

Highly multiplexed simultaneous detection of RNAs and proteins in single cells

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To enable the detection of expression signatures specific to individual cells, we developed PLAYR (proximity ligation assay for RNA), a method for highly multiplexed transcript quantification by flow and mass cytometry that is compatible with standard antibody staining. When used with mass cytometry, PLAYR allowed for the simultaneous quantification of more than 40 different mRNAs and proteins. In primary cells, we quantified multiple transcripts, with the identity and functional state of each analyzed cell defined on the basis of the expression of a separate set of transcripts or proteins. By expanding high-throughput deep phenotyping of cells beyond protein epitopes to include RNA expression, PLAYR opens a new avenue for the characterization of cellular metabolism.

The ability to measure single-cell gene expression in complex samples, such as heterogeneous cancers or tissues undergoing an immune response, can lead to a better understanding of system-wide cellular interactions and function.

Single-cell RNA-seq is currently a method of choice for studying transcript expression in up to thousands of individual cells in a single experiment^{1–12}. However, aspects of sample handling (including the separation of live cells before lysis) have been shown to induce significant alterations in the transcriptome¹³. Moreover, RNA-seq does not allow simultaneous detection of protein epitopes and transcripts, and throughput is limited by cost, protocol complexity and available sequencing depth. These limitations notwithstanding, the combination of genome-wide expression and precise quantification through the use of unique molecular identifiers makes single-cell RNA-seq an exceptionally promising technology¹⁴.

A complementary approach is to quantify a smaller number of transcripts while increasing the number of cells that can be analyzed. Flow cytometry allows multiple parameters to be measured in hundreds to thousands of cells per second. Fluorescence *in situ* hybridization (FISH) protocols have been adapted to quantify gene expression on cytometry platforms^{15–20}. Such experiments require bright FISH signals with excellent signal-to-noise ratios, as flow cytometry does not provide the subcellular imaging

resolution necessary to distinguish individual RNA signals from diffuse background. DNA padlock probes in combination with rolling circle amplification^{21,22} (RCA) and branched DNA technology²³ have been used to generate strong hybridization signals. Recently the branched DNA approach was successfully applied to flow cytometry²⁴, but the availability of only three non-interfering branched DNA amplification systems and the spectral overlap of fluorescent reporters complicate multiplexing.

Our PLAYR system addresses these limitations by enabling the simultaneous detection of protein epitopes and multiple RNA targets routinely in thousands of cells per second. The method preserves the native state of input cells; detects transcripts without the need for cDNA synthesis; and is compatible with flow cytometry, mass cytometry and microscope-based imaging systems. Making use of the different measurement channels of mass cytometry²⁵, this approach enables the simultaneous quantitative acquisition of more than 40 different proteins and RNAs. We expect that PLAYR will lead to a better understanding of stochastic processes in gene expression^{26–28} and allow for deeper insights into complex cell populations.

RESULTS

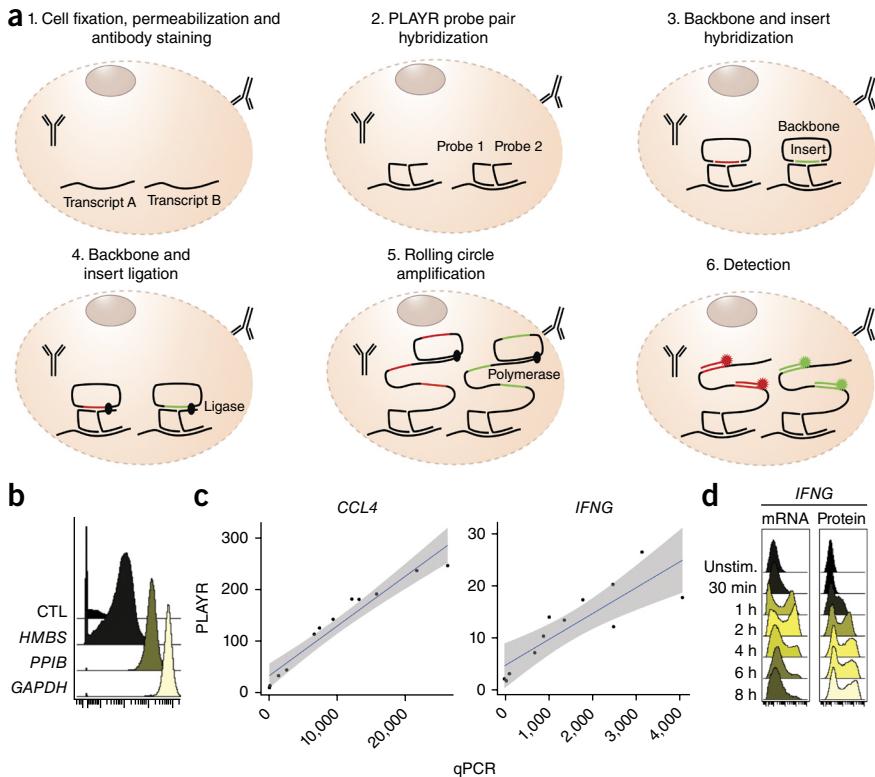
Overview of PLAYR technology and probe design

PLAYR uses the concept of proximity ligation^{29,30} to detect individual transcripts in single cells (Fig. 1a) and is compatible with immunostaining. Pairs of DNA oligonucleotide probes are designed to hybridize to two adjacent regions of target transcripts in fixed and permeabilized cells. Each probe includes one region to selectively hybridize to its cognate target RNA sequence and a second region to act as a template for the binding and circularization of two additional oligonucleotides (termed backbone and insert). When hybridized to an adjacent probe pair, backbone and insert oligonucleotides can be ligated and amplified through RCA by phi29 polymerase to produce concatenated complementary copies of the original circle³¹. The amplified product of any probe pair can then be detected using an oligonucleotide labeled with fluorescence (for flow cytometry) or a metal (for mass cytometry) using a CyTOF instrument²⁵.

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Figure 1 | PLAYR enables the simultaneous quantification of specific transcripts and proteins in single cells. **(a)** The main steps of the PLAYR protocol. **(b)** Mass cytometry detection of transcripts for three housekeeping genes that span a wide abundance range in U937 cells. CTL, control. **(c)** Quantification of *CCL4* and *IFNG* mRNA by PLAYR and qPCR in NKL cells after stimulation with PMA-ionomycin. Measurements were performed at four time points in three replicates. Linear regression is shown (blue lines) along with the 95% confidence interval (gray shading); black circles represent individual data points. **(d)** Simultaneous quantification of *IFNG* mRNA and protein by mass cytometry in NKL cells at the indicated time points after stimulation with PMA-ionomycin.



