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Laser particle activated cell sorting in microfluidics†

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Laser particles providing bright, spectrally narrowband emission renders them suitable for use as cellular barcodes. Here, we demonstrate a microfluidic platform integrated with a high-speed spectrometer, capable of reading the emission from laser particles in fluidic channels and routing cells based on their optical barcodes. The sub-nanometer spectral emission of each laser particle enables us to distinguish individual cells labeled with hundreds of different laser colors in the near infrared. Furthermore, cells tagged with laser particles are sorted based on their spectral barcodes at a kilohertz rate by using a real-time field programmable gate array and 2-way electric field switch. We demonstrate several different flavors of sorting, including isolation of barcoded cells, and cells tagged with a specific laser color. We term this novel sorting technique laser particle activated cell sorting (LACS). This flow reading and sorting technology adds to the arsenal of single-cell analysis tools using laser particles.

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Introduction

The biological behavior of a tissue, organ or organism arises from the complex interactions of numerous distinguishable cell subpopulations. To discern each subpopulation's different role, it is desirable to tag each group in a manner that renders each distinguishable. In the most ideal and extreme case, each tagged subpopulation comprises of just a single cell thereby allowing the relevant biological dynamics to be analyzed at a single cell level. Tags in the form of optical barcodes offer several advantages allowing real-time, nondestructive, and repeatable readout using a relatively simple optical reader. Recently a new form of optical barcode termed laser particles (LPs) has been developed that enables a high degree of multiplexing due to the large number of unique emission signatures LPs can form.^{1–3} Upon optical excitation, each LP emits coherent radiation of sub-nanometer spectral linewidth providing an ultra-pure, distinguishable laser color. With a wavelength interval of 1

nm, about 400 colors from 1200 to 1600 nm have been achieved,^{1,2} which could in principle be scaled to millions of barcodes by using combinations of LPs to tag each cell. The scalability of LPs offers distinct advantages over conventional fluorescence-based tagging techniques, such as multi-color fluorophores,⁴ nanoparticles,⁵ and microbeads,⁶ which typically permit only a small number of cells or cell-groups (<10 to 100) to be labelled. Laser-based optical barcoding promises to make it possible to tag single cells at the scales (>10 000) commonly used in single-cell analysis through imaging, flow cytometry, and sequencing. Data acquired at different times and in different instruments can be aligned to individual cells based on their unique optical barcodes allowing for multi-dimensional analysis of the cell population with single cell resolution.⁷ In our previous work, we demonstrated tracking of LP tagged cells both in 2D and 3D cell cultures and *in vivo*¹ by integrating a high-resolution optical spectrometer capable of reading LP barcodes into a conventional fluorescence microscope.

In this paper, we extend the ability to read LP barcodes to LPs flowing in microfluidic channels and demonstrate a flow sorter capable of routing specific LPs and LP-tagged cells. Fluidics represents a versatile platform for analyzing and processing single cells at high throughput. Flow cytometers and fluorescence-activated cell sorters (FACS) are widely used instruments for various biological and biomedical applications.^{4,8,9} FACS is commonly used to isolate cells in a high throughput manner for downstream utilities for adoptive cell transfer, cell therapy development, drug screening, and

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lens, the pump laser focus was shaped into a line ($10\text{ }\mu\text{m} \times 50\text{ }\mu\text{m}$, FWHM), covering the entire $50\text{ }\mu\text{m}$ channel width. These parameters were chosen to ensure reliable LP detection across the entire wavelength bandwidth based on our previous threshold and lasing measurements.¹⁴ The optical emission from the pumping zone was directed into a home-built grating-based spectrometer (resolution $0.5\text{--}1.0\text{ nm}$) equipped with a 2048-pixel InGaAs linear camera typically operated at a speed of approximately 25 000 lines per s. The flow rate was set to approximately $40\text{ }\mu\text{L min}^{-1}$, corresponding to a mean flow speed of $\sim 0.5\text{ m s}^{-1}$ within the flow channel. Given the parabolic profile of laminar flow, cells travelled at slightly different speeds depending on their location in the channel. Each cell traversed the pump's excitation spot in 20 to $40\text{ }\mu\text{s}$. We set the spectrometer camera exposure time to $35\text{ }\mu\text{s}$ that allow the lasing event to be captured within 1–2 frames.

