol for protein detection can be separated into three steps: (i) synthesis of particles via microfluidic flow lithography at a rate of 16,000 particles per hour; (ii) a 3–4-h assay in which protein targets are captured and labeled within particles using an antibody sandwich technique; and (iii) a flow scanning procedure to detect bar codes and quantify corresponding targets at rates of 25 particles per s. By using the techniques described, single- or multiple-probe particles can be reproducibly synthesized and used in customizable multiplexed panels to measure protein targets over a three-log range and at concentrations as low as 1 pg ml^{-1}.

INTRODUCTION

Monitoring individual protein markers or panels of targets provides valuable information for diagnosis of illness, monitoring of disease states and detection of contaminants[1–5]. Unlike genetic profiling, protein measurements offer insight into the active processes that determine phenotype and the mechanisms by which drug treatments achieve success. It is highly likely that focused panels of protein biomarkers will soon provide the earliest warnings of disease, even enabling a diagnosis before a person develops symptoms[6].

Although the genomic revolution has led to the development of a wide variety of effective tools for nucleic acid analysis, similar technologies for protein detection have progressed much more slowly because of the physical and chemical heterogeneity of the targets. Novel high-throughput platforms for the sensitive and multiplexed quantification of medically relevant proteins in complex biological samples are needed to narrow the large gap that currently separates academic discussions of proteomic analysis from the realities of the clinical setting[7,8]. The enzyme-linked immunosorbent assay (ELISA) is the most widely used technique for precise protein measurement. Developed in the early 1970s, the ELISA leverages immunology to detect a wide range of targets[9]. In common 'sandwich' applications, paired antibodies are used, one to capture or 'pull-down' the target and a second to report the capture event through fluorescent, chemiluminescent, colorimetric or radiation signaling. Although it remains a central method for protein quantification, the standard ELISA cannot meet the stringent demands of emerging applications in the biomedical field, as it is not easily adapted to multiplexed or high-throughput biomolecule analysis. In recent years, the antibody-based sandwich approach of the ELISA has been successfully adapted to a number of other detection schemes, thereby enhancing throughput and multiplexing capacity to levels suitable for use in a clinical setting[7,8,10].

Multiplexed protein measurement

Two primary classes of technologies currently exist to measure protein panels in a single assay: planar arrays and particle-based arrays[11]. Planar arrays (also referred to as microarrays) use fixed panels of hundreds to thousands of different probes spatially segregated on a slide for a single assay. Although this allows for massively multiplexed measurement, it provides limited flexibility for probe-set modification, it consumes large quantities of reagents and its array read-off is time consuming and requires expensive, specialized equipment[12,13]. Particle-based detection platforms offer multiple advantages over conventional planar arrays as they feature improved scalability, shorter assay times and the ability to rapidly change probe sets to meet evolving assay demands. Particle-based systems are particularly well-suited for the efficient quantification of focused panels of biomolecules; rather than consuming large quantities of valuable reagents and samples for the simultaneous measurement of a fixed set of hundreds or even thousands of targets, encoded particles for single or small groups of targets can be quickly combined for tailored assays, addressing only the targets of interest in an economical manner.

The bar-coded hydrogel approach

The bar-coded hydrogel technology demonstrated here leverages the flexibility of particle-based arrays while also allowing the immobilization of one or more probes within the hydrated, 3D binding environment of a single gel particle[14,15]. The permeable poly(ethylene glycol) (PEG) hydrogel particles are composed of spatially segregated regions containing a graphical barcode (consisting of unpolymerized holes in the wafer structure of the gel particle) and one or more separate probe strips for multiplexed quantification of targets. Hydrogel particles allow the bulk immobilization of capture probes, thereby providing enhanced binding capacity over surface-functionalization techniques. The flexibility of the hydrogel matrix reduces steric constraints on binding and has been shown to enhance binding kinetics when adapted to a planar array format[16–18].

Particles are synthesized with stop-flow lithography (SFL), a method that uses co-flowing streams of UV-curable monomer solutions and automated control of UV exposure to rapidly synthesize, encode and functionalize particles at a typical rate of

16,000 particles per h with microscope-based illumination in a single channel (**Supplementary Movie 1**)[19]. This robust manufacturing strategy produces particles with highly uniform encoding and detection properties, a crucial feature in the development of versatile, high-performance bioassay tools. It should be noted that SFL throughput can be greatly augmented to rates up to 10^6 particles per h through the use of hydrodynamic focusing lithography, parallel exposure of multiple synthesis channels and alternative illumination setups that use freestanding optics[20–22]. The microfluidic SFL process is efficient and cost effective, as it consumes only small amounts of valuable reagents such as biological probes and fluorescent dyes. Briefly, the monomer used for synthesis is composed of different ratios of PEG diacrylate (PEG-DA), PEG porogen, UV photoinitiator, Tris-EDTA (TE) buffer and the relevant acrylate-modified reactive species (fluorophore for the code region, biological capture entity for the probe region, none for the blank region). These solutions are then introduced into a polydimethylsiloxane (PDMS) microfluidic device where a laminar co-flow is established. Flow is momentarily halted, at which point a brief UV exposure of the streams through a negative mask of the bar-coded particle shape causes selective polymerization within the streams. The flow is then restarted, removing the polymerized particles from the device into a collection tube and re-establishing the laminar co-flow of streams for the next synthesis event. Adhesion of the particles to the top and bottom faces of the device is prevented by the permeation of oxygen at the fluid-device interface that locally inhibits polymerization and thus provides a lubricating liquid layer above and below the particles.

Comparison with existing particle-based arrays

Particle-based arrays, such as the commercially available Luminex system, provide rapid protein detection through enhanced mass transfer during incubation steps, as well as the use of flow cytometers for post-assay particle analysis. Rather than graphically encoding a particle, the Luminex system uses a set of dyes to spectrally identify beads and the probes they bear on their surfaces. The latest version offers about 500 unique codes, but it can encounter spectral overlap issues that limit choices for detection fluorophores. The graphical particle-encoding scheme described here is based on a series of unpolymerized holes of distinct sizes that can be used as coding 'bits', having values of 0, 1, 2 or 3. With five bits (one of which is used to provide information on the orientation of the particle during analysis), 192 unique codes are possible. Through the addition of more code bits and the use of multiple fluorescent intensities in the code region, the pool of unique codes can easily be expanded to over 10^5 without affecting target detection performance. This high coding capacity offers advantages even when interrogating small panels of targets, as it enables the pooling of particles from many different assays for a single, efficient scanning-analysis step. For such pooling, the code would serve to identify the embedded probe(s) as well as the sample with which the particle was incubated.

Although the Luminex system is the most mature particle-based array available for multiplexed protein analysis, several emerging encoded-particle technologies are being developed to surpass Luminex in one or more metrics of performance. To date, most of the effort by other groups has focused on expanding the number of available codes. However, many of the new encoded particles are fabricated from standard photoresist materials, such as SU-8, that foul easily and are not well adapted to bioassays, resulting in poor sensitivity and large coefficients of variation (CVs) for target-binding signal[23,24]. High degrees of signal variability are also observed on bar-coded chips because of limitations and challenges in the manufacturing process[25]. A recently developed class of metallic bar-coded rods has shown promise as a novel bioassay platform, but few protein immunoassays have been developed so far for this system, and its demonstrated limit of detection (LOD) is roughly two orders of magnitude higher than that of ELISA[26].