Because both PLAYR probes must hybridize independently in order for the ligation and RCA to take place, the approach yields low background binding and high specificity. Nonspecific, off-target binding of single probes does not result in a signal. This is in contrast to FISH approaches, in which nonspecific binding of individual probes can lead to background signals that cannot be distinguished from specific RNA signals by flow cytometry. PLAYR can be multiplexed via the use of designed oligonucleotides with different insert regions that act as cognate barcodes for given transcripts³². Different insert sequences are designed to have the same melting temperatures and base compositions and form RCA products with similar efficiency (Supplementary Fig. 1). To ensure that RCA products uniquely barcode a particular transcript, the insert sequences do not have common substrings longer than 4 bases.

We developed an open-source, user-friendly R software package for the rapid design of PLAYR probes (Supplementary Fig. 2 and Supplementary Software) that includes a graphical user interface. Candidate probe pairs with similar thermodynamic properties are produced using Primer3 (ref. 33), and the application displays their location along the target transcript. BLAST matches to other transcripts or repetitive genomic sequences³⁴ and the position of nonconstitutively spliced exons are also given (additional comments regarding probe design can be found in Supplementary Fig. 2). For each gene, the user can then use these features to guide manual selection of the best probe pairs, in combination with one of the PLAYR insert systems for multiplexing. The software then outputs complete probe sequences for detecting transcripts of interest (a list of all probe and backbone-insert system sequences used in this study is presented in Supplementary Table 1).

Simultaneous single-cell quantification of proteins and RNAs
We first designed probe pairs for the β -actin gene (*ACTB*). Applying PLAYR with these probes led to a flow cytometry signal that was well above background in Jurkat T cells (Supplementary Fig. 3). No signal was observed if any component of signal generation or amplification was omitted. Similarly, no signal was observed when sense probes, probes with the same half of the insert-targeted sequence, or combinations of probes targeting different genes were used (Supplementary Fig. 3).

To further demonstrate specificity, we used one or several probe pairs designed to detect *CD10* (*MME*) and *CD3E* transcripts, which are expressed in pre-B cells and T cells, respectively (Supplementary Fig. 4). As expected, signal intensities for these transcripts increased when multiple PLAYR probe pairs were used simultaneously. In certain cases the signal increase was more than additive, which might have been a result of the formation of RCA products from nonadjacent probes on the same transcript. For instance, bound probes may be brought into proximity in unexpected manners by the structure of RNA molecules in three dimensions. Supporting this, we observed an inverse relationship between the strength of the signal and the distance in the target hybridization regions of two probes (Supplementary Fig. 4).

Using a mixture of more than one probe pair for each transcript increases signal and improves sensitivity. Using probe pairs that target different regions of a transcript also makes the results for individual genes more reproducible, as it limits variability due to differences in probe accessibility to target sites, secondary RNA structure and alternative splicing. We recommend using four or five probe pairs whenever possible, because we found that this number of probe pairs per gene generally led to reliable and robust detection of both rare and highly abundant transcripts. Fewer probes can be used if a transcript is known to be highly abundant.

Because PLAYR is used primarily to investigate the abundance of a given transcript across different biological conditions, rather than to compare expression levels of different transcripts, varying numbers of probe pairs can be used to simultaneously detect different transcripts with different expression levels. Compared to single-molecule FISH and branched DNA approaches, PLAYR uses a small number of probes, which allows for the selection of probes with ideal properties and for targeting of short transcripts. We note that carefully designed probe pairs could be used to

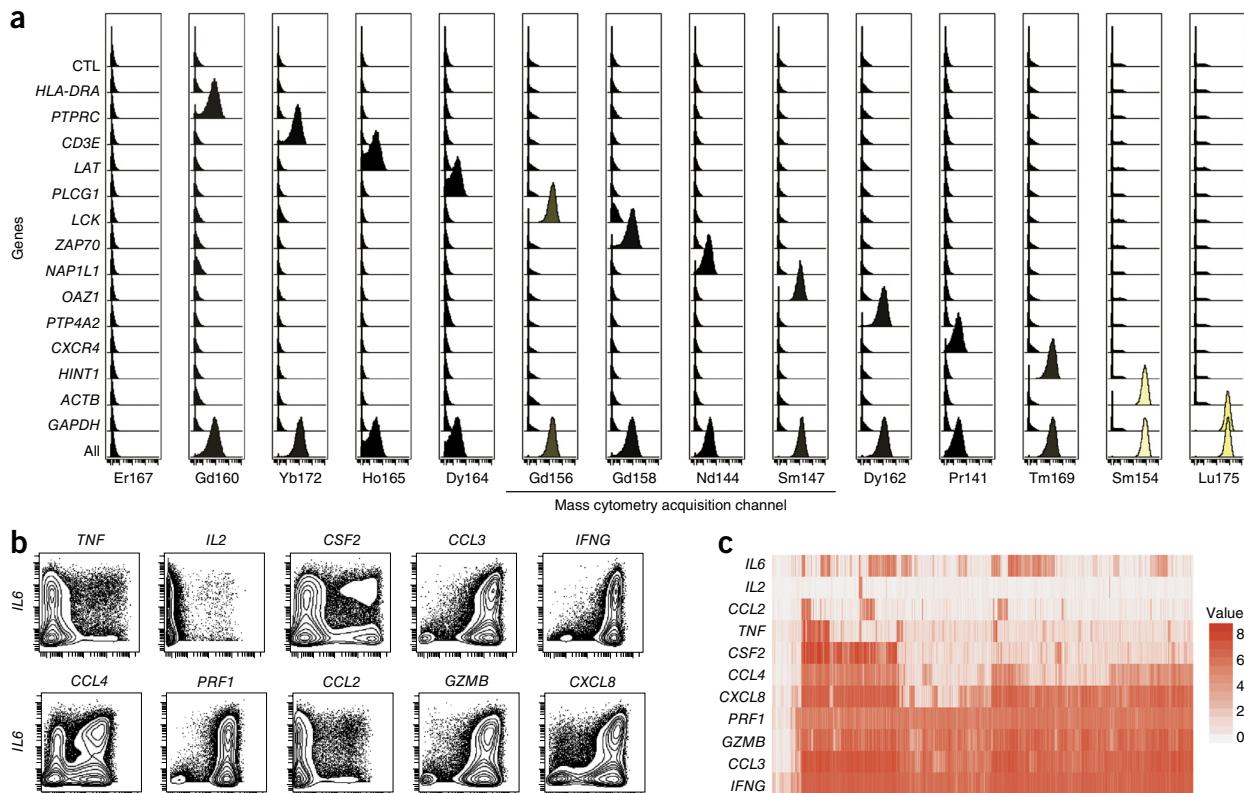


Figure 2 | Highly multiplexed measurement of different transcripts in single cells. **(a)** Mass cytometry detection of 14 transcripts encoding T cell surface markers, signaling molecules and housekeeping proteins of different abundance levels (or not expressed (*HLA-DRA*)) in Jurkat cells. Each row represents a sample to which probe pairs for the given gene or for all genes simultaneously (bottom row) were added. CTL, noncognate control probes that use the same insert system. **(b)** Contour plots showing coexpression of NKL effector transcripts in NKL cells primed with IL-2, IL-12 and IL-18 and stimulated with PMA-ionomycin for 3 h as measured by mass cytometry. **(c)** Transcript expression from a random sample of 10,000 cells from **b**. Each column corresponds to a single cell, and rows denote effector transcripts. Rows and columns are clustered for visual clarity (dendograms not shown). Experiments were run multiple times, and representative examples are shown.

delineate splice-variant complexities and genomic translocations in genes of interest.