Co-culture of LPs with various cell types results in their spontaneous uptake in a manner consistent with Poisson statistics.^{1,15} Efficient LP uptake has been demonstrated in numerous cell types, including primary cells.^{1,2} We tagged HeLa cells by overnight incubation with LPs with a low LP to cell ratio so that cells are tagged with an average one LP per cell. 500 000 cells suspended in 1 mL cell media were injected into the input end of the microfluidic chip. Fig. 1a shows high-speed video images (14 μ s exposure, 10 k frames per second) of an LP-tagged cell that is traversing the pump excitation. As it flows through this readout region, a distinct signal is clearly observed on a few pixels of the spectrometer's linescan camera corresponding to a wavelength, in this case, of 1387 nm (Fig. 1b). The recorded linewidth $\delta\lambda$ was 0.72 nm FWHM, limited by the spectrometer resolution. Importantly, this narrow linewidth means that the detection system can easily identify and resolve large numbers of laser colors. Fig. 1c shows 256 representative recorded spectra from 256 LPs across a bandwidth $\Delta\lambda$ of \sim 400 nm from 1200 nm to 1600 nm, the detection range of our spectrometer. Fig. 1d shows the histogram of over 460 000 LPs across the bandwidth. The distribution itself reflects the diameters of the recorded LPs. The relatively fewer lasing events at wavelengths greater than 1450 nm is likely due to a relatively lower number of lasing LPs in the spectral range.

Unlike organic fluorophores, semiconductor LPs of different emission wavelengths can be excited with a single laser pump source because of the characteristic band-induced absorption of semiconductor gain media. We estimated that each LP emitted $\sim 10^7$ photons per pump pulse, $>1\%$ of which is detectable by the spectrometer. These bright signals from LPs above lasing threshold are critical to the high-speed spectral readout. The identity, or color, of an LP is represented by the peak wavelength, independent of its recorded intensity (Fig. S2†). This spectral information is in general far more robust than intensity-based measurement and particularly critical for the LPs that typically have direction-dependent emission intensity.¹⁶ In theory, the flow LP reader presented here can

We used disk-shaped InGaAsP LPs with a diameter of $\sim 2\text{ }\mu\text{m}$ and thickness of $0.2\text{ }\mu\text{m}$.¹ The diameter of each particle determines the emission wavelength of its lasing peak with a tuning coefficient of $\sim 1\text{ nm nm}^{-1}$ in wavelength to disk radius changes. This wavelength is remarkably stable over a range of typical excitation pump intensities (Fig. S1†). LPs of different diameter can each emit their own narrowband radiation within a 75–100 nm full width at half maximum (FWHM) spectral gain bandwidth determined by the semiconductor alloy composition. We used 9 different compositions of $\text{In}_{1-x}\text{Ga}_x\text{As}_y\text{P}_{1-y}$ ranging from ($x = 0.26$, $y = 0.55$) to ($x = 0.44$, $y = 0.95$) to produce LPs over 1150 nm to 1650 nm.¹⁴

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Fig. 1 Readout of LP emission in the flow. **a**, Image frames showing a cell containing a single LP (arrow), as it traverses the pump laser focus (dashed ellipse). **b**, Recorded spectra at the corresponding time. A narrow lasing peak is observed on the spectrometer at the frame corresponding to the cell traversing this point. **c**, Collection of lasing spectra observed during a single experimental run. **d**, Histogram showing recordings of lasing wavelengths from a near half million LPs measured in a single flow experiment.

resolve up to 550 colors ($\Delta\lambda/\delta\lambda$). We note that many more cells can be uniquely tagged if they are tagged with more than a single LP. For example, with 3 or more LPs per cell, the number of unique identifiers could be as many as ${}_{550}C_3 = 25$ million. This set would allow 1 million cells to be uniquely tagged with a low duplicate error of $\sim 4\%$.