In addition to offering greater coding options, the hydrogel particle–based platform is a more versatile tool for biomolecule analysis than existing particle systems because of the bio-inert, nonfouling and customizable properties of the PEG gel matrix[27,28]. Most notably, the polymer is easy to work with: it is water soluble, and particles can be simultaneously synthesized, encoded and functionalized with only a single UV illumination step. Biological entities can be mixed directly into the PEG precursor solutions, allowing 3D covalent incorporation into the resulting network. This is in sharp contrast to the surface functionalization of current particle-based systems and planar arrays. The porous, 3D gel matrix has been shown to improve the reaction/capture kinetics in nucleic acid systems[29,30], and the polymer offers a distinct advantage over the rigid attachment used in existing planar and particle arrays by providing a flexible presentation of the capture antibody, reducing steric limitations and enhancing the loading capacity of capture agents. Improved loading capacity potentially extends the upper LOD over surface-binding techniques that are easily saturated. In addition, the bio-inert nature of PEG allows samples with other biomolecule contaminants, such as serum, to be assayed without the addition of blocking proteins such as bovine serum albumin[31].

Bar-coded hydrogel microparticles offer a range of tunable parameters that can be used to optimize assay performance[32,33]. Pore size, for example, can easily be modulated by varying the relative amounts of the cross-linking agent and porogen in the precursor monomer solution. Control of this parameter can be used to tune the rate of target capture, as well as to exclude high-molecular-weight background proteins found in complex biological media such as serum from interacting with embedded probes. The ability to synthesize geometrically complex gel particles with multiple distinct adjacent chemistries provides a valuable degree of flexibility in assay design. The ease with which alternative capture molecules can be incorporated allows for the detection of DNA, RNA and proteins; it also allows the ability to provide adhesion to cells[34]. Depending on the desired application, additional chemical and physical properties that facilitate assay processing can be incorporated into the hydrogel particles. For example, magnetically addressable regions can be added through physical entrapment of magnetic beads within a desired portion of the gel particle, thus enabling alternative options for filtering, separation and manipulation[35,36]. More complex particles with multidimensional anisotropy can also be created with advanced microfluidic designs to provide a construct for size-based target restriction and filtering within the particles themselves[20,37–39].

A major shortcoming of next-generation particle-based arrays is the lack of companion technologies to rapidly decode particles and quantify target binding in a high-throughput manner suitable for applications in research and clinical settings[26,40,41]. Even the current flow-through analysis approach used with commercially available Luminex beads is suboptimal, as it limits assay flexibility by constraining the multiplexing capacity and offering only simple intensity measurements for gate-based analysis. To expand the utility of our bar-coded hydrogel particle system, we

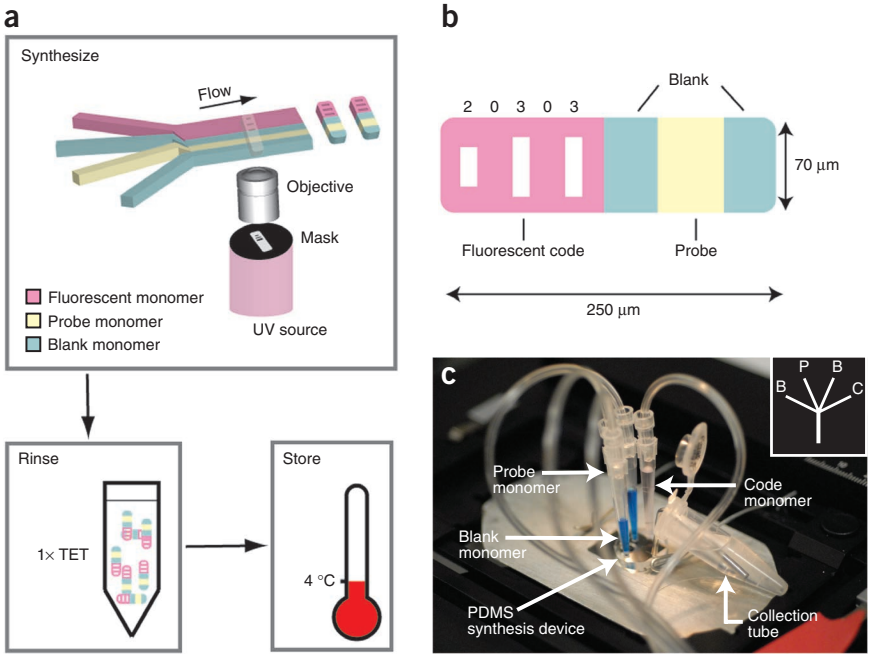


Figure 1 | Synthesis device and particle design. (**a**) Schematic diagram of the synthesis of single-probe particles. Stop-flow cycles are synchronized with pulses of UV illumination to simultaneously synthesize, encode and functionalize. Particles are collected and rinsed before storage at 4 °C. (**b**) A bar-coded particle with code 20303. The thickness of the particle is defined by the height of the microfluidic channel used for synthesis, usually about 37.5 μm. (**c**) A photograph of the synthesis device mounted on the microscope. Blue food coloring allows easy identification of the blank monomers and precise adjustment of stream widths during synthesis. B, blank; P, probe; C, code.

have developed and demonstrated the versatility of a companion microfluidic flow scanner that is capable of accurately decoding particles and quantifying the amount of bound target at rates up to 25 particles per s. A multistage flow-focusing motif is combined with abrupt contraction points to coerce the soft-gel microparticles into a single-file procession traveling at velocities up to 0.5 m s^{-1} in the center of a PDMS device. Particle and device design have been optimized to ensure the precise orientation and positioning of particles without deformation so that laser-induced fluorescence from the graphical code and the probe strip can be captured and recorded by a microscope-based slit-scan scheme. Temporal fluorescence signatures can then be analyzed with automated algorithms to extract target amounts for each probe type. This scanning system represents a key step toward developing a practical quantification platform because it allows for the rapid collection of data from large groups of samples. Moreover, the ability to spatially segregate different chemistries on a single particle, when combined with the graphical code scheme, enables the implementation of a relatively simple and inexpensive 'single-color', morphology-based scanning strategy that requires only one excitation laser and one photomultiplier tube (PMT) detector. This is in contrast to the bulky arrays of excitation and detection instruments required for the Luminex system and other cytometric strategies that use optical encoding[42,43]. The particle platform described in this protocol is open source, in the sense that assays based on this platform can be run on a wide variety of common laboratory instrumentation, from microarray scanners to microscopes. Because of the reduced number of colors necessary for encoding, our system can be used on inexpensive readers.

This protocol describes a system for creating and analyzing bar-coded particles with a single probe strip for the capture and quantification of an individual protein target. With minimal alteration to the synthesis steps through the incorporation of additional target streams, a multiprobe particle can also be created for intraparticle multiplexing. Each particle will have a unique graphical bar code specific to the probe strip (or strips) it contains, allowing for automated readout and target identification using a microfluidic scanning system. Multiple particle types can be combined in a single assay to measure a panel of different proteins at one time. Furthermore, by replacing capture antibodies in the probe strips with nucleic acid constructs, a variety of genetic targets can be detected with high sensitivity and specificity[19,44].

Experimental design

The production and use of bar-coded hydrogel particles require instrument construction, microfluidic design and assay development. The instrumentation essential to the synthesis of hydrogel particles using SFL is described explicitly in Dendukuri *et al.*[45], the equipment for pressure control of streams is examined in Bong *et al.*[46], and the scanning system (built on the same microscope used for synthesis) is discussed in detail in two references from Chapin *et al.*[44,47] The protein assay itself is composed of three primary parts: synthesis of bar-coded particles, capture and labeling of protein targets on the particles and final read-off of bar codes and quantification of bound protein targets.

Microparticle design considerations and synthesis. While the particles described here conform to a standard design, it is certainly possible to modify particle porosity, rigidity, layout and coding capacity to suit various assay needs. However, it is important to understand the design principles that led to our standard particles so that variations in synthesis do not lead to mechanical instability, reduced sensitivity or increases in background signal.

