Bioassays

https://doi.org/10.1038/s41551-023-01165-4

Multipass high-dimensional flow cytometry

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Barcoding cells with microparticles that emit near-infrared laser enables the use of flow cytometry to track the dynamics of single cells by using more markers and fewer colours.

When cells in suspension are analysed by flow cytometry, single cells can be distinguished according to their physical parameters, their capability to bind fluorescent probes or their protein expression as detected by fluorescently conjugated monoclonal antibodies. This is akin to deciphering graffiti writing — the complex lettering outlined in different shapes and filled with colours by using spray cans and markers — by recognizing the tag or signature of the writers and their style.

Flow cytometry has long stood as a cornerstone in biomedical research, owing to the technique's ability to simultaneously measure multiple fluorescent parameters[1]. Conventional flow cytometry can now detect up to 30 fluorescent markers[2]. But one longstanding challenge has been the spectral overlap and spectral 'spillover' between fluorophores, which make the development of panels of fluorophores cumbersome and time-consuming[3]. Although measurements have been extended to 50 or more parameters by using spectral unmixing to separate overlapping spectra[4], this capability is hindered by the limited number of commercially available fluorescently conjugated antibodies. Recent efforts have sought to address this via multiplexing through optical barcoding, whereby barcoding cells with laser-light-emitting nanoparticles (or laser particles; LPs) allows enhanced cell identification and classification during analysis[5]. By building on such multiplexed optical barcoding, Sheldon Kwok, Seok-Hyun Yun and colleagues now report in *Nature Biomedical Engineering* a technique to indelibly 'tag' cells for multipass flow cytometry[6].

Kwok and co-authors' procedure involved tagging each cell with a random assortment of LPs (Fig. 1). The LPs were made of InGaAsP microdiscs[5] and had six distinct compositions that emitted light within the wavelength range of 1,150–1,550 nm, which leaves the visible and 700–900-nm near-infrared (NIR) windows available for standard fluorescence labelling. The LPs were coated with a SiO_2 layer and functionalized with polyethylenimine[7] so that they could attach quickly (within 15 min) to live human peripheral blood mononuclear cells (PBMCs). The cells were processed in a custom flow cytometer that had an NIR laser in addition to four standard lasers to excite fluorophores commonly used in traditional flow cytometry. The authors' machine was also equipped with a collection device to recover the cells after analysis and to enable multiple cycles of photobleaching (essentially, erasing the standard fluorophores but maintaining the barcodes) before re-staining and reanalysis. The authors show that the collection and cleansing procedures retain about 95% of live human PBMCs, even after multiple cell-capture cycles (the authors considered a cell to be barcoded if it had three or more LPs), and that the technique can tag approximately 50% of PBMCs. Lymphoid cells generally kept the LPs on their surfaces, whereas myeloid and epithelial cells tended to absorb them through micropinocytosis[5,7,8]. By combining three random LPs per cell one could, in theory, yield 8.5×10^7 unique spectral barcodes, which would allow researchers to track up to a million cells with minimal margin of error from duplication.

Moreover, the authors observed minimal reductions in cell viability (from 93.2% to 91.6% after LP tagging, which further decreased to 89.1% after the LP-tagged samples were stored for 5 h at 4 °C). The technique did not promote cell aggregation, and no substantial differences in the expression of major immune markers were detected between control and LP-tagged samples. Notably, a slight decrease in fluorescence intensity in the cells could be observed with higher LP-tag counts, indicating a possible fluorescence-quenching effect. The authors confirmed the effectiveness of the technique by staining LP-tagged PBMCs from three different donors with key immune markers, running them twice in their custom cytometer, and then comparing the fluorescence data of the cells with matching LP barcodes. The fluorescence intensities across the two cycles correlated for about 98.5–99.4% of the cells.

A major limitation of flow cytometry is that it does not differentiate between alterations in the markers arising from phenotypic changes in the original population and those arising from the expansion or death of specific cell types, especially after perturbations following cell division or the treatment or stimulation of the cells. By using multipass flow cytometry, Kwok and co-authors show that time-resolved measurements can be used to track changes in marker expression in individual cells across different cycles. To prove this, the authors conducted an experiment using human T cells treated with phorbol myristate acetate and ionomycin, a pharmacological stimulation generally used to induce cytokine production and that causes the specific loss of CD4 expression on the cells' surfaces[9]. The cells were initially stained with releasable antibodies[10] and analysed for baseline phenotyping before undergoing stimulation, after which they were re-stained for surface markers and intracellular cytokines. The time-resolved strategy enabled cell gating based on pre-stimulation markers, including the identification of cytokine-secreting $CD4^+$ cells for downstream analyses. The authors also confirmed that barcoding did not adversely affect the secretion of specific cytokines from the T cells.