Laser particle activated sorting

Traditional FACS uses a gating strategy based on fluorescence biomarker intensities to sort cells in order to enrich for certain, desired subpopulations. To realize single-cell sorting based on their LP barcoding signals, we adopted a previously established water-in-oil droplet based sorting technique¹⁷ and fabricated a 2-way microdroplet sorting device (Fig. 2a and S3†). This device

is comprised of three inlets and two outlets. In one of the inlets, cells suspended in cell media were flowed. A fluorinated oil, immiscible with the aqueous cell media was flowed into the other two inlets. The first of these inlets pinched the aqueous flow, generating 50 μm diameter aqueous droplets. The second inlet was used to space the droplets at intervals of approximately 500 μm to ensure that each droplet was independently sorted. The central sorting zone had a channel size (width \times depth) of $\sim 55 \mu\text{m} \times 25 \mu\text{m}$ and a mean flow speed of $\sim 0.5 \text{ m s}^{-1}$. Droplets entered the flow channel at a rate of approximately 1.5 kHz.

Fig. 2b shows a schematic of the experimental setup. The pump laser is focused just upstream of the sorting junction, enabling the possible presence of an LP emission signal to be registered. Data from the spectrometer is streamed in real



Fig. 2 The microfluidic system. **a**, Schematic of a microfluidic chip. Pump laser focus location shown in inset (red arrow). **b**, Schematic of the cell sorting setup. A spectrometer reads LP laser emission. Decision hardware in a field programmable gate array (FPGA) triggers the computer to send high voltage pulses to electrodes to deflect cell-containing droplets into the (+) outlet.



In the future, binary sorting based on the presence or absence of an LP could be helpful to isolate an LP-tagged subpopulation of cells for downstream experiments such as cell-tracking imaging.^{1,2} Alternatively, LP tags have been proposed as single cell sequencing probes, in which the emission signatures allow individual cells to be tracked

a Time-lapse fluorescence microscopy images of the device at 0 μs , 200 μs , 600 μs , and 1000 μs . The images show the evolution of the fluorescence signal (red) in response to a voltage pulse. The regions are labeled (i), (ii), and (iii). The scale bar indicates 100 μm .

b Fluorescence spectra of the device. The spectra show the intensity of fluorescence as a function of wavelength (nm) from 1300 to 1500 nm. The spectra are labeled (i), (ii), and (iii).

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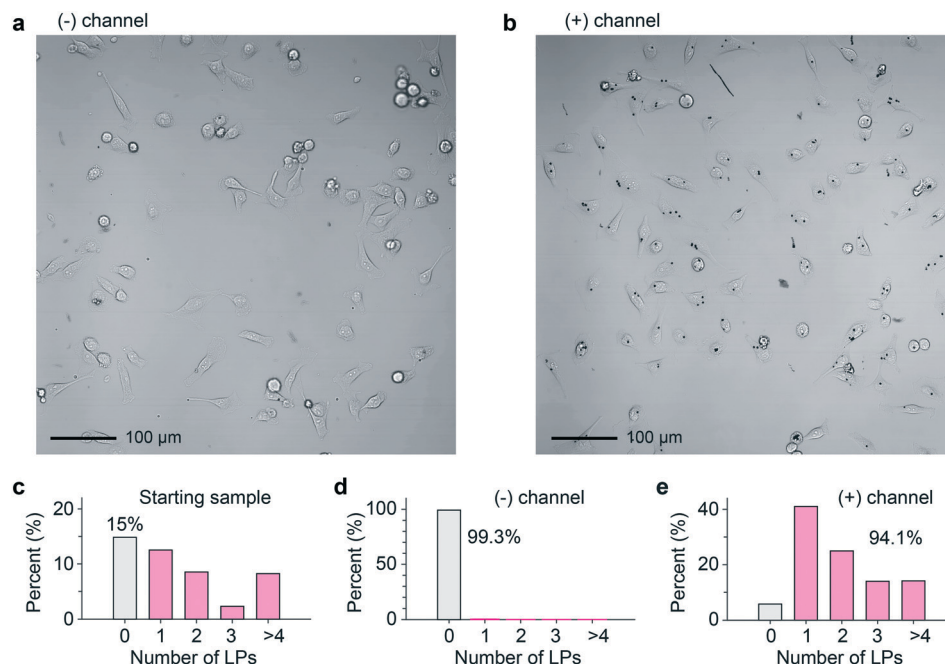