The most important consideration in particle synthesis is pre-polymer composition, as this directly controls the porosity, flexibility and loading capacity of both probe and fluorescent coding dye. In general, increasing the relative amount of cross-linking agent (PEG-DA) and decreasing the relative amount of porogen (PEG) leads to gel particles with lower porosity, greater rigidity and higher probe/dye incorporation. We have optimized our pre-polymers such that probe regions are more porous than code and blank regions, thereby allowing the efficient penetration of target and labeling reagents during assay. The rigidity imparted by the code and blank regions has a crucial role in preventing deformation of particles and their coding holes during assay manipulation and high-speed scanning. It should also be noted that different pre-polymer compositions can lead to different swelling properties, depending on the buffer used.

SFL enables the creation of multifunctional particles with different adjacent chemistries (**Fig. 1** and **Supplementary Movie 1**). Careful

design of particle layout is required to ensure that captured target amount and probe identity can be determined rapidly and accurately with single-color fluorescence-based flow-through analysis. If it is placed too closely to the rhodamine-doped code region, the probe strip can produce a fluorescent signal that will interfere with the last bit of the code signature, particularly when the corresponding target molecule is present in large amounts. Similar bleed-over from the code can drown out low signal levels on a proximally located probe strip. To obtain reliable code reads and highly sensitive target detection, we have inserted a 30-μm blank region to separate the code and probe (**Fig. 1b**). The size of this blank spacer is dictated by the dynamic response properties of the detector, the level of rhodamine used in the code and the size of the slit used to generate the excitation beam in the scanning channel. A similar challenge arises when multiple probe strips are incorporated into the same particle and then scanned at high velocity. In past work, reliable scans have been achieved with such particles by adding similar spacers between probes[5]. It should be noted that the use of different reporting colors for adjacent regions and/or deconvolution methods could be used in future iterations of the system to eliminate the need for spacers and make more efficient use of particle layout. Finally, it has proven useful to incorporate a blank spacer at the end of the particle to ensure symmetrical exposure of probe to carrier solutions. If not used, the portion of the probe at the end of the particle will typically register a higher target signal than the portion of the probe joined to the rest of the gel; the resulting asymmetry is geometry dependent and can complicate signal analysis and quantification.

During the synthesis procedure, it is important to take certain steps to ensure uniform particle production and successful antibody incorporation. The alignment of the synthesis channel with the particle photomask and the adjustment of synthesis stream widths are performed manually at the microscope with an ×20 objective and a charge-coupled device (CCD) camera. For the creation of multiple batches of particles for a multiplex study, we recommend creating a transparency that marks off the desired dimensions of each chemical strip on the particle. To provide a greater degree of reproducibility over the course of multiple synthesis sessions, this can be overlaid on the computer monitor that displays the CCD image of the synthesis channel. If care is not taken in setup, then it is possible for capture performance to differ from batch to batch. Although we have demonstrated relatively low target-signal CVs using this manual alignment procedure, the implementation of a feedback control loop linked to the video feed of synthesis streams and pressure regulation could potentially result in higher-throughput and more uniform synthesis[31,44].

Maintaining the solubility of the capture antibody throughout the synthesis process can be challenging because of the high concentrations of antibody used. Two-phase flow in the probe stream during synthesis is evidence of separation and results in nonuniform, spotty signal patterns when the particles are used in assays. Four practices were used to eliminate this separation: (i) coupling the antibodies to the heterobifunctional PEG linker in a separate step prior to synthesis; (ii) thorough yet gentle mixing during probe pre-polymer preparation (~1 min of agitation); (iii) centrifugation of the probe pre-polymer for 5 min before synthesis; and (iv) use of only the top half of the centrifuged probe pre-polymer to load the synthesis device.

Alternatives to the PEG-linker conjugation scheme described here are available. For example, incorporating streptavidin into the particle provides a flexible option for the post-synthesis attachment of biotinylated capture antibodies, but it requires alteration of the reporter antibody and the fluorophore because they use the same linker[48]. Direct attachment to amine or carboxyl groups is also possible, but this strategy cannot be used to create multiprobe particles for intrabead multiplexing without the introduction of multiple, distinct reporter colors[49]. The use of DNA-encoded antibody linkers would enable both intrabead multiplexing and the development of a 'generic' particle that could be functionalized at any point after synthesis with a range of different antibodies[50]. Following the covalent incorporation of an acrydite-modified DNA address in each probe region during synthesis, particles could be functionalized via incubation with antibodies conjugated to a DNA sequence that is complementary to the gel-embedded address DNA.

The successful UV polymerization of gel particles within a microchannel requires proper choices of both exposure time and optical focus. The exposure time should be selected such that the fluid-particle interfaces appear sharp and the code holes maintain a rectangular appearance. Insufficient polymerization can lead to nonspecific adsorption of reporter antibody and fluorescent label during assay, whereas overpolymerization can limit the diffusion of molecules within the polymer matrix. The optical focus should be adjusted until synthesized particles show the maximum contrast gradient at fluid-particle interfaces upon increasing the intensity of the microscope's halogen lamp. Improper focus can prevent the coding holes from penetrating all the way through the particle, which can complicate flow-through decoding. During synthesis device construction, a thin PDMS layer is applied to the supporting glass slide to allow oxygen permeation for the prevention of particle adhesion. It should be noted that this layer does not substantially affect the fidelity of pattern transfer; under optimal focus conditions, microparticles with pattern features as small as 3 μm can be reliably created.

Bar-coded particles can be produced far in advance of the capture and read-off steps, as the antibody-containing particles are stable for at least 4 months. Using typical synthesis conditions, 1 h of semi-continuous SFL can produce enough particles for more than 300 separate assays. When protein measurement is desired, particles can be drawn from the stock storage tubes (kept at 4 °C) and inserted into the desired chamber for assay, which takes about 3–4 h. The particles can then be immediately scanned in the particle reader for quantification, a process that takes about 3 min per sample.

Sandwich assay procedure and workflow considerations. We made an effort to keep the assay workflow similar to that of existing particle-based techniques[51]. The assay involves assembling an antibody sandwich around the target protein by exposing particles to the sample, adding a biotinylated reporter antibody and labeling the bound reporters with a streptavidin-phycoerythrin (SAPE) complex (**Fig. 2**). The use of a 96-well filter plate streamlines this procedure by eliminating time-consuming centrifugation steps during the frequent washes, although the particles described here are mechanically robust enough to withstand reasonable centrifugation and vortexing speeds. Mixing on the 96-well plate has been performed at speeds up to 1,000 r.p.m. without any noticeable deformation or degradation of the particles. The particles tend to settle in most biological buffers over the period of several minutes, and thus particles should be gently vortexed or agitated before extraction from a microcentrifuge tube or well.

If necessary, the reporter antibody and SAPE concentrations can be modulated to reduce background fluorescence on the particles.

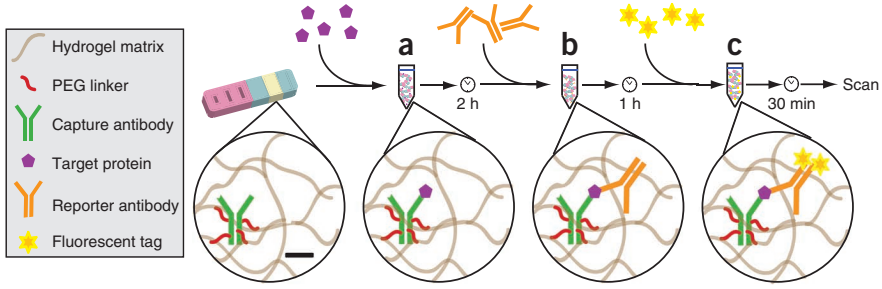


Figure 2 | Schematic of the sandwich assay. (**a**) Particles are combined with the sample, and target proteins are allowed to bind to the capture antibodies. (**b**) Reporter antibody is added and binds to separate epitopes of the target. (**c**) Fluorescent label is added and binds to the reporter antibody. Scale bar in the expanded view of the polymer matrix is 5 nm.