Kwok and colleagues also describe the use of LP barcoding to facilitate the acquisition of different markers across multiple cycles and to measure additional markers on identical cells without the need for additional fluorophores, which often complicates the design of fluorophore panels, owing to spectral-spillover errors. To this end, isolated live human T cells were first barcoded with LPs and subsequently stained with a five-marker panel of releasable antibodies. Then the cells were acquired and captured, the releasable antibodies detached, and the cells subsequently stained with a fresh five-marker panel before a second acquisition. Out of ten fluorescence markers, only 7.8% of the fluorophore pairs required fluorescence compensation greater than 1%. Remarkably, 50 pairs showed no spillover whatsoever, and 19 pairs had minimal compensation, notably less than is typical in conventional ten-marker panels using ten distinct fluorophores.

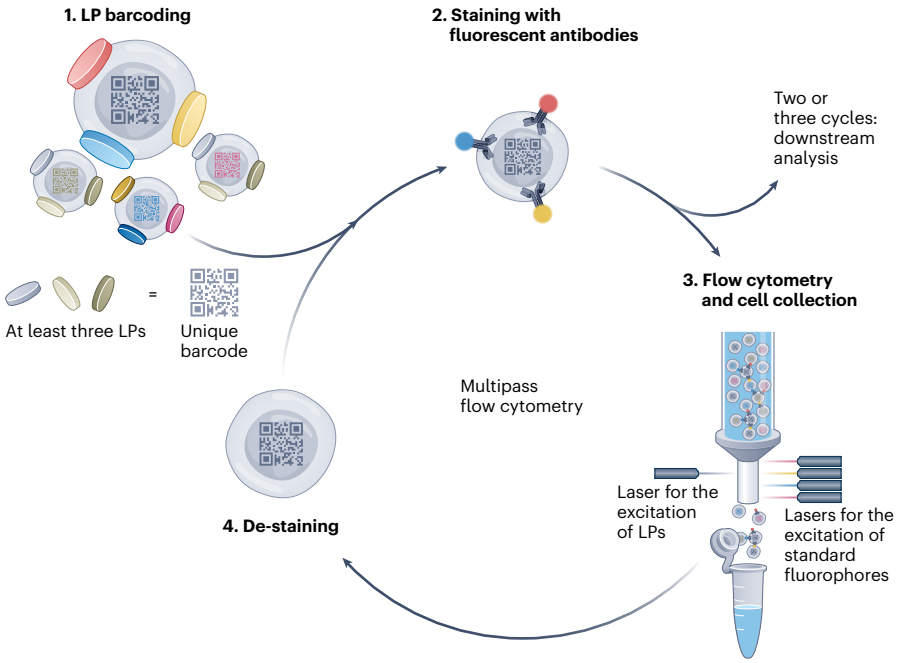

Fig. 1 | High-dimensional multipass flow cytometry via barcoded cells. Cell suspensions are first barcoded with LPs (1), followed by staining with fluorescently conjugated antibodies (2) and analysis using a custom-made flow cytometer equipped with both an NIR laser and conventional lasers (3). Cells are then collected and photobleached with lasers to remove antibody-emitted fluorescence (de-staining; 4). For high-dimensional measurements, the staining protocol and analysis are repeated multiple times.

To overcome limitations in the availability of chemically releasable antibodies, Kwok and co-authors introduced a photobleaching technique that is compatible with live cells. The authors designed a device that illuminates cell samples and that actively cools them to near 4 °C, minimizing cell mortality during the process. Broadband light-emitting diodes photobleached a variety of frequently used antibody-conjugated fluorophores within a span of 3 to 25 minutes. After photobleaching, the fluorescence signals resembled those of an unstained sample. When the authors used ten antibody-conjugated fluorophores simultaneously, there was only a slight decline in cell viability (from 97.2% to 93.4% after one bleach and down to 91.1% after two rounds of bleach). The authors noticed that fluorophores tied to markers with higher antigen density typically bleached at a slower pace, probably owing to a restricted local supply of oxygen. Immunophenotyping analysis of a sample after photobleaching showed no substantial differences between cell types, indicating that the approach is reliable.