Using five probe pairs per gene, we detected the housekeeping genes *HMBP*, *PPIB* and *GAPDH* in U937 cells by mass cytometry (Fig. 1b), demonstrating a dynamic range of detection from high-abundance (*GAPDH*) to low-abundance transcripts (*HMBP*, detected at about ten copies per U937 cell using branched DNA technology²⁴). To investigate the relationship between PLAYR signal and transcript abundance, we compared PLAYR and reverse-transcription quantitative PCR (RT-qPCR) results for the induction of the cytokines interferon- γ (encoded by *IFNG*) and chemokine ligand 4 (encoded by *CCL4*) in NKL cells at different time points after stimulation with PMA (phorbol 12-myristate 13-acetate)-ionomycin. Measurements obtained using the two platforms were correlated (R^2 values of 0.93 (*CCL4*) and 0.72 (*IFNG*)), indicating that PLAYR can be used to assess relative changes in transcript abundance across different biological conditions (Fig. 1c).

To use PLAYR for the detection of both transcripts and proteins, we optimized the protocol using conditions that preserve antibody binding. The best results were obtained when antibody staining was performed immediately after cell fixation and was followed by amine-to-amine cross-linking using the BS3 (bis(sulfosuccinimidyl)suberate) cross-linker to prevent antibodies from being washed away. This cross-linking step made PLAYR compatible with the majority of antibodies tested. We found that

transient permeabilization by the addition of 0.2% saponin in the presence of RNase inhibitors during antibody staining preserved RNA integrity (Supplementary Fig. 5) and allowed intracellular proteins to be stained with antibodies.

Using this protocol, we stimulated NKL cells with PMA-ionomycin in the presence of protein-secretion inhibitors and determined changes in interferon- γ protein and transcript levels as a function of time (Fig. 1d). Thus PLAYR allowed us to study the dynamic nature of transcription and translation at the single-cell level. Both RNA and protein expression consistently showed bimodal distributions, which suggests that not all cells in this supposedly homogeneous cell line responded equally to stimulation and highlights the potential of PLAYR to assess the functional capacity of individual cells in complex populations.

Highly multiplexed protein and transcript detection

With the insert-based multiplexing strategy (Fig. 1a), multiple targets can be detected simultaneously within individual cells. We designed probes to target 14 different transcripts and evaluated them by mass cytometry first individually and then simultaneously in Jurkat T cells (Fig. 2a). Combinations of noncognate probe pairs included as controls did not lead to observable signals even in the presence of insert-backbone oligonucleotides. Furthermore, the signal amplitude for any given target in the multiplexed sample was not affected by the presence of oligonucleotides

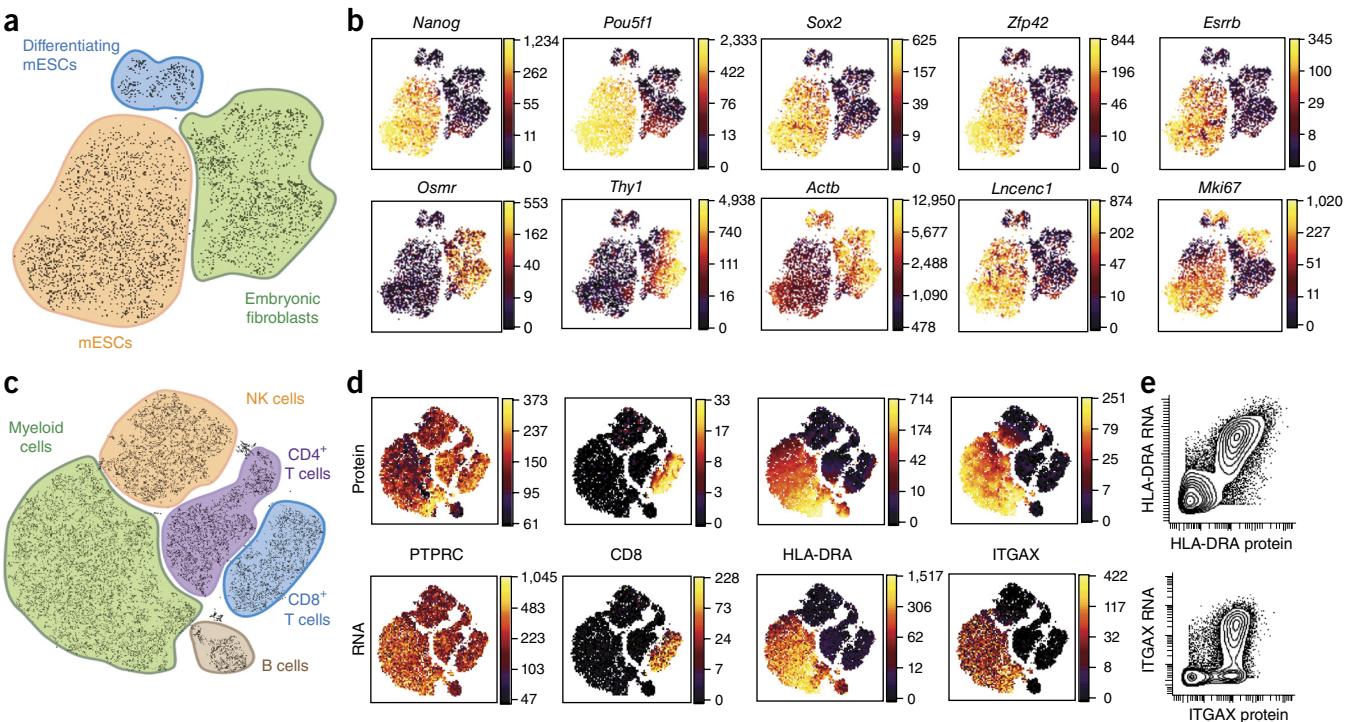


Figure 3 | Highly multiplexed measurement of transcripts in cell types defined by other transcripts or protein epitopes. **(a)** viSNE analysis of mESCs, differentiating mESCs, and embryonic fibroblasts on the basis of expression of 15 transcripts (*Cd44*, *Mki67*, *Cdh1*, *Cd47*, *Klf4*, *Esrrb*, *Actb*, *Sox2*, *Lncenc1*, *Zfp42*, *Sall4*, *Cd9*, *Pou5f1* (*Oct4*), *Thy1* and *Nanog*) with overlays showing the locations of the three cell populations. **(b)** Color-coded expression levels of selected transcripts used to construct the viSNE map in **a**. **(c)** viSNE analysis of PBMCs on the basis of expression of ten surface protein markers (CD19, CD4, CD8, CD20, protein tyrosine phosphatase receptor type C (PTPRC; also known as CD45), PTPRCRA (CD45RA), CD33, integrin- α X (ITGAX; also known as CD11c), CD3 and HLA-DRA) with overlays showing the locations of major cell populations. **(d)** Expression of selected proteins and the corresponding transcripts overlaid on the viSNE map shown in **c** and color-coded by signal intensity. **(e)** Contour plots showing correlations of protein and transcript levels for HLA-DRA and ITGAX in individual PBMCs. Experiments were run multiple times, and representative examples are shown.