Recent unpublished work (R.L.S.) suggests that the reporter antibody concentrations can be reduced by 50% to 67% from the amounts cited in the PROCEDURE section, thereby improving the signal-to-noise ratio (SNR). Although it is important to supply the reporter antibody in sufficient excess of the expected number of target-probe complexes at equilibrium, beyond a critical concentration there is negligible gain in signal, yet there is a substantial increase in nonspecific binding of the reporter antibody to the PEG scaffold of the particles. This critical concentration depends on the reporter antibody. To determine the optimal concentration, we advise running an assay in which the reporter antibody is titrated down from a starting reaction concentration of 5–1.25 ng μl^{-1}. To satisfy requirements across the desired dynamic range, it is necessary to test each reporter concentration in at least two separate reactions: one in which the target spiked in is at the upper end of the dynamic range, and one in which there is no target spiked in. The former will ensure that there is no appreciable signal loss as a result of the change in reporter antibody concentration, and the latter will provide information about the degree of nonspecific binding in the assay. SAPE concentration can be modulated in a similar way, although we have found that modifying reporter antibody concentration is more effective at reducing nonspecific fluorescence.

The majority of the time required for the sandwich assay is dedicated to target and/or reagent incubation. To increase efficiency, we suggest that the experimenter complete additional required assay tasks during these incubation periods; these tasks include buffer preparation, reagent thawing and equipment setup. For example, as the reporter antibody is binding to the captured targets, the various components of the scanning system should be turned on and aligned using the methods described in the PROCEDURE section.

Intraparticle multiplexed analysis on a multiprobe particle can be achieved with slight modification to the experimental protocol detailed below. To synthesize a multiprobe particle, it is necessary to use a microfluidic device that has additional inlets to accommodate the additional probe monomer streams. The assay can then be carried out in the same manner as that described below for interparticle multiplexing.

Microparticle scanning. The single-color flow-through scanning system described here has previously been used for multiplexed protein and microRNA detection[31,44]. A thin excitation window was created by passing a collimated 100-mW, 532-nm laser through a chrome-coated glass slit mask that is inserted into the field stop of the microscope (**Fig. 3**). It is important not to use a transparency mask for this purpose, as the laser will degrade the mask within minutes of exposure. The width of the excitation beam can be adjusted by using various masks. A wider slit provides a larger excitation region and thus increases signal intensity. This should be balanced by the consideration that a wider slit reduces the spatial resolution of the scan. For the coding scheme, particle layout and optical train we describe, a slit width of 4 µm in the focal plane produced the best combination of sensitivity and resolution. In future iterations of the system, it would be most efficient to use a cylindrical lens to focus the laser beam to a line rather than blocking a majority of the light with a mask.

The PMT in our system was selected to provide high sensitivity at the desired fluorescence emission wavelengths, as well as a sufficient linear response range to cover expected target concentrations. In practice, the detector range covered nearly four orders of magnitude above the observed LOD. Other PMT instruments could expand this range with a minimal effect on sensitivity. The rise time of the PMT is an important factor that can affect scan resolution. With a 4-µm–wide slit, we have observed that a PMT rise time of 1 ns produces scans with excellent feature resolution, even at particle velocities as high as 0.5 m s^{-1}. Similar considerations regarding resolution must

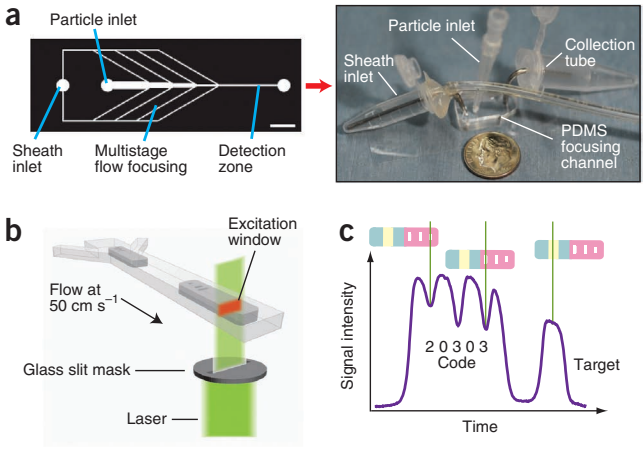


Figure 3 | Scanning device and data analysis. (**a**) Diagram of the microfluidic device used for flow alignment of bar-coded particles, along with a photograph of the device with sheath inlet, particle inlet and collection tube attached. The central channel of the device features a series of abrupt contraction points that serve to focus and align the soft gel microparticles for efficient and accurate analysis. Side streams merge with the central channel at these contraction points and provide impinging flows of a sheath fluid for further focusing. The particle detection zone is 125 µm wide and 2.3 mm long, with the excitation laser and photomultiplier tube (PMT) positioned ~750 µm from the outlet of the channel. Scale bar, 1 mm. (**b**) Schematic of precisely aligned particles passing through the laser excitation window established in the detection zone of the device. A 532-nm laser is used for fluorescent excitation. The rhodamine acrylate incorporated in the code region emits at a wavelength of 580 nm and the SAPE used for labeling of complexes emits at a wavelength of 575 nm. Signal is measured using a PMT (not shown). (**c**) Actual particle scan illustrating the fluorescence signature of a particle with code 20303 as it passes through the line illumination. Total passage time of the particle is ~500 µs.

be made when selecting a method for amplifying and filtering the PMT signal. We constructed a homemade amplifier setup (gain of ~20) that included a low-pass filter with a cutoff frequency of 100 kHz to exclude high-frequency noise while still retaining the lower-frequency signal variations that were crucial for decoding and quantification. To obtain the highest SNR possible, it is also important to eliminate background sources of light by setting up an enclosure around the microscope or by turning off all nearby lights.

Target quantification. Before an unknown sample can be quantified, a calibration curve should be constructed for each target type to be investigated. Optimally, the curve should have multiple points for target concentrations spanning the range of expected values. For example, with the instruments, settings and particles used in Appleyard *et al.*[31] for interleukin (IL)-2, a range between 3 and 800 pg ml^{-1} was used with calibration points at 0, 6, 9, 30, 60 and 800 pg ml^{-1}. A blank sample (one without target added) should be run in order to subtract a background fluorescence intensity from each of the spike-in calibration points. Sample conditions for the calibration curve should closely match those of the anticipated unknown sample. When quantifying the unknown sample, a blank must again be run to allow for data normalization. To ensure that targets fall within the dynamic range of the assay, it may be necessary in some cases to dilute samples or to adjust instrument sensitivity.

MATERIALS

REAGENTS

- Polydimethylsiloxane (PDMS; Dow Corning, Sylgard 184)
- IL-2 capture antibody, lyophilized (R&D Systems, cat. no. MAB602)
- IL-2 target protein, lyophilized (R&D Systems, cat. no. 202-IL)
- IL-2 reporter antibody, lyophilized (R&D Systems, cat. no. BAF202)
- Heterobifunctional 2-kDa poly(ethylene glycol) linker (ACRL-PEG-SCM-2000, Laysan Bio)
- Poly(ethylene glycol) diacrylate, molecular weight (MW) 700 g mol^{-1} (PEG-DA 700; Sigma-Aldrich)
- Poly(ethylene glycol), MW 200 g mol^{-1} (PEG 200; Sigma-Aldrich)
- Poly(ethylene glycol), MW 400 g mol^{-1} (PEG 400; Sigma-Aldrich)
- Darocur 1173 (Sigma-Aldrich)
- Tris-EDTA (1×, 5×), pH 8.0 (TE; USB)
- Rhodamine acrylate (Polysciences)
- Food coloring (Durkee)
- Nuclease-free water (USB)
- Fetal bovine serum (FBS; Sigma-Aldrich, cat. no. F2442)
- Phosphate-buffered saline (PBS; Cellgro)
- Streptavidin-phycoerythrin (SAPE; Invitrogen)
- Tween-20 (Sigma-Aldrich)
- (Tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (fluorosilane, United Chemical Technologies)