Fig. 4 a and b, Representative bright-field images of HeLa cells after sorting as collected from the (-) and (+) outlets. c-e, Statistics of the fraction of cells that contain a specified number of disks, analyzed from >500 cells in all acquired images.

through an RNA sequencing workflow to enable knowledge of each cell's spatial location to be tied to information regarding its genetic expression.⁷ In some single cell sequencing protocols, cells are commonly sorted prior to sequencing by traditional FACS to target a desired subpopulation to be sequenced.^{18,19} Similarly, binary LACS would be able to enrich for cells suitable for sequencing by selecting tagged cells.

Spectrally selective LP activated cell sorting

We then explored sorting based on spectral barcodes. First, we tested a gating strategy for short-pass or long-pass

wavelength sorting. The spectrometer-linked FPGA was programmed to identify, in real time, the pixel of highest intensity each time a lasing event was registered. If this pixel corresponded to a wavelength within the sorting window, a high-voltage electric field was applied to the electrodes to deflect the traversing droplet toward the appropriate outflow channel. In cases in which more than one lasing peak emanated from a droplet, possibly because of the presence of two cells or a single cell with two LPs in the droplet, the gating condition was set such that the sorting signal is fired only if all the emission peaks fell within the sorting window. In the experiment, we used a short-wavelength gating

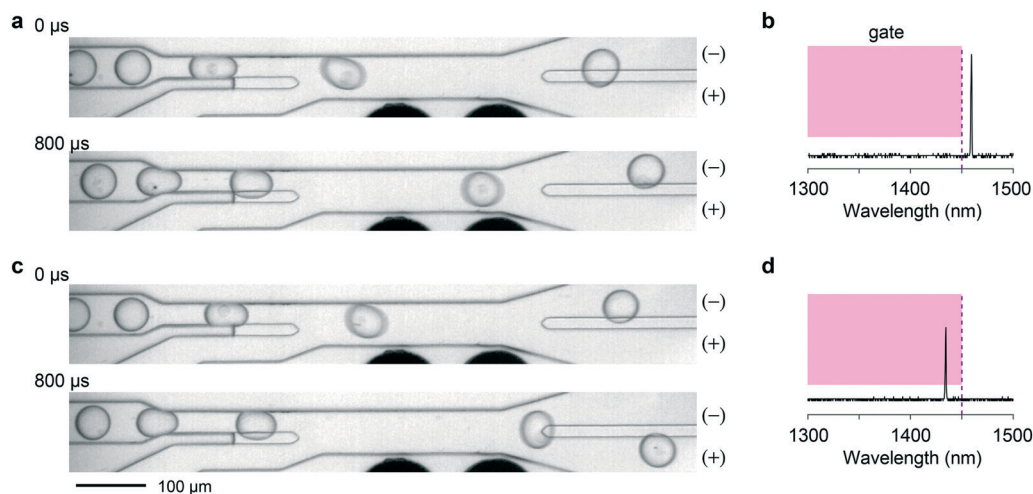


Fig. 5 Short-wavelength sorting. **a**, A negative cell ($\lambda > 1450$ nm) flowing into the (–) channel. **b**, The LP emission spectrum of the negative cell. The magenta box indicates the gating condition used. **c**, A positive cell ($\lambda < 1450$ nm) directed into the (+) channel. **d**, The LP spectrum of the positive cell.