against noncognate targets and corresponding amplification products. This suggests that the number of transcripts that can be quantified within a single cell is limited only by the number of reporters that can be conjugated to detection oligonucleotides and analyzed simultaneously with a given platform.

We used the multiplexing capability of PLAYR to simultaneously detect the transcripts of 11 different cytokines and other effector molecules in NKL cells that had been primed with three cytokines (interleukin-2 (IL-2), IL-12 and IL-18) and stimulated with PMA-ionomycin. Instead of a uniform cellular response, simultaneous transcript quantification revealed complex combinatorial RNA expression patterns in this supposedly homogeneous clonal cell line (Fig. 2b). To identify functional NKL subpopulations, we clustered cells on the basis of expression of induced effector transcripts, which revealed a remarkable complexity of cellular responses (Fig. 2c). A number of studies^{35,36} have shown that supposedly homogeneous cell populations in primary samples also express such a remarkable diversity of cytokine combinations. Although the functional implications are still poorly understood, the study of any such combinatorial phenomenon clearly benefits from the increased parameterization enabled by PLAYR.

Transcript measurements can be used to both define cell types and study gene expression. Experiments measuring only RNA can be set up at a fraction of the cost associated with antibody-based experiments and are not limited by the availability of antibodies for genes of interest. We analyzed an artificial mixture of cells that contained mouse embryonic fibroblasts, mouse embryonic stem cells (mESCs)

and differentiating mESCs on the basis of expression of 15 different transcripts. We then visualized the data using viSNE³⁷ and found three populations of cells in the mixture that were clearly defined on the basis of RNA expression (Fig. 3a). We were subsequently able to study different markers of pluripotency (e.g., *Nanog*), differentiation (e.g., *Thy1*), proliferation (*Mki67*) and pluripotency-associated long intergenic noncoding RNAs (*Lncenc1*) in the context of this cellular system (Fig. 3b). In an independent experiment with mESCs, we measured 22 different transcripts including negative controls in 16 technical replicates. The results demonstrated the very high reproducibility of PLAYR for both low-abundance and high-abundance transcripts (Supplementary Fig. 6).

To demonstrate the ability of PLAYR to uncover differences in RNA and protein expression and to compare the use of each data type for cell-type detection, we analyzed primary human peripheral blood mononuclear cells (PBMCs) for ten cell-surface proteins and corresponding transcripts. In this case, protein markers were first used to visualize the major cell types in peripheral blood in a viSNE³⁷ analysis (Fig. 3c). Overlaying data for the corresponding transcripts demonstrated remarkable cell-type specificity in mRNA expression patterns (Fig. 3d). In general, transcripts showed more gradual differences than the corresponding proteins, and we found protein but essentially no transcript for ITGAX in a distinct subpopulation of cells (Fig. 3e). Such discrepancies can be explained by the longer half-lives of proteins compared to transcripts, post-transcriptional regulation and the fact that transcription has been observed to occur in