EQUIPMENT

- Synthesis microscope: inverted microscope (Zeiss Axio Observer), UV light source (Prior Lumen 200), ×20 0.5 NA objective (Zeiss Plan Neofluar, cat. no. 1004-072)—the objective needs maximal UV transmission between 350 and 360 nm, where the photoinitiator absorbs and the lumen excites, UV filter set (cat. no. 11000v3, Chroma), CCD camera (Andor Clara; **Fig. 4**)
- Scanning microscope: inverted microscope (Zeiss Axio Observer), 100-mW, 532-nm laser (Dragon Lasers), slit mask (Advance Reproductions), ×20 0.5 NA objective (Zeiss Plan Neofluar), SAPE dichroic set (cat. no. XF101-2, Omega), PMT (Hamamatsu, cat. no. H7422-40; **Fig. 4**)
- Computer (Lenovo), data acquisition board and software (National Instruments, cat. no. USB-6251)
- Pressure system: building supply air regulator (150 psi range, Dayton), tubing (1/4-in inner diameter, 3/8-in outer diameter, VWR), solenoid valve (Model 6014, Burkert), T & L junctions (size 8, VWR), needle valve (1/8-in, Swagelock), Luer syringe connector (Becton Dickinson). See Bong *et al.*[8] for expanded information.
- Plate shaker (VWR)
- Vortex (VWR)
- Centrifuge (VWR)
- Microscope cover slips (22 × 60 mm, VWR)
- Microcentrifuge tubes (1.5 ml and 0.6 ml, VWR)
- Petri dish, 6-in (BD Falcon)
- Aluminum foil
- Filters (0.2 µm; cat. no. 4652, Pall Life Sciences)
- Scalpel
- Luer stub adapter (15 and 18 gauge, Becton Dickinson)
- Filter plate (cat. no. MSBVN1210, Millipore)
- Vacuum manifold for filter plate (cat. no. 16003-836, VWR)
- Pipette tips (10 and 200 µl, Molecular BioProducts)
- Tygon tubing (S-50-HL 1/32-in inner diameter, 3/32-in outer diameter, VWR)
- Metal tubing
- Sonicator (VWR)
- Vacuum chamber (VWR)
- Wafers and masks (see EQUIPMENT SETUP)

REAGENT SETUP

PEG-DA pre-polymer mixture (20%, vol/vol) Combine 20% (vol/vol) PEG-DA 700, 40% PEG 200, 5% Darocur 1173 and 35% 3× TE. ▲ **CRITICAL** PEG and Darocur stocks are viscous, and careful pipetting is required to obtain accurate quantities. ▲ **CRITICAL** This and other pre-polymer mixtures must be used within 2 weeks of preparation.

PEG-DA pre-polymer mixture (35%, vol/vol) Combine 35% (vol/vol) PEG-DA 700, 20% PEG 200, 5% Darocur 1173 and 40% 3× TE.

Code pre-polymer mixture Mix a 1:9 (vol/vol) ratio of rhodamine acrylate at 0.06 mg ml^{-1} in 1× TE with 35% pre-polymer mixture.

Blank pre-polymer mixture Mix a 1:9 (vol/vol) ratio of 20% (vol/vol) food coloring in 1× TE with 35% pre-polymer mixture.

TET (1×) Combine 1× TE with 0.05% (vol/vol) Tween-20; filter through a 0.2-µm filter. Use within 2 months of preparation. ▲ **CRITICAL** Filtering will help prevent clogs and reduce background noise.

PBST (1×) Combine PBS with 0.05% (vol/vol) Tween-20; filter through a 0.2-µm filter. Use within 2 months of preparation.

FBST (1×) Combine FBS with 0.05% (vol/vol) Tween-20. Use within 2 months of preparation.

Reporter antibody mixture Mix 1× PBST with IL-2 reporter antibody at 5 ng $µl^{-1}$. Freshly prepare before use.

SAPE mixture Mix streptavidin-phycoerythrin diluted in 1× PBST to 4 ng $µl^{-1}$. Freshly prepare before use.

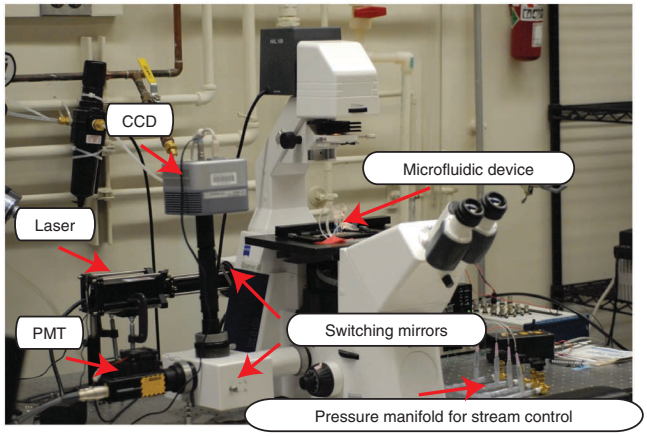


Figure 4 | Microscope setup for synthesis and scanning. Two separate switching mirrors allow for selection of the illumination source (laser for scanning, UV for synthesis) and detection system (PMT for scanning, CCD for synthesis and alignment).

PTET Combine 5× TE with 25% (vol/vol) PEG 400 and 0.05% (vol/vol) Tween-20; filter through a 0.2-μm filter. Use within 2 months of preparation.

EQUIPMENT SETUP

Master wafer Devices are formed with standard soft-lithography molding from a 4-inch-diameter master wafer designed using AutoCAD (see **Supplementary Methods** for a design containing the channels used in this article) and ordered from a vendor such as the Stanford Microfluidics Foundry (http://www.stanford.edu/group/foundry). Alternatively, for the simple single-layer devices used here, suitable master wafers can be fabricated in a clean room after only about 1 week of training.

Particle masks Use AutoCAD software to design negative field-stop masks for the bar-coded particles. Owing to size reduction by the objective, particle features on the mask should be appropriately scaled to produce the desired feature size in the synthesis plane. The area in the synthesis plane receiving uniform UV illumination (~700 μm by 700 μm for a Prior Lumen 200 source and the optical train described earlier) should be used to guide mask design/layout. To increase throughput, duplicate particles on a single mask are recommended; see **Supplementary Methods** for example masks. Masks can be ordered from FineLine Imaging (http://www.thinmetalparts.com/fineline) and can be reused indefinitely.

PROCEDURE

Fabrication of devices for synthesis and scanning ● TIMING 1 week

1| Silanize the master wafer to promote easy release of PDMS by taping the wafer to the bottom of an open Petri dish and leaving it in a vacuum chamber along with an open vial containing 50 μl of fluorosilane. Allow it to sit for 1 h and then remove it from the chamber.

! CAUTION Fluorosilane is toxic and should be handled and discarded appropriately.

2| For the first use of the wafer, mix 6.5 g of curing agent with 65.0 g PDMS base for 30 s and allow the mixture to sit for 45 min to remove bubbles. Pour PDMS over the wafer to a height of ~7 mm. Cure the PDMS overnight in an oven at 65 °C.

▲ CRITICAL STEP Bubbles in the PDMS can hamper performance by attaching to features on the wafer. An additional vacuum step before curing can help remove these if present.

3| Use a scalpel to remove the PDMS that lies on top of the wafer but leave the PDMS that covers the peripheral area of the Petri dish.

4| For future molds, a mixture of 3.5 g of curing agent and 35.0 g of base will be sufficient to cover the exposed 4-inch wafer.

5| Cut out the individual devices from the excised PDMS, leaving at least a 7-mm edge around the channel features.

6| Punch inlet and outlet holes using an 18-gauge Luer stub adapter. Punch through from the channel side of the PDMS to give a clean interface.

7| Clean off the extracted and punched channels by sonicating in ethanol for 5 min, rinsing with ethanol, rinsing with water and drying with argon.