Fig. 6 Band-pass sorting. a, A bright-field image of replated cells collected from the (–) outlet. b, The LP lasing emissions from the LPs labelled i, ii and iii. c and d, Image and emission spectra of cells collected from the (+) outlet, showing three LPs (iv–vi).

condition with a cutoff wavelength of 1450 nm. Exemplary events are shown in Fig. 5 and Videos S6–S8.†

As a more sophisticated demonstration, we implemented band-pass sorting. A 5 nm-wide sorting window centered around 1285 nm was applied as the selection criterion. We used LPs obtained from a single wafer with $\text{In}_{0.75}\text{Ga}_{0.25}\text{As}_{0.54}\text{P}_{0.46}$ as active material, which covers a spectral range from 1245 to 1340 nm. The LPs were co-cultured with HeLa cells and flowed through the sorting chip. Cells collected from each outlet were re-plated on a culture well plate, and the LP emission from the cells were measured using the previously modified confocal microscope employing a high-resolution spectrometer.^{1,2} Fig. 6 shows representative images and LP emission spectra obtained from both channels. The cells harvested from the (+) outlet contained LPs that predominantly lased at wavelengths within the predefined 5 nm window close to 1285 nm (Fig. S7†). The routing accuracy was consistent with that obtained in the above wavelength-independent binary sorting (ESI† Note 3).

Wavelength-based LP sorting allows specific cells identified from an imaging experiment to be isolated for further analysis. Since our LPs emit at infrared wavelengths, they do not exhibit crosstalk with common fluorophores, making LPs compatible with established fluorescence-based technologies. For example, LACS could be used to isolate cells based on their LP barcodes that have been previously identified by techniques such as microscopy or flow cytometry, that can associate individual cells with phenotypic features, such as protein-tagged fluorescence expression. By reading LP signatures at each step of a workflow, comprehensive cell profiles could ultimately be built and isolated as desired.

Conclusions

Cell sorters are indispensable instruments used in biological research and medical research for isolating specific cellular subpopulations with specific phenotypes. FACS are the

amongst the most common types, which divert and collect cells into different vials based on the fluorescence signals related to immuno-stained biomarkers, viability, and reporter-gene expressions.⁸ Light scattering-based sorting is also used for label-free purification of cell subpopulations such as lymphocytes and pancreatic islet cells.^{20,21} Magnetic-activated cell sorters (MACS) are also increasingly used to purify engineered cells for cell therapy.²² Fluorescence image-based cell sorting has recently been developed to enable selective isolation of single cells with unique spatial and morphological traits.²³ In all these techniques, the sorting signals or routing decision are related to the cellular phenotypes measured *in situ*. Here we have demonstrated a new type of cell sorter based on laser-emitting cellular barcodes, which we term LP activated cell sorting (LACS).

Besides cell sorting, the technology may be used to sort LPs, either with or without cells, inside droplets. Currently, LPs are fabricated such that each particle has a random wavelength, distributed over the gain bandwidth of the semiconductor. However, for some experiments it would be useful to be able to produce a batch of LPs with an identical wavelength, just like fluorophores with identical fluorescence spectrum. By repeated wavelength-selective sorting, batches of LPs at different colors could be created for various applications such as highly multiplexed cell-type labeling for *in vivo* imaging or drug screening, which is currently performed using fluorescent cell barcoding.^{24,25}

Future improvements in sorting accuracy should also be emphasized and can likely be realized most easily by using alternative sorting mechanisms^{26–28} that can deflect droplets in the air and into multiple channels, such as those used in commercial machines (2- to 6-way sorting). Increased sorting device sophistication could enable sorting rates as high as tens of thousands of cells per second.

In conclusion, the demonstrated flow technology enabling cell barcode identification and sorting based on laser emission spectra will prove useful in the emerging single-cell applications of LPs.



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