HHS Public Access

Author manuscript

Nat Methods. Author manuscript; available in PMC 2016 July 25.

Published in final edited form as:

Nat Methods. 2016 March ; 13(3): 269–275. doi:10.1038/nmeth.3742.

Highly multiplexed simultaneous detection of RNAs and proteins in single cells

Andreas P Frei[1,4], **Felice Alessio Bava**[1,4], **Eli R Zunder**[1,2], **Elena WY Hsieh**[1,3], **Shih-Yu Chen**[1], **Garry P Nolan**[1], and **Pier Federico Gherardini**[1]

[1]Baxter Laboratory in Stem Cell Biology, Department of Microbiology and Immunology, Stanford University, Stanford, California, USA

[2]Department of Biomedical Engineering, University of Virginia, Charlottesville, Virginia, USA

[3]Department of Pediatrics and Department of Immunology and Microbiology, University of Colorado Denver, Denver, Colorado, USA

Abstract

Precise gene expression measurement has been fundamental to developing an advanced understanding of the roles of biological networks in health and disease. To enable detection of expression signatures specific to individual cells we developed PLAYR (Proximity Ligation Assay for RNA). PLAYR enables highly multiplexed quantification of transcripts in single cells by flow- and mass-cytometry and is compatible with standard antibody staining of proteins. With mass cytometry, this currently enables simultaneous quantification of more than 40 different mRNAs and proteins. The technology was demonstrated in primary cells to be capable of quantifying multiple gene expression transcripts while the identity and the functional state of each analyzed cell was defined based on the expression of other transcripts or proteins. PLAYR now enables high throughput deep phenotyping of cells to readily expand beyond protein epitopes to include RNA expression, thereby opening a new venue on the characterization of cellular metabolism.

Introduction

Biological systems operate through the functional interaction and coordination of multiple cell types. Whether one is trying to delineate the complexity of an immune response, or characterize the intrinsic cellular diversity of cancer, the ability to perform single-cell measurements of gene expression within such complex samples can lead to a better understanding of system-wide interactions and overall function.

Correspondence should be addressed to G.P.N. (; Email: gnolan@stanford.edu) or P.F.G. (; Email: pfg@stanford.edu)

[4]These authors contributed equally to this work.

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.

Statement of Competing Financial Interests

G.P.N. has a personal financial interest in the company Fluidigm, the manufacturer of the mass cytometer used in this manuscript.

A current method of choice for study of transcript expression in individual cells is single-cell RNA-seq. This approach involves physical separation of cells, followed by lysis and library preparation with protocols that have been optimized for small amounts of input RNA[1–11]. Barcoding of physically separated cells before sequence analysis makes possible the analysis of thousands of individual cells in a single experiment[12]. However, sample handling (such as separation of live cells before lysis) has been shown to induce significant alterations in the transcriptome[13]. Moreover RNA-seq requires cDNA synthesis and does not enable simultaneous detection of protein epitopes and transcripts. The complexity of protocols and the associated costs further limit the applicability of this technology in studies where sample throughput is essential. Finally, the number of cells that can be analyzed is limited by the overall sequencing depth available. These limitations notwithstanding, the possibility of taking a genome-wide approach to the study of gene expression in single cells, coupled with precise quantification through the use of Unique Molecular Identifiers, make single-cell RNA-seq an exceptionally promising technology[14].

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. For such a purpose, fluorescence *in situ* hybridization (FISH) protocols have been adapted to quantify gene expression on cytometry platforms[15–20]. In such experiments bright FISH signals with excellent signal-to-noise ratios are necessary since flow cytometry does not provide the subcellular imaging resolution necessary to distinguish individual RNA signals from diffuse background. Different techniques have been adapted for the generation and amplification of specific hybridization signals including DNA padlock probes in combination with rolling circle amplification (RCA)[21,22] or branched DNA technology[23]. Recently the branched DNA approach has been successfully applied to flow cytometry[24] but the availability of only three non-interfering branched DNA amplification systems and the spectral overlap of fluorescent reporters complicates multiplexing. What was missing for higher parameter purposes was a technology that allowed full access to the parameterization enabled by mass cytometry[25] and also allowed for protein epitopes to be simultaneously measured.