! CAUTION Handle the scalpel and Luer stub carefully to prevent injury.

8| Mix 1.0 g curing agent with 10.0 g base for 30 s and allow the mixture to sit for 45 min to remove bubbles.

9| Prepare a cover slip with a thin layer of PDMS by placing about 50 μl of the degassed mixture on a cover slip.

10| Sandwich the PDMS between a second cover slip and allow the polymer to spread. Shear the cover slips apart repeatedly to obtain an even distribution of the PDMS (1–2 mm thick).

11| Cure the cover slips for 20–25 min at 65 °C until the polymer is highly viscous but not yet fully set.

▲ CRITICAL STEP If the PDMS is not sufficiently cured, it will flow into and obstruct the microfluidic channels. If it is cured for too long, the device will not fully seal to the cover slip and, consequently, leaks may occur during synthesis.

12| Place a clean synthesis or scanning device on the PDMS layer of the cover slip, channel side down. Cure the completed devices overnight at 65 °C.

▲ CRITICAL STEP Ensure that the device seals along all of the channel edges. Gentle pressure can be used to improve sealing and remove trapped air pockets.

■ PAUSE POINT Completed devices can be stored for months before use. Scanning channels can be reused indefinitely and synthesis channels can be used up to ten times.

13| Inspect the channels on a microscope for obvious clogs, leaks or contamination after construction or use. This helps to prevent unexpected experimental loss.

Construction of microfluidic attachments ● TIMING 2–3 h

14| Construct loading tips by cutting a 10-µl pipette tip about 7 mm from the wide end and inserting it into a 200-µl tip that has been cut at the same point. Insert the end of a ~70-cm piece of Tygon tubing into the wide end of the tip assembly and attach an 18-gauge Luer stub adapter to the other end of the tube[46].

▲ **CRITICAL STEP** A tight fit between tip and tubing is essential to prevent leakage during synthesis or scanning.

15| Build a synthesis and scanning collection reservoir for gathering particles by removing the cap from a 1.5-ml microcentrifuge tube and punching two holes inside the sealing ring of the cap with a 15-gauge Luer stub. Cut a 3-cm length of metal tubing and bend it in the middle to form a 70° angle. Insert the metal tubing into one of the cap holes and attach the assembly to a new 1.5-ml microcentrifuge tube. See **Figure 3** for a constructed collection reservoir.

16| Assemble a sheath reservoir for scanning by punching two holes in the removed cap of a 0.6-ml microcentrifuge tube with a 15-gauge Luer stub. Again, cut a 3-cm length of metal tubing and bend it in the middle to form a 70° angle. Insert the metal tubing into one of the holes and put one end of a ~70-cm length of Tygon tubing into the other. Attach an 18-gauge Luer stub to the other end of the Tygon tubing. Seal the connections with a generous application of PDMS to the outside of the cap. Cure the PDMS at 65 °C for 45 min and then attach a clean 0.6-ml microcentrifuge tube. See **Figure 3a** for the completed sheath reservoir.

17| Before use, rinse the loading tips (separated from the Tygon tubing), collection cap and sheath reservoir cap with ethanol and then deionized water. Dry all with a stream of argon. Reattach the Tygon tubing to the loading tips. The tips and caps can be reused indefinitely.

Hydrogel synthesis ● TIMING 4–5 h

18| Functionalize the capture antibodies by combining 25 µg μl^{-1} antibody in PBS (1×) with 50 µg μl^{-1} heterobifunctional PEG linker at a 4:1 (vol/vol) ratio. Place on a shaker running at 1,000 r.p.m. at room temperature (25 °C) and incubate for 3 h.

▲ **CRITICAL STEP** The PEG linker should be combined with the antibody as quickly as possible to avoid loss of functionality of the reactive ester in aqueous solution.

19| Prepare a synthesis device for use by sonicating in ethanol for 5 min, then rinsing thoroughly with ethanol, then with water; dry with argon. Clean four loading tips and a collection tube with ethanol, water and argon. Attach tubing connectors to the four tips.

▲ **CRITICAL STEP** Loading tips must be dry; otherwise, separation or contamination of the pre-polymer mixture may occur during synthesis.

20| Select a mask and load it into the field stop of the microscope. Place ~12.5 µl of blank pre-polymer on a cover slip and load it onto the microscope. Visualize the sample through a CCD camera and perform a 75-ms UV exposure to determine the orientation of the mask. Mark the polymerized particle edges on the computer monitor for channel alignment purposes.

! CAUTION UV light is dangerous, and thus exposure of eyes or skin should be avoided.

21| Centrifuge the code, blank and 20% pre-polymer mixtures at 2,000*g* for 5 min at room temperature.

▲ **CRITICAL STEP** Minimize light exposure of the code pre-polymer to limit photobleaching of the rhodamine dye.

22| Combine the functionalized antibodies with 20% PEG-DA pre-polymer mixture (REAGENT SETUP) at a 1:9 (vol/vol) ratio and vortex rapidly for 5 min at room temperature to form the probe pre-polymer. After vortexing, centrifuge the probe pre-polymer for 5 min at 2,000*g*.

▲ **CRITICAL STEP** Add the PEG-DA 20% pre-polymer mixture into the functionalized antibody solution to improve mixing.

23| Use a 1-ml syringe fitted to the tubing connectors to fill a loading tip with ~25 µl of code pre-polymer solution, drawing from the top of the sample tube. Gently insert the tip into the top inlet of the synthesis device, being careful to push only until resistance is felt. Pushing the tip too hard can separate the device from the cover slip and lead to device failure. Repeat the process, using the inlets from the top down and inserting the blank pre-polymer, probe pre-polymer and blank pre-polymer.

24| Wrap the inlet tips with a small piece of aluminum foil to prevent UV exposure of the polymer solutions inside. Add 950 µl of 1× TET to the collection tube and attach it to the synthesis device outlet.

25| Place the synthesis device on the microscope and align the channel with the particle edges marked in Step 15. Optimal synthesis occurs at approximately one-third of the distance from the outlet.

26| Attach the inlet tubes to the pressure system, verify that the pressure is ~3.5 psi and initiate the flow for 5 min to remove any air bubbles that may be trapped in the system. While purging, adjust the pressure valves for each inlet tube to produce the desired stream widths. See Bong *et al.*[46] for additional information.

27| Stop the flow and perform a single UV exposure. Adjust the microscope focus and exposure time to produce properly polymerized particles, which can be identified by visible texture on the surfaces, sharp interfaces and lack of film formation in the bar code holes.
▲ **CRITICAL STEP** Proper polymerization is critical to optimal particle function and consistent results. Increasing the intensity of the microscope's halogen lamp can aid in particle inspection, as particle texture, interface quality and film presence become more evident in brighter conditions. However, once proper polymerization has been achieved, reduce the halogen intensity to prevent unwanted polymerization in the pre-polymer loaded into the pipette tips.

28| Initiate the automated flow control with the desired synthesis settings. A flow period of ~500 ms will quickly re-establish laminar co-flow of streams at a forcing pressure of 3.5 psi. A stop time of ~300 ms will allow the flow to stop completely and prevent 'smearing' of particles. An exposure time of ~75 ms and a subsequent final hold period of ~150 ms allows for complete polymerization with high feature resolution. These times may need to be adjusted for different equipment setups to achieve desired particle characteristics.
? TROUBLESHOOTING

29| Begin automated synthesis and allow it to cycle for up to 45 min (**Supplementary Movie 1**). Monitor pre-polymer levels and watch for particles that stick to the synthesis channel. If particles begin to stick, pause the operation and move to a different position in the channel before resuming synthesis.
▲ **CRITICAL STEP** Maintaining the same focus and synthesis settings throughout the process will produce the most consistent particles and result in more reliable experimental data.