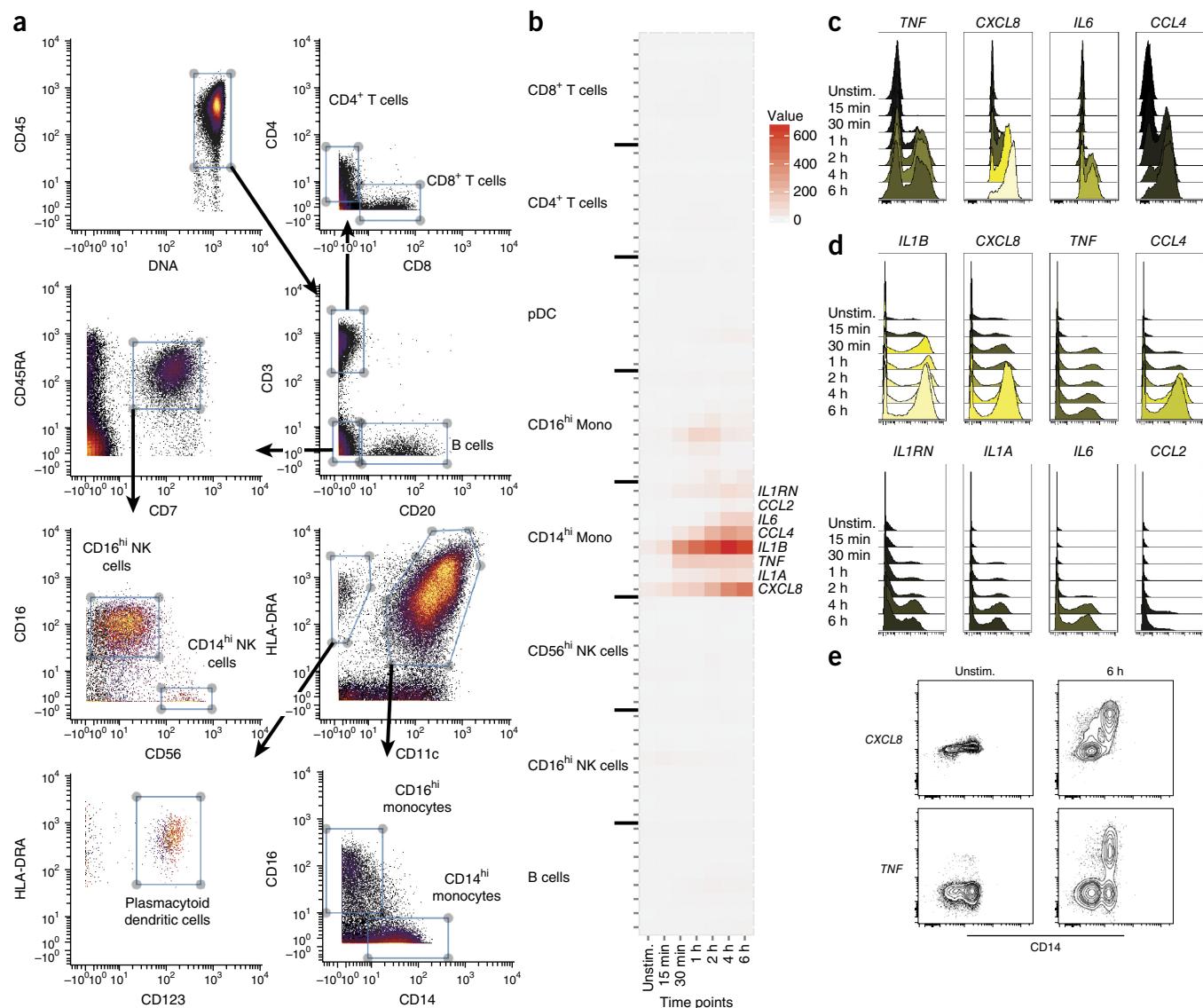


Figure 4 | Measurements of cytokine transcript induction in human PBMCs by mass cytometry and fluorescence flow cytometry. **(a)** Mass cytometry gating strategy for human PBMCs. **(b)** Mean expression values of cytokine transcripts at different time points after stimulation with LPS. pDC, plasmacytoid dendritic cells; Mono, monocytes. **(c,d)** Cytokine transcript expression as measured by flow cytometry **(c)** and mass cytometry **(d)** in CD14⁺ monocytes stimulated for the indicated lengths of time. Unstim., unstimulated. **(e)** Contour plots showing CXCL8 and TNF transcript expression in CD14⁺ monocytes as measured by flow cytometry. Experiments were run multiple times, and representative examples are shown.

bursts^{26–28}. Accordingly, this type of analysis demonstrates some of the challenges of defining cell types on the basis of individual transcripts and the potential of PLAYR for studying the relationships of transcripts and proteins within individual cells in complex primary samples.

Profiling of cytokine transcript induction in primary samples

We next used PLAYR to monitor cytokine transcript induction in PBMCs after stimulation with lipopolysaccharide (LPS) to correlate protein marker expression with the functional capacity of individual cells. Cytokine expression in single cells is traditionally evaluated on the protein level by flow cytometry after treatment with secretion inhibitors that lead to accumulation of cytokines in the cells. This approach precludes the study of (and is complicated by) paracrine effects, such as intercellular communication and feedback loops. We profiled protein markers

in human PBMCs to distinguish cell populations and monitored the expression of a panel of cytokine transcripts using PLAYR with both flow cytometry and mass cytometry. The fluorescence experiment detected four transcripts and four surface markers, whereas mass cytometry allowed for the simultaneous quantification of eight transcripts and 18 protein epitopes.

In both experiments, antibody staining enabled gating of distinct cell populations (gating is shown for mass cytometry in **Fig. 4a** and for flow cytometry in **Supplementary Fig. 7**). As expected, cytokine production was restricted to the monocyte compartment and therein was confined mostly to cells that expressed the LPS coreceptor CD14 (**Fig. 4b**). Moreover, different cytokines consistently exhibited distinct expression dynamics. For example, tumor necrosis factor- α (encoded by *TNF*) and IL-8 (encoded by *CXCL8*) were induced early, and expression of the former peaked between 2 and 4 h after induction, whereas expression of the latter continued

Figure 5 | Single-cell-resolution map of cytokine induction in human PBMCs. PBMCs were stimulated with LPS and analyzed after 4 h. Cells were analyzed with antibodies to cell-surface proteins (CD19, CD38, CD4, CD8, CD7, CD14, IL-3RA (CD123), PTPRC, PTPRCRA, CD33, ITGAX, FCGR3A (CD16), CD3, CD20, HLA-DRA, NCAM1 (CD56) and phosphorylation sites phospho-p38 MAPK (pT180/pY182) and pERK1/2 (pT202/pY204)). (a) viSNE map based on cell-surface marker expression with overlays showing the locations of major cell populations. (b) Selected protein markers used to define myeloid cell populations and MAPK signaling, color-coded by expression level. (c) Measurements for eight different cytokine transcripts overlaid and color-coded by expression level. The experiment was run multiple times, and a representative example is shown.

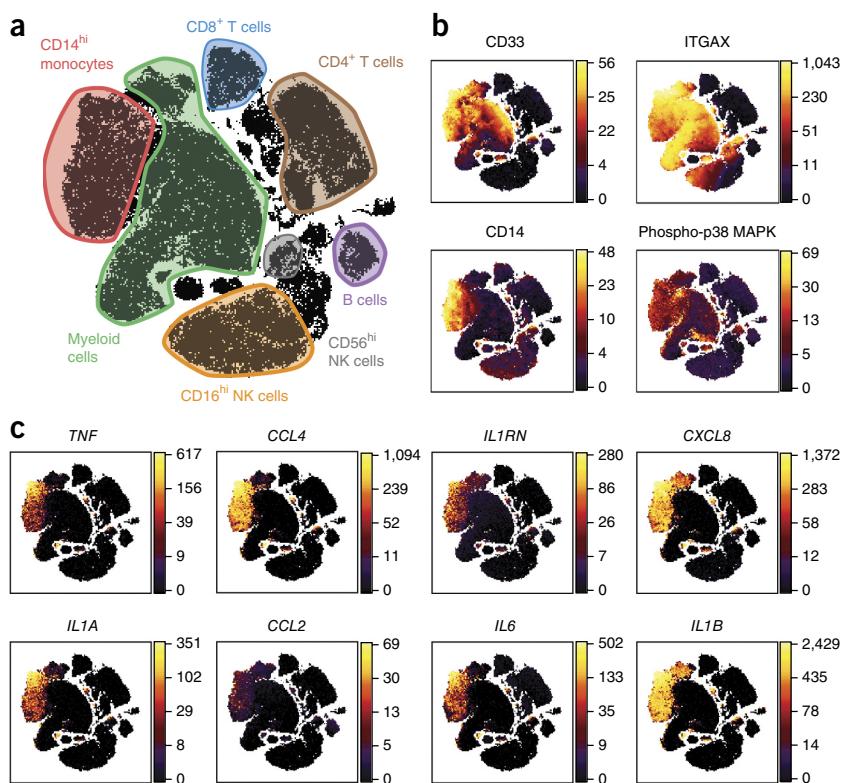
to increase during the entire time course. Conversely, expression of IL-6 (encoded by *IL6*) was strongly induced only after 4 h (Fig. 4c,d). These results recapitulated previous individual observations^{38,39} and confirmed that PLAYR effectively detects RNA expression in specific cellular subpopulations. Interestingly, whereas CXCL8 at its peak was expressed in the entire CD14⁺ monocyte compartment, a distinct population of CD14⁺ cells did not express TNF (Fig. 4e), underscoring the utility of protein and RNA codetection for identifying functional differences.