The Proximity Ligation Assay for RNA (PLAYR) system as described here addresses these limitations by enabling routine analyses of thousands of cells per second by flow cytometric approaches and simultaneous detection of protein epitopes and multiple RNA targets. The method preserves the native state of cells in the first step of the protocol, detects transcripts in intact cells without the need for cDNA synthesis, and is compatible with flow cytometry, mass cytometry, as well as microscope-based imaging systems. Making use of the different measurement channels of mass cytometry, this enables the simultaneous quantitative acquisition of more than 40 different proteins and RNAs. Thus, PLAYR adds a unique and flexible capability to the growing list of technologies that merge ‘omics datasets (transcript, protein, and signaling levels) in single cells. 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 the technology and PLAYR probe design

PLAYR uses the concept of proximity ligation[29,30] to detect individual transcripts in single cells, as shown schematically in Fig. 1a, and is compatible with immunostaining. Pairs of DNA oligonucleotide probes (probe pairs) are designed to hybridize to two adjacent regions of target transcripts in fixed and permeabilized cells. Each probe in a pair is composed of two regions with distinct function. The role of the first region is to selectively hybridize to its cognate target RNA sequence. The second region, separated from the first by a short spacer, acts as template for the binding and circularization of two additional oligonucleotides (termed *backbone* and *insert*). When hybridized to an adjacent probe pair, the backbone and insert oligonucleotides form a single-stranded DNA circle that can be ligated. The ligated, closed circle is then amplified through rolling circle amplification by phi29 polymerase initiated by the 3′ OH of one member of the probe pair. As phi29 continues to polymerize, it creates a linear molecule that contains hundreds of concatenated complementary copies of the original circle[31]. Then, using a labeled oligonucleotide that is complementary to the insert region of the amplicon, one can detect any given probe pair through binding to the amplified product. For analysis by flow cytometry fluorescently labeled oligonucleotides are used for detection. Alternatively, metal-conjugated oligonucleotides enable mass cytometric analyses using a CyTOF instrument[25].

Lowering of background binding events and increased specificity result from the fact that both PLAYR probes must hybridize independently to adjacent locations of a target RNA in order for the two independent ligation events and subsequent RCA to take place. Non-specific, off-target binding of single probes does not result in a signal. This is in contrast to FISH approaches, where many different labeled oligonucleotides bind to transcripts directly and any unspecific binding of individual probes can lead to background signals that cannot be distinguished from specific RNA signals using flow cytometry platforms. PLAYR can be multiplexed by designing oligonucleotides with different insert regions that act as cognate barcodes for given transcripts[32]. Different insert sequences are designed to have the same melting temperatures and base compositions and have been found to lead to the formation of RCA products with similar efficiency (Supplementary Fig. 1). To ensure that the resulting RCA products uniquely barcode a particular transcript the insert sequences do not have common substrings longer than 4 bases.

An open-source R software package has been developed for rapid design of PLAYR probes (Supplementary Fig. 2 and Supplementary Software). The package comes with point-by-point installation instructions and includes a graphical user interface front end for user-friendly probe design. Candidate probe pairs with similar thermodynamic properties are first produced using the Primer3 software[33]. The application then displays the location of the probes along the target transcript sequence and other characteristics including BLAST matches to other transcripts or to repetitive sequences of the genome[34] and the position of non-constitutively spliced exons (see Supplementary Fig. 2 for additional comments regarding probe design). These features are used to guide the selection of specific probe pairs. For each gene, the user can then manually select the best probe pairs in combination

with one of the PLAYR insert systems for multiplexing. Based on these selection criteria the software outputs the complete sequences of probes that can be used to detect transcripts of interest. The sequences of all probes and backbone-insert systems used in this manuscript can be found in Supplementary Table 1.