30| Stop the synthesis and allow the pre-polymer solution to flow for an additional 5 min to push all of the particles into the collection microcentrifuge tube.

31| Remove the collection tube, disconnect the attached microcentrifuge and add 350 µl of 1× TET to the microcentrifuge tube. Centrifuge the contents for 5 min, remove the supernatant down to the 100-µl marker, and then resuspend the particles in 400 µl of 1× TET. Repeat the rinsing process five times before storing the particles at 4 °C in 1× TET.

32| To determine the final concentration of particles, place five 2-µl aliquots of particles onto a cover glass, place it on the microscope, count the particles in each aliquot and average. Before drawing the particles out of solution, vortex gently to resuspend the particles.
▲ **CRITICAL STEP** Avoid vortexing vigorously (above 1,000 r.p.m.) as this can cause particles to break or deform.

33| Clean the microfluidic device in the same manner as described in Step 14, then store in a Petri dish for reuse.

Protein assay ● TIMING 3–4 h
34| Set up the protein assay in a filter plate such that each well contains a total volume of 50 µl and ~50 particles with 0.05% (vol/vol) Tween-20 in addition to the unknown sample or calibration target. If possible, add the particles to the well last. In the experiments described in Appleyard *et al.*[29], calibration targets were diluted in 1× FBST. Cover the plate with aluminum foil and incubate for 2 h at room temperature on a shaker running at 600 r.p.m.

35| Using a vacuum manifold, draw the carrier liquid through the filter until the well bottom begins to dry. Resuspend the particles in 200 µl of 1× PBST. Repeat the wash procedure with 200 µl of 1× PBST a total of three times, and on the final resuspension use 50 µl of the reporter antibody mixture (REAGENT SETUP). Re-cover the plate and incubate it on the shaker at room temperature for an additional 1 h at 600 r.p.m.
▲ **CRITICAL STEP** Excessive suction can cause particles to deform.

36| Wash the particles again with three 200-µl volumes of 1× PBST. On the final resuspension, use 50 µl of SAPE solution (REAGENT SETUP). Re-cover the plate and incubate it on the shaker at 600 r.p.m. for 30 min at room temperature.
▲ **CRITICAL STEP** Particles should be scanned within 1 h of the final rinse, and thus the preparation for scanning should start during the SAPE incubation. Limit the exposure of SAPE solution to light to avoid photobleaching the reporter.

37| In a final wash, use the vacuum manifold to pull three 200-µl volumes of 1× PBST through each filter well. Resuspend the particles in 50 µl of recently sonicated PTET (REAGENT SETUP).
▲ **CRITICAL STEP** Attempt to minimize light exposure during the wash.

Particle scanning ● TIMING 1 h
38| Prepare the microscope for scanning by turning on the laser, PMT, data acquisition board and loading the automation software. Set the pressure at 7–8 psi. Place an excitation line mask in the field stop of the microscope.
! **CAUTION** Use appropriate protective gear and procedures to avoid exposure to the laser.

39| Clean a microfluidic scanning device by sonicating it in ethanol for 5 min and then rinsing with ethanol and water; dry with argon. Load the sheath tube with 400 µl of recently sonicated PTET and prepare the collection tube with 750 µl of PTET. Attach a tubing connector to one of the modified pipette tips.

40| Place a small amount of SAPE solution on a cover slip and load it onto the microscope. Open the laser shutter and image the microscope slide with the CCD camera, marking the location of the excitation line on the computer monitor.

41| Gently shake the samples to ensure that the particles are in solution. Draw ~40 µl of a particle sample into the loading pipette tip using a 1-ml syringe. To prevent clogging/poor alignment during scanning, particle concentration should not exceed 18 particles per µl.

42| Place the tip into the sample inlet on the scanning device. Transfer the device to the microscope and attach the sample and sheath tubing to the pressure feed. Use the CCD camera to orient the device such that the marked laser line is positioned in the middle of the channel about one-third of the distance from the outlet and such that the laser line is perpendicular to the direction of flow. Adjust the focus until the walls of the channel come into sharp focus in the CCD image.

43| Turn off all lights, turn on the PMT, turn on the laser, initiate data acquisition, and then start the flow by opening the pressure valve. Just before the sample has been exhausted from the loading tip, close the pressure valve and turn off the laser and PMT.
! **CAUTION** The PMT can be damaged by exposure to ambient light; ensure that the PMT sensor is on only during scanning.
▲ **CRITICAL STEP** Isolating the scanning system from outside light sources will help reduce background noise.
? **TROUBLESHOOTING**

44| Rinse the loading tip with 1× TE and repeat Steps 41–43 to scan the remaining samples.
▲ **CRITICAL STEP** If particles or debris become stuck in the channel, flush it with 40 µl PTET to clear the device.

45| Clean the device by sonicating in ethanol and rinsing in ethanol and water; dry in argon. Store in a Petri dish for reuse.

46| Scan data consists of a time series of detector voltages. Individual particle events can be extracted most efficiently from the series by using a simple, automated script in MATLAB that searches for events of appropriate magnitude and length. If desired, particle events can be manually plotted in Excel and visually decoded. The target signal is measured by taking the average of the detector response in the probe region. Alternatively, the automated script can be adapted to decode particles and extract signal data[44].
? **TROUBLESHOOTING**

47| Particle analysis involves identifying the probe region in the scan and quantifying the target level by using an averaging window centered on the probe strip. The width of the averaging window will be a function of the particle design; we have found that a 15-µm window works well. The background signal, obtained from the control (no target) particles, is subtracted from the data. Normalization between runs can be achieved by scaling by the code height or through the use of a standard external control particle that is expected to register the same target level regardless of sample.

48| Particles can also be analyzed individually by imaging them on a fluorescence microscope using the appropriate excitation and emission filters for the SAPE dye. Imaging particles separately can be a valuable method for troubleshooting the synthesis and assay.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
28	Clogged synthesis channel	Stray polymerization is occurring in the inlet tips; monomer was not properly centrifuged before use	Readjust the aluminum foil around the inlet tips to better prevent UV exposure. Ensure that the aluminum foil reaches all the way down to the PDMS. If possible, move the synthesis area closer to the outlet
	Not all inlet streams are present	There may be a clog or a loose pressure connection to the inlet	Carefully check all inlet pressure tubing. If all are properly connected and the issue persists, then increase the manifold pressure to 5 psi to push out potential clogs
	Film formation in holes	Overexposure or poor focus	Readjust the focus, aiming for sharp particle interfaces and clearly defined texture. Reduce exposure time
28, 43	Leaky channel	The PDMS channel did not fully seal onto the microscope slide	Make new channels, using a shorter oven incubation time (1–2 min less) when curing the slide with the thin PDMS layer
43	Particle breakup during scanning	Underexposure during synthesis; excessive force while mixing	Vortex or shake the particles gently, only enough to keep them suspended in solution. Consider increasing the exposure time during synthesis
46	Noisy data	Particulate matter is attached to the particles	Image the particles on a fluorescence microscope (Step 39) to verify that the noise is coming from contamination. Ensure that all buffers are filtered and that particles are not underpolymerized

● TIMING

Steps 1–13, Fabrication of devices for synthesis and scanning: 1 week
Steps 14–17, Construction of microfluidic attachments: 2–3 h
Steps 18–33, Hydrogel synthesis: 4–5 h
Steps 34–37, Protein assay: 3–4 h
Steps 38–48, Particle scanning: 1 h

ANTICIPATED RESULTS

Synthesis

As outlined in **Figure 5**, well-polymerized particles will show surface texture in bright-field images in addition to sharp, well-defined code holes and interfaces. The ability to produce such high-quality hydrogel particles functionalized with capture antibodies can be attained through several (2–4) trial rounds of synthesis during which exposure times and optical focus should be adjusted until optimal results are achieved. Rounded corners and buckled edges in the code holes are indicative of insufficient polymerization (exposure time is too short), whereas the appearance of a thin film in the code holes is indicative of overpolymerization (exposure time is too long).