Analysis of the high-dimensional CyTOF data for the cytokine-induction experiment demonstrated that all major PBMC populations clustered in distinct areas of a viSNE map (Fig. 5a) and could be identified on the basis of the restricted expression of canonical markers (Fig. 5b). Similarly, MAP kinase signaling could be monitored on the basis of p38 MAP kinase phosphorylation and was restricted to the myeloid compartment. When we overlaid cytokine transcript expression on the map, we noted that cells that responded to LPS were mostly restricted to the region of CD14⁺ monocytes (Fig. 5c). Thus this analysis provided a single-cell-resolution map of cytokine induction and MAP kinase signaling in PBMCs for transcriptional network analysis.

DISCUSSION

PLAYR enables highly multiplexed measurement of gene expression in hundreds to thousands of intact cells per second. On the protein level, single-cell measurements have prognostic and diagnostic value in multiple clinical settings^{40–43}. PLAYR extends such analyses to include transcript measurements and can supplement the use of antibodies, especially where exon-specific expression is concerned and no relevant antibody reagents exist. Immediate measurement of mRNA can overcome issues introduced with *ex vivo* processing of live cells, and experimental artifacts are minimized, as PLAYR detects RNA molecules directly without the need for cDNA synthesis⁴⁴.

PLAYR can be used to characterize the interplay between transcription and translation at the single-cell level. Other applications include the clustering of complex cellular populations purely



on the basis of transcript abundance, which could be particularly useful when high-quality antibodies are unavailable. We believe that such an approach will help to define new cell populations that share patterns of temporally or spatially regulated RNA expression. Of relevance to this last point, PLAYR can be deployed for index sorting^{45,46} and imaging approaches such as fluorescence microscopy and multiplexed ion beam imaging⁴⁷, making it a flexible tool for studying gene expression in single intact cells on a variety of platforms.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the [online version of the paper](#).

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AUTHOR CONTRIBUTIONS

A.P.F., F.-A.B. and P.F.G. conceived the work, performed experiments, analyzed data and wrote the manuscript. E.R.Z. provided help with mouse embryonic stem

cell experiments. E.W.Y.H. and S.-Y.C. provided help with cytokine induction experiments. G.P.N. supervised the work and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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1. Jaitin, D.A. *et al.* Massively parallel single-cell RNA-seq for marker-free decomposition of tissues into cell types. *Science* **343**, 776–779 (2014).
2. Hashimshony, T., Wagner, F., Sher, N. & Yanai, I. CEL-Seq: single-cell RNA-Seq by multiplexed linear amplification. *Cell Rep.* **2**, 666–673 (2012).
3. Islam, S. *et al.* Highly multiplexed and strand-specific single-cell RNA 5' end sequencing. *Nat. Protoc.* **7**, 813–828 (2012).
4. Islam, S. *et al.* Quantitative single-cell RNA-seq with unique molecular identifiers. *Nat. Methods* **11**, 163–166 (2014).
5. Ransköld, D. *et al.* Full-length mRNA-seq from single-cell levels of RNA and individual circulating tumor cells. *Nat. Biotechnol.* **30**, 777–782 (2012).
6. Sasagawa, Y. *et al.* Quartz-Seq: a highly reproducible and sensitive single-cell RNA sequencing method, reveals non-genetic gene-expression heterogeneity. *Genome Biol.* **14**, R31 (2013).
7. Shalek, A.K. *et al.* Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. *Nature* **498**, 236–240 (2013).
8. Wu, A.R. *et al.* Quantitative assessment of single-cell RNA-sequencing methods. *Nat. Methods* **11**, 41–46 (2014).
9. Deng, Q., Ransköld, D., Reinius, B. & Sandberg, R. Single-cell RNA-seq reveals dynamic, random monoallelic gene expression in mammalian cells. *Science* **343**, 193–196 (2014).
10. Picelli, S. *et al.* Full-length RNA-seq from single cells using Smart-seq2. *Nat. Protoc.* **9**, 171–181 (2014).
11. Tang, F., Lao, K. & Surani, M.A. Development and applications of single-cell transcriptome analysis. *Nat. Methods* **8**, S6–S11 (2011).
12. Fan, H.C., Fu, G.K. & Fodor, S.P.A. Expression profiling. Combinatorial labeling of single cells for gene expression cytometry. *Science* **347**, 1258367 (2015).
13. Dvinge, H. *et al.* Sample processing obscures cancer-specific alterations in leukemic transcriptomes. *Proc. Natl. Acad. Sci. USA* **111**, 16802–16807 (2014).
14. Grün, D. & van Oudenaarden, A. Design and analysis of single-cell sequencing experiments. *Cell* **163**, 799–810 (2015).
15. Bauman, J.G., Bayer, J.A. & van Dekken, H. Fluorescent in-situ hybridization to detect cellular RNA by flow cytometry and confocal microscopy. *J. Microsc.* **157**, 73–81 (1990).
16. Patterson, B.K. *et al.* Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. *Science* **260**, 976–979 (1993).
17. Belloc, F. & Durrieu, F. Detection of mRNA species by flow cytometry. *Methods Cell Biol.* **42**, 59–69 (1994).
18. Borzi, R.M. *et al.* A fluorescent in situ hybridization method in flow cytometry to detect HIV-1 specific RNA. *J. Immunol. Methods* **193**, 167–176 (1996).
19. Lalli, E., Gibellini, D., Santi, S. & Facchini, A. In situ hybridization in suspension and flow cytometry as a tool for the study of gene expression. *Anal. Biochem.* **207**, 298–303 (1992).
20. Just, T., Burgwald, H. & Broe, M.K. Flow cytometric detection of EBV (EBER snRNA) using peptide nucleic acid probes. *J. Virol. Methods* **73**, 163–174 (1998).
21. Larsson, C., Grundberg, I., Söderberg, O. & Nilsson, M. *In situ* detection and genotyping of individual mRNA molecules. *Nat. Methods* **7**, 395–397 (2010).
22. Weibrech, I. *et al.* *In situ* detection of individual mRNA molecules and protein complexes or post-translational modifications using padlock probes combined with the *in situ* proximity ligation assay. *Nat. Protoc.* **8**, 355–372 (2013).
23. Player, A.N., Shen, L.P., Kenny, D., Antao, V.P. & Kolberg, J.A. Single-copy gene detection using branched DNA (bDNA) *in situ* hybridization. *J. Histochem. Cytochem.* **49**, 603–612 (2001).
24. Porichis, F. *et al.* High-throughput detection of miRNAs and gene-specific mRNA at the single-cell level by flow cytometry. *Nat. Commun.* **5**, 5641 (2014).
25. Bendall, S.C. *et al.* Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science* **332**, 687–696 (2011).
26. Lin, Y., Sohn, C.H., Dalal, C.K., Cai, L. & Elowitz, M.B. Combinatorial gene regulation by modulation of relative pulse timing. *Nature* **527**, 54–58 (2015).
27. Chubb, J.R., Trcek, T., Shenoy, S.M. & Singer, R.H. Transcriptional pulsing of a developmental gene. *Curr. Biol.* **16**, 1018–1025 (2006).
28. Cai, L., Friedman, N. & Xie, X.S. Stochastic protein expression in individual cells at the single molecule level. *Nature* **440**, 358–362 (2006).
29. Fredriksson, S. *et al.* Protein detection using proximity-dependent DNA ligation assays. *Nat. Biotechnol.* **20**, 473–477 (2002).
30. Söderberg, O. *et al.* Direct observation of individual endogenous protein complexes *in situ* by proximity ligation. *Nat. Methods* **3**, 995–1000 (2006).
31. Lizardi, P.M. *et al.* Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat. Genet.* **19**, 225–232 (1998).
32. Leuchowius, K.-J. *et al.* Parallel visualization of multiple protein complexes in individual cells in tumor tissue. *Mol. Cell. Proteomics* **12**, 1563–1571 (2013).
33. Untergasser, A. *et al.* Primer3—new capabilities and interfaces. *Nucleic Acids Res.* **40**, e115 (2012).
34. Jurka, J. *et al.* Repbase Update, a database of eukaryotic repetitive elements. *Cytogenet. Genome Res.* **110**, 462–467 (2005).
35. O’Gorman, W.E. *et al.* Single-cell systems-level analysis of human Toll-like receptor activation defines a chemokine signature in patients with systemic lupus erythematosus. *J. Allergy Clin. Immunol.* **136**, 1326–1336 (2015).
36. Newell, E.W., Sigal, N., Bendall, S.C., Nolan, G.P. & Davis, M.M. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8+ T cell phenotypes. *Immunity* **36**, 142–152 (2012).
37. Amir, A.-D. *et al.* t-SNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nat. Biotechnol.* **31**, 545–552 (2013).
38. DeForge, L.E. & Remick, D.G. Kinetics of TNF, IL-6, and IL-8 gene expression in LPS-stimulated human whole blood. *Biochem. Biophys. Res. Commun.* **174**, 18–24 (1991).
39. Agarwal, S., Piesco, N.P., Johns, L.P. & Riccelli, A.E. Differential expression of IL-1 beta, TNF-alpha, IL-6, and IL-8 in human monocytes in response to lipopolysaccharides from different microbes. *J. Dent. Res.* **74**, 1057–1065 (1995).
40. Irish, J.M. *et al.* Single cell profiling of potentiated phospho-protein networks in cancer cells. *Cell* **118**, 217–228 (2004).
41. Gaudilliére, B. *et al.* Clinical recovery from surgery correlates with single-cell immune signatures. *Sci. Transl. Med.* **6**, 255ra131 (2014).
42. Jaye, D.L., Bray, R.A., Gebel, H.M., Harris, W.A.C. & Waller, E.K. Translational applications of flow cytometry in clinical practice. *J. Immunol.* **188**, 4715–4719 (2012).
43. Kaleem, Z. *et al.* Flow cytometric analysis of acute leukemias. Diagnostic utility and critical analysis of data. *Arch. Pathol. Lab. Med.* **127**, 42–48 (2003).
44. Picelli, S. *et al.* Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nat. Methods* **10**, 1096–1098 (2013).
45. Schulte, R. *et al.* Index sorting resolves heterogeneous murine hematopoietic stem cell populations. *Exp. Hematol.* **43**, 803–811 (2015).
46. Wilson, N.K. *et al.* Combined single-cell functional and gene expression analysis resolves heterogeneity within stem cell populations. *Cell Stem Cell* **16**, 712–724 (2015).
47. Angelo, M. *et al.* Multiplexed ion beam imaging of human breast tumors. *Nat. Med.* **20**, 436–442 (2014).