Simultaneous quantification of proteins and RNAs in single cells

In a first experiment probe pairs specific for *beta-actin* (*ACTB*) were designed. In Jurkat T cells that had been fixed and permeabilized, the PLAYR protocol led to a signal that was detected well above background by flow cytometry (Supplementary Fig. 3). No signal was observed if any component of the signal generation or amplification cascade 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 the specificity of the approach, the protocol was used with one or several probe pairs designed to detect *CD10* and *CD3E* transcripts, which are known to be 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. Interestingly, the resulting signal increase was in certain cases more than additive. This may be due to formation of RCA products generated from probes of two different pairs on the same transcript even though the target regions of the two probes are not adjacent as they would be for a cognate pair. For instance, bound probes may be brought into proximity in unexpected manners by the structure of RNA molecules in three dimensions. Supporting this there was an inverse relationship between the strength of the signal and the distance in the target hybridization regions of two probes (Supplementary Fig. 4). Using more than one probe pair per transcript simultaneously as a mixture leads to an increase in signal and improves the sensitivity of the method. Using multiple probe pairs that target different regions of a transcript also makes 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.

In general we found that a mixture of four or five probe pairs per gene led to reliable and robust detection of both rare and highly abundant transcripts and we recommend using four or five probe pairs whenever possible. Fewer probes can be used if a transcript is known to be highly abundant. Since PLAYR is primarily used to compare 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, the small number of probes used in PLAYR experiments allows for selection of probes with ideal properties and for targeting of short transcripts. Though we did not apply the principle in this report, we note that careful design of probe pairs could be used to delineate splice variant complexities and genomic translocations in genes of interest.

Using 5 probe pairs per gene we detected the three housekeeping genes *HMBS*, *PPIB*, and *GAPDH* in U937 cells by mass cytometry (Fig. 1b). This application demonstrates a dynamic range of PLAYR that enables the detection of highly abundant transcripts (*GAPDH*) as well as low abundant transcripts (*HMBS*) that have been detected at only about

10 copies per U937 cell using branched DNA technology[24]. To further investigate to what extent PLAYR signals correlate with the underlying abundance of a transcript, results obtained with PLAYR and with RT-qPCR were compared for the induction of the cytokines interferon-γ (*IFNG*) and chemokine ligand 4 (*CCL4)* in NKL cells at different time points after stimulation with PMA-ionomycin. As shown in Fig. 1c, PLAYR and qPCR measurements 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.

An additional requirement in the PLAYR protocol was the simultaneous detection of transcripts and proteins. The protocol was therefore optimized using conditions that preserve binding of antibodies. Best results were obtained when antibody staining was performed immediately after cell fixation (i.e. at the beginning of the protocol). After antibody staining, amine-to-amine crosslinking using the BS^3 crosslinker was used to prevent antibodies from being washed away during the procedure. This crosslinking step made PLAYR compatible with the majority of antibodies tested. We found that transient permeabilization of cells by the addition of 0.2% saponin in the presence of RNase inhibitors during antibody staining greatly enhanced the preservation of RNA integrity (Supplementary Fig. 5). Furthermore, this transient permeabilization can be leveraged to stain intracellular proteins with antibodies. Using this protocol NKL cells were stimulated with PMA-ionomycin, in presence of protein-secretion inhibitors, and changes in IFNG protein and transcript levels were determined as a function of time (Fig. 1d). Thus, PLAYR allows studies of the dynamic nature of transcription and translation at the single-cell level. Both RNA and protein expression consistently showed bimodal distributions, which suggested that not all cells in this supposedly homogeneous cell line responded equally to stimulation and highlighted the potential of PLAYR to assess the functional capacity of individual cells within complex populations.

Highly multiplexed detection of specific proteins and transcripts

Using the insert-based multiplexing strategy illustrated in Fig. 1a, multiple targets can be detected simultaneously within individual cells. We designed probes to target 14 different transcripts and first evaluated them individually and then simultaneously in Jurkat T cells by mass cytometry (Fig. 2a). For this experiment cells were incubated either with probes against individual transcripts or with a mixture of all probes. Appropriate control combinations of non-cognate probe pairs were included to demonstrate probe pair specificity. Critically, the presence of insert-backbone oligonucleotides did not lead to observable signals if corresponding cognate probes were not also present in the reaction. Furthermore, the signal amplitude for any given target in the multiplexed sample was not affected by the presence of oligonucleotides against non-cognate targets and corresponding amplification products. This suggests that the number of transcripts that can be quantified within the same cell is only limited by the number of reporters that can be conjugated to detection oligonucleotides and analyzed simultaneously with a given platform.