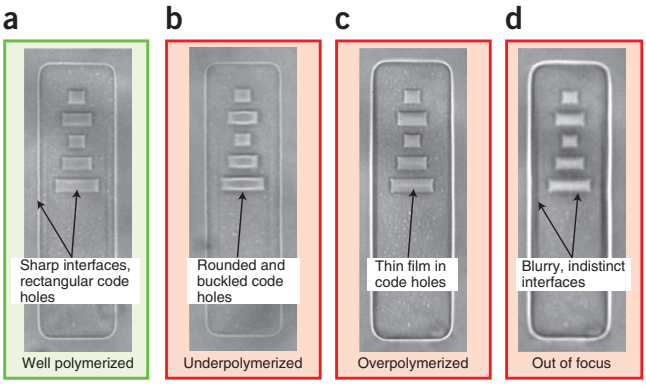


Figure 5 | Optimization of polymerization conditions. (**a**) A well-polymerized particle with sharp interfaces, rectangular code holes and notable texture on the particle. (**b**) Underpolymerized particle with rounded and buckled code holes. (**c**) Overpolymerized particle with a thin film in the code holes. (**d**) A particle that was polymerized with an appropriate exposure time, but with improper focus.

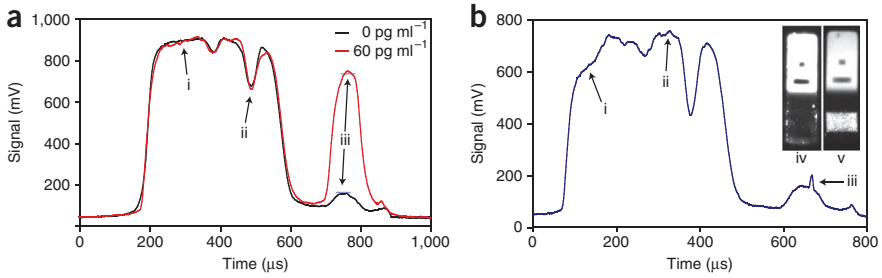
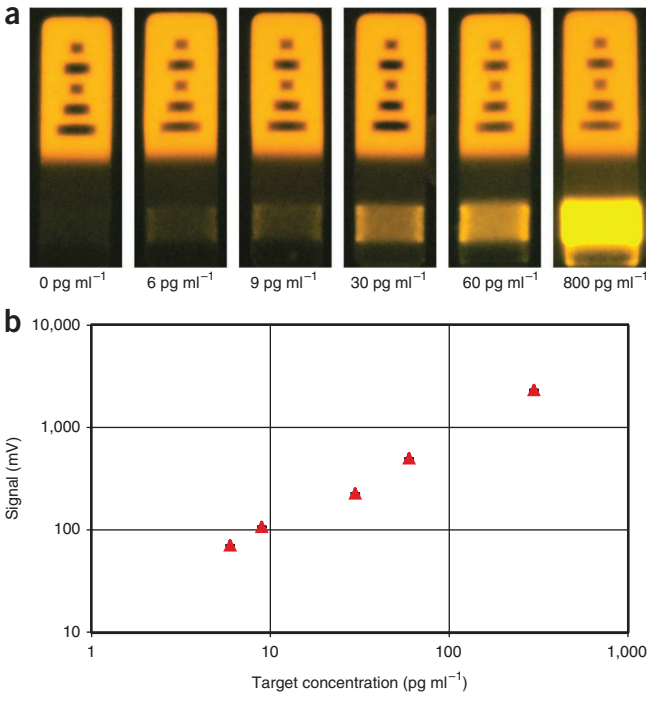


Figure 6 | Quality of scan data. (**a**) Overlay of two IL-2 scans, one with a target concentration of 60 pg ml^{-1} and the other a control (no target) for particles with code 00103. (i) A low-noise, nearly flat code region. (ii) A clean '3' hole dropping to a level approximately 25% of the full ('0') code fluorescence level, making it easily decodable. (iii) A 15-µm window over which the signal from the probe region is averaged for target quantification purposes. (**b**) A poorly synthesized and scanned particle with code 00103. (i) Failure to reach maximum code level at the edge of the particle, possibly because of a defect in synthesis or deformation during scanning. (ii) Noisy signal in the code, likely because of debris binding to the particle. (iii) A spike in probe strip read-off that may indicate immiscibility of antibody with monomer precursor during synthesis. (iv) A fluorescent image of a particle with bound debris/nonspecific adsorption. (v) A fluorescent image of a particle in which the probe strip exhibits 'spotty' signal patterns as a result of antibody aggregation during synthesis.

Scanning

Particles that are well synthesized will yield easily quantifiable and decodable scans. The characteristics of a good scan are shown in **Figure 6a**. First, scans should show a distinct code signature in which the depth of the trough in the fluorescent profile indicates the type of code hole. For example, the '3' hole in the displayed scan shows a signal drop of 25% relative to a region with no holes; the '1' hole on the same particle shows a smaller signal drop. Second, the blank region between the code and the probe regions should show no signs of fluorescence from either the code region or the probe region. Finally, a control particle should display minimal fluorescence from the probe region. The signal intensity of the control probe strip will sometimes differ from the neighboring blank regions of the particle because of differences in the pore sizes of the regions.

A poor scan can result from a number of different factors. **Figure 6b** shows some of these causes. Sudden signal spikes in a scan are indicative of debris nonspecifically binding to the particle, or of potential miscibility problems that may have arisen during synthesis and caused uneven patches of signal along the probe region. Fluctuation in signal along the baseline of the scan (between particle events) is caused by laser instability/misalignment or by improper shielding/grounding of the electronic components used for data acquisition, amplification and filtering. In addition, clogs or debris in the channel can lead to particles deforming or changing velocity abruptly during the scanning process, which can also lead to poor scans. Following the guidelines laid out in the PROCEDURE section should mitigate these issues and allow the efficient collection of high-quality scans that can easily be decoded.



Overall

Careful performance of the assay using the steps outlined here should yield accurate scan data for each concentration in the calibration curve (**Fig. 7**). The background-subtracted signal can be plotted as a function of target concentration and used to quantify unknown samples of the same target type. The LOD for the target can be found by plotting the SNR (background-subtracted target signal divided by the standard deviation of the control particle signal) as a function of the target concentration. The target concentration at which a line fit to these data intersects with an SNR of three is taken to be the LOD. With high-affinity antibodies, an LOD between 1 and 10 pg ml^{-1} is attainable with an intratrial CV in measurements below 9% using only five particles[31].

Figure 7 | Single-probe particles used in a calibration of IL-2 detection. (**a**) Images of each particle at various calibration concentrations taken using a Nikon D200 attached to the microscope (1-s exposure). Note that the overall image intensity has been uniformly increased to allow for visualization of probe strips at the 6 and 9 pg ml^{-1} levels. (**b**) The calibration curve constructed from flow-through scans of IL-2 particles using target concentrations from 6 pg ml^{-1} through 800 pg ml^{-1}. Each point represents the background-subtracted average of five particles.

Fluorescent images of post-assay IL-2 particles and a background-subtracted calibration curve from flow-scanned IL-2 particles are provided as a reference in **Figure 7**. Notably, the PEG particles show very little nonspecific binding even in a complex 90% (vol/vol) FBST buffer. In the FBST trials, the code region produces the expected fluorescence profiles, and the blank regions are clear of nonspecific signal. Furthermore, the fluorescence intensity in the probe region increases with increasing target concentration over a dynamic range of 3 logs and the LOD is determined to be 1.1 pg ml^{-1} (ref. 22). Because the reaction between target and probe occurs on a time scale that is much smaller than the characteristic time for target diffusion within the gel, a stronger signal is observed at the edges of the probe strip.

Note: Supplementary information is available via the HTML version of this article.

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