ONLINE METHODS

Tissue culture. Jurkat E6-1 (ATCC; TIP-152), NALM-6 (DSMZ; ACC128), and NKL (a gift from Dr. Lewis Lanier, UCSF) cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS (Omega Scientific), 100 U/mL penicillin (Life Technologies), 100 µg/mL streptomycin (Life Technologies) and 2 mM L-glutamine (Life Technologies) at 37 °C with 5% CO₂. In addition, 200 U/ml of rhIL-2 (NCI Biological Resources Branch) was added to NKL cell cultures. For measurements of individual cytokine transcripts (Fig. 1), NKL cells were treated with 1× protein transport inhibitor cocktail (eBioscience) and 1× cell stimulation cocktail (eBioscience). For combinatorial measurements of cytokine transcripts (Fig. 2), NKL cells were primed with 200 U/ml of rhIL-2, 10 ng/mL rhIL-12 (Peprotech), and 20 ng/mL rhIL-18 (R&D Systems) for 24 h and treated with 150 ng/ml PMA (Sigma-Aldrich) plus 1 µM ionomycin (Sigma-Aldrich) for 3 h in the presence of 1× Brefeldin A (eBioscience) and 1× monensin (eBioscience). Mouse embryonic fibroblasts were prepared as described elsewhere⁴⁸ and cultured in DMEM (Life Technologies), 10% FBS, 2-mercaptoethanol (Sigma-Aldrich), 1 mM sodium pyruvate (Life Technologies), 1× non-essential amino acids (Life Technologies), 100 U/mL penicillin and 100 µg/mL streptomycin. Mouse embryonic stem cells (ATCC; CRL18-21) were grown on gelatin-coated plates in DMEM, 10% FBS, 2-mercaptoethanol, 1 mM sodium pyruvate, 1× non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, 1,000 U/mL LIF (ESGRO, EMD Millipore), and 1× 2i (MEK/GSK3 inhibitor supplement, EMD Millipore). Differentiation of embryonic stem cells was induced by withdrawal of 2i and LIF from the culture medium for 2 d. Human peripheral blood was purchased from the Stanford Blood Bank and was collected according to a Stanford University Institutional Review Board-approved protocol. PBMCs were separated from whole blood with Ficoll (Thermo) and cryopreserved in liquid nitrogen. For analysis, PBMCs were thawed, washed with complete RPMI medium, and allowed to rest for 30 min at 37 °C under 5% CO₂ in complete RPMI medium. PBMCs were stimulated with LPS (InvivoGen) at a concentration of 10 ng/mL in complete RPMI medium under gentle agitation. All cell lines used in this study were obtained from the indicated suppliers and were not further tested for mycoplasma contamination.