We made use of 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 (IL2, IL12 and IL18) and stimulated with PMA-ionomycin. Instead of a uniform cellular response, simultaneous transcript quantification revealed complex combinatorial RNA expression patterns in this supposedly homogenous clonal cell line (Fig. 2b). Based on such multiplexed measurements, high-dimensional analysis methods can be leveraged to identify functional NKL subpopulations based on transcript expression profiles. To that end we clustered cells based on the 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 homogenous cell populations in primary samples also express such a remarkable diversity of cytokine combinations. While the functional implications of this observation are still poorly understood, the study of any such combinatorial phenomenon clearly benefits from the increased parameterization enabled by PLAYR.

The increased multiplexing capabilities of PLAYR also enable RNA-only experiments, where transcript expression is used to define cell types in which expression patterns of other transcripts can then be studied. Such experiments can be set up at a fraction of the costs typically 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 (MEFs), mouse embryonic stem cells (mESCs), and differentiating mESCs based on the expression of 15 different transcripts. We then visualized the data using viSNE[37], an algorithm that maps high-dimensional cytometry data onto two dimensions in a manner that best separates cell populations from the original high-dimensional space. This type of analysis clearly defined the three different populations of cells in the mixture based on RNA expression (Fig. 3a). Subsequently, different markers of pluripotency (e.g. *Nanog*), differentiation (e.g. *Thy1*), proliferation (*Mki67*), as well as pluripotency-associated long intergenic non-coding RNAs (*Lincenc1*) could be studied in the context of this cellular system (Fig. 3b). In an independent experiment with mESCs, 22 different transcripts including negative controls were measured in 16 technical replicates, which demonstrated the very high reproducibility of PLAYR for low as well as highly abundant transcripts (Supplementary Fig. 6).

We further validated this approach by making use of the protein co-detection and multiplexing capabilities of PLAYR. For this experiment we analyzed primary human peripheral blood mononuclear cells (PBMCs) for 10 cell surface proteins and corresponding transcripts. In contrast to the previous experiment, antibody stained protein markers were used to create a viSNE[37] analysis, which enabled the visualization of the major cell types in human peripheral blood (Fig 3c). Subsequent addition of the data on expression of corresponding transcripts demonstrated remarkable cell-type specificity in mRNA expression patterns (Fig 3d). In general, transcripts showed more gradual differences in expression levels than the corresponding proteins and the analysis revealed a clear discrepancy in the case of ITGAX, for which the protein was detected whereas essentially no transcript was observed in a distinct subpopulation of cells (Fig. 3e). Such differences can be explained by longer half-lives of proteins compared to transcripts, post-transcriptional regulation, and the fact that transcription has been observed to occur in bursts[26–28]. Accordingly, this type of analysis demonstrates some of the challenges when defining cell

types based on individual transcripts and the potential of PLAYR to study the relationship of transcripts and proteins in individual cells within complex primary samples.

Profiling of cytokine transcript induction in primary samples

We next used PLAYR to monitor cytokine transcript induction in PBMCs upon 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 used antibodies against surface markers to distinguish different cell populations within human PBMCs while monitoring the expression of a panel of cytokine genes at the transcript level with PLAYR. Similar experiments were performed using fluorescence-based flow cytometry and mass cytometry. The fluorescence experiment involved the detection of four transcripts and four surface markers, whereas mass cytometry allowed for the simultaneous quantification of 8 transcripts and 18 protein epitopes.

In both experiments, antibody staining enabled gating of distinct cell populations (gating for mass cytometry shown in Fig 4a, see Supplementary Fig. 7 for flow cytometry). As expected cytokine production was restricted to the monocyte compartment and therein confined mostly to individual cells that expressed the LPS co-receptor CD14 (Fig 4b). Moreover, different cytokines consistently exhibited distinct expression dynamics. For example, tumor necrosis factor alpha (*TNF*) and interleukin 8 *(CXCL8)* were induced early and the former peaked between 2 and 4 hours, while the latter continued to increase during the entire time course. Conversely, expression of interleukin 6 (*IL6*) was only strongly induced after 4 hours (Fig 4c and 4d). These results recapitulated previous individual observations[38,39] and confirmed that PLAYR effectively detects RNA expression in specific cellular subpopulations. Interestingly, while *CXCL8* at its peak is expressed in the entire $CD14^+$ monocyte compartment, there was a distinct population of $CD14^+$ cells that did not express *TNF* (Fig 4e). This observation underscores the utility of protein and RNA co-detection in identifying functional differences in cellular populations.