To test the potency of the technique for high-dimensional measurements, Kwok and colleagues developed a three-cycle, 32-marker deep-immunophenotyping panel for human PBMCs that used 10–13 fluorophores per cycle. Live human PBMCs were used for data collection, going through cycles of staining, acquisition and photobleaching. About 50% of the barcoded cells acquired in the last cycle were successfully matched with those of the previous cycles. The process showed consistency and reproducibility when tested on samples from multiple donors, with a noticeable reduction in spillover spreading[3,11] when fewer colours were acquired over multiple passes, thereby resulting in improved resolution of the co-expressing markers in high-parameter panels.

The broad applicability of the technology may be impeded by a few limitations. First, the technique requires a flow cytometer equipped with an unconventional NIR laser and a cell-collection device. Second, LP tagging is efficient for only approximately 50% of cells. This could be a substantial drawback for rare samples or when sample amounts are limited. Third, the cyclic workflow inherently lengthens the analysis time, owing to the addition of several intermediate steps, such as LP tagging, photobleaching and re-staining. Hence, the technique will be competitive with existing technologies only if it combines iterative staining cycles of 25–30 markers per cycle. In this way, one could potentially reach more than 100 markers in a single analysis in a cost-effective manner. Algorithms and web tools for the rapid and automated analysis of such complex data are currently available[12,13].

An exciting application of Kwok and co-authors' technique is in vivo cell tracking. Individual cells can be barcoded, phenotyped by flow cytometry and then re-infused into an animal. The trafficking and lifespan of individual cells (that are not dividing) could be measured by recovering the cells after re-circulation for hours or days. Although the recovery of re-infused cells would be challenging, the identification of even a few barcoded cells would yield a wealth of information. Challenges notwithstanding, the authors' work overcomes a long-standing problem in flow cytometry: the need for multiplexed staining without substantial interference and without loss of data quality. By developing a photobleaching technique tailored for live cells, the authors have crafted a means to circumvent the limitations imposed by the current pool of commercially available and chemically releasable antibodies. This allows multiple rounds of staining and measurement, which is particularly beneficial for comprehensive

immunophenotyping. The technique's adaptability and compatibility with existing flow-cytometry setups pave the way for its integration into standard laboratory workflows. By reducing the spillovers experienced in conventional single-pass flow cytometry, the technique enhances data accuracy and resolution. Such detailed cellular analyses could offer powerful insight into intricate cellular networks and cellular interactions, potentially enhancing the understanding of various immune responses, disease states and cellular dynamics during therapeutic interventions.

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Published online: 20 December 2023

References

1. Shapiro, H. A. *Practical Flow Cytometry* (Wiley, 2003).
2. Brummelman, J. et al. *J. Exp. Med.* **215**, 2520–2535 (2018).
3. Brummelman, J. et al. *Nat. Protocols* **14**, 1946–1969 (2019).
4. Park, L. M., Lannigan, J. & Jaimes, M. C. *Cytometry A* **97**, 1044–1051 (2020).
5. Martino, N. et al. *Nat. Photon.* **13**, 720–727 (2019).
6. Kwok, S. J. J. et al. *Nat. Biomed. Eng.* https://doi.org/10.1038/s41551-023-01144-9 (2023).
7. Xia, T. et al. *ACS Nano* **3**, 3273–3286 (2009).
8. Patiño, T., Soriano, J., Barrios, L., Ibáñez, E. & Nogués, C. *Sci. Rep.* **5**, 11371 (2015).
9. Karlsson, F. & Hassan-Zahraee, M. *Curr. Protoc. Cytom.* **71**, 6.35.1–6.35.7 (2015).
10. Pankratz, J. et al. *Cancer Res.* **79**, 4048 (2019).
11. Nguyen, R., Perfetto, S., Mahnke, Y. D., Chattopadhyay, P. & Roederer, M. *Cytometry A* **83A**, 306–315 (2013).
12. Liechti, T. et al. *Nat. Immunol.* **22**, 1190–1197 (2021).
13. Puccio, S. et al. *Nat. Commun.* **14**, 5102 (2023).

Acknowledgements

This work was supported by the grant Giovani Ricercatori GR-2016-02363315 (to E.L.) from the Italian Ministry of Health, and by the Intramural Research Program of the Vaccine Research Center, NIAID, NIH (to M.R.).

Competing interests

E.L. serves as a consultant for BD Biosciences. The other authors declare no competing interests.