Cell fixation, permeabilization and antibody staining. Cells at a density of ~1 × 10⁶/mL were fixed in RPMI medium without serum in 1.6% paraformaldehyde (Electronic Microscopy Sciences) for 10 min at room temperature under gentle agitation as described previously⁴⁹. For detection of protein epitopes, cells were stained with antibodies in PBS (Life Technologies) supplemented with 5 mg/mL UltraPure BSA (Life Technologies), 0.2% saponin (Sigma-Aldrich), 2.5% vol/vol polyvinylsulfonic acid (Polysciences), and 40 U/mL RNasin (Promega) for 30 min at room temperature. After washing, antibodies were cross-linked to the cells with 5 mM bis(sulfosuccinimidyl) suberate (Pierce) in a buffer containing PBS, 0.2% saponin, and 40 U/mL RNasin for 30 min at room temperature at a density of ~20 × 10⁶ cells/mL. Glycine was added to a final concentration of 100 mM, and samples were incubated for 5 min. Cells were pelleted and permeabilized with ice-cold methanol for at least 10 min on ice. Once in methanol, cells can be stored at -80 °C for several weeks without

loss of antibody signal or RNA degradation. For detection of RNA only, cells were permeabilized in ice-cold methanol immediately after fixation with paraformaldehyde. For mass cytometry, antibodies to the following were purchased from Fluidigm: CD19 (HIB19), CD38 (HIT2), CD4 (RPA-T4), CD8 (RPA-T8), CD7 (CD7-6B7), CD14 (RMO52), CD123 (6H6), CD45 (HI30), CD45RA (HI100), CD33 (WM53), CD11c (Bu15), CD16 (3G8), CD3 (UCHT1), CD20 (2H7), HLA-DRA (L243), CD56 (NCAM 16.2) and phosphorylation sites pP38 MAPK (pT180/pY182) and pERK1/2 (pT202/pY204). Antibodies to the following were used for flow cytometry: CD3 (UCHT1, Bv510, BioLegend), CD7 (M-T701, Alexa700, BD), CD16 (3G8, Bv605, BioLegend), CD14 (HCD14, Bv421, BioLegend), BrdU (Bu20a, PE, BioLegend) and biotin (streptavidin, PE-Cy7, BD).

PLAYR protocol. PLAYR probes were designed using the PLAYRDesign software developed in-house (available at <https://github.com/nolanlab/PLAYRDesign>). PLAYR probes were synthesized at the Stanford Protein and Nucleic Acid Facility and resuspended in diethylpyrocarbonate (DEPC)-treated water at a concentration of 100 µM. The carrier solution for most of the protocol steps, including washes, was PBS, 0.1% Tween (Sigma-Aldrich) and 4 U/mL RNasin. Paraformaldehyde-fixed and methanol-permeabilized cells (see above) were pelleted by centrifugation at 600g for 3 min. Hybridizations with PLAYR probes were performed in a buffer based on DEPC-treated water (Life Technologies) containing 1× saline-sodium citrate (SSC) (Affymetrix), 2.5% vol/vol polyvinylsulfonic acid, 20 mM ribonucleoside vanadyl complex (New England BioLabs), 40 U/mL RNasin, 1% Tween, and 100 µg/mL salmon sperm DNA (Life Technologies). PLAYR probes for all target transcripts of an experiment were mixed and heated to 90 °C for 5 min. Probes were then chilled on ice and added to cells in hybridization buffer at a final concentration of 100 nM. Cells were incubated for 1 h at 40 °C under vigorous agitation and subsequently washed three times. Cells were then incubated for 20 min in a buffer containing PBS, 4× SSC and 40 U/mL RNasin at 40 °C under vigorous agitation. Samples to be analyzed by mass cytometry were barcoded at this step as described previously⁵⁰. After two washes, cells were incubated with 100 nM insert/backbone oligonucleotides in PBS, 1× SSC and 40 U/mL RNasin for 30 min at 37 °C. After two washes, cells were incubated for 30 min with T4 DNA ligase (Thermo) at room temperature with gentle agitation and then for 2 h (flow cytometry) or 4 h (mass cytometry) with phi29 DNA polymerase (Thermo) at 30 °C under agitation. Longer amplification (up to 16 h) generally increases signal intensity. Both enzymes were used according to their respective manufacturers' instructions, with the addition of 40 U/mL RNasin. For flow cytometry, cells were incubated with detection oligonucleotides at a concentration of 5 nM for 30 min at 37 °C in PBS, 1× SSC, 0.1% Tween and 40 U/mL RNasin. Two fluorophore-conjugated (Alexa Fluor 488 and Alexa Fluor 647) oligonucleotides were used as detection probes. Also used were a biotinylated oligonucleotide and an oligonucleotide labeled with a single BrdU nucleotide at the 5' end; cells were then incubated with phycoerythrin-labeled Cy7-streptavidin or a BrdU-phycoerythrin antibody conjugate as appropriate. For mass cytometry, cells were incubated with metal-conjugated detection oligonucleotides at a concentration of 10 nM for 30 min at 37 °C in PBS, 5 mg/mL BSA, 0.02% sodium azide. After washing,

cells were either processed immediately on a fluorescence-based flow cytometer or further processed for CyTOF acquisition as described elsewhere⁵⁰.

Preparation of metal-conjugated detection oligonucleotides.

Maleimide-activated Maxpar metal-chelating X8 polymers (Fluidigm, Maxpar labeling kit) were loaded with metals and purified using centrifugal filters as per the manufacturer's instructions. Detection oligonucleotides carrying a 5' Thiol-Modifier C6 S-S (Glen Research) were synthesized at the Stanford Protein and Nucleic Acid Facility. Oligonucleotides were resuspended in DEPC-treated water at 250 μM, and the thiol was reduced by treatment with 50 mM TCEP (Pierce) for 30 min at room temperature. After ethanol precipitation, oligonucleotides were resuspended in C buffer (Fluidigm, Maxpar labeling kit), and conjugation reactions were performed with 2 nmol of oligonucleotide per reaction with X8 polymer. After 2 h at room temperature, TCEP was added to a final concentration of 5 mM, and samples were incubated for 30 min to reduce unconjugated oligonucleotides. Conjugates were filtered through 30-kDa centrifugal filter

units (EMD Millipore) in a total of 500 μl of water, spun at 14,000g for 12 min, and washed twice with DEPC-treated water (Life Technologies). Purified detection oligonucleotide conjugates were resuspended in DEPC-treated water at a concentration of 1 μM and stored at 4 °C.

RT-qPCR. RNA was extracted using an RNeasy Plus micro kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using the SuperScript III first-strand synthesis system (Life Technologies) according to the manufacturer's instructions. PCR was carried out in a LightCycler 480 II (Roche) using SYBRGreen I Master (Roche).

48. Wernig, M. *et al.* A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. *Nat. Biotechnol.* **26**, 916–924 (2008).
49. Krutzik, P.O. & Nolan, G.P. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry A* **55**, 61–70 (2003).
50. Zunder, E.R. *et al.* Palladium-based mass tag cell barcoding with a doublet-filtering scheme and single-cell deconvolution algorithm. *Nat. Protoc.* **10**, 316–333 (2015).