viSNE[37] analysis using the CyTOF data for the cytokine induction experiment demonstrated that all major PBMC populations clustered in unique areas of the viSNE map (Fig. 5a) and could be identified by observing the restricted expression of canonical markers (Fig. 5b). Similarly, MAP kinase signaling as measured by p38 MAP kinase phosphorylation could be monitored and was restricted to the myeloid compartment. When cytokine transcript expression was overlaid on the map, cells that responded to LPS were mostly restricted to the $CD14^+$ monocytes region (Fig. 5c). This analysis provides a single-cell resolution map of cytokine induction and MAP kinase signaling in PBMCs, highlighting the potential of PLAYR in combination with mass cytometry for system-wide analyses of transcriptional networks in complex samples.

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 been shown to have prognostic and diagnostic value in multiple clinical settings[40–43]. PLAYR extends such analyses to include measurements on the transcript level and can supplement the use of antibodies especially where exon-specific expression is concerned and no relevant antibody reagents exist. Immediate measurement of mRNA as enabled by PLAYR can overcome issues introduced with *ex vivo* processing of live cells and experimental artifacts are further minimized since PLAYR assays for RNA molecules directly without the need for cDNA synthesis[44].

PLAYR can simultaneously measure transcripts and their encoded proteins, thus enabling the characterization of the interplay between transcription and translation at the single-cell level. Other applications will include clustering of complex cellular populations purely on the basis of transcript abundance, which could be particularly useful when the availability or quality of antibodies is limiting. We believe that such an approach will help in the definition of yet undetermined cellular populations, which share specific patterns of temporal or spatial regulation of 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 imaging[47], making it a flexible tool to study gene expression in single intact cells on a variety of platforms.

Online Methods

Tissue culture

Jurkat E6-1 (ATCC TIP-152), NALM-6 (DSMZ ACC128), and NKL (gift from Dr. Lewis Lanier, UCSF) cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Omega Scientific), 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies), and 2 mM L-glutamine (Life Technologies) at 37 °C with 5% CO_2. In addition, 200 U/ml of rhIL-2 (NCI Biological Resources Branch) was added to NKL 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 hours and treated with 150 ng/ml PMA (Sigma-Aldrich) plus 1 μM ionomycin (Sigma-Aldrich) for 3 hours in the presence of 1× Brefeldin A (eBioscience) and 1× Monensin (eBioscience). All cell lines used in this study were obtained from the indicated vendors and not further tested for mycoplasma. Mouse embryonic fibroblasts were prepared as described elsewhere[48] and cultured in DMEM (Life Technologies), 10% fetal bovine serum, 2-mercaptoethanol (Sigma Aldrich), 1mM 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% fetal bovine serum, 2-mercaptoethanol, 1mM sodium pyruvate, 1× non-essential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, 1000 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 two days. Human peripheral blood was purchased from the Stanford Blood Bank and was collected according to a Stanford University IRB-approved protocol. PBMCs were separated from whole blood using Ficoll (Thermo) and cryopreserved in liquid nitrogen. For analysis, PBMCs were thawed, washed with complete RPMI medium, and rested for 30 min at 37 °C under 5% CO_2 in complete RPMI medium. PBMCs were stimulated with LPS (InvivoGen) at a concentration of 10 ng/mL in complete RPMI medium under gentle agitation.

Cell fixation, permeabilization, and antibody staining

Cells at a density of ~1×10^6/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 previously[49]. 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% v/v polyvinylsulfonic acid (Polysciences), and 40 U/mL RNasin (Promega) for 30 min at room temperature. After washing, antibodies were crosslinked 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 ~20×10^6 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. Antibodies used for mass cytometry were purchased from Fluidigm Corporation: 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), pERK1/2 (pT202/pY204). Antibodies 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), 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 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 600 g for 3 min. Hybridizations with PLAYR probes were performed in a buffer based on DEPC-treated water (Life Technologies) containing 1× SSC (Affymetrix), 2.5 % v/v 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, 40 U/mL RNasin at 40 °C under vigorous agitation. Samples to be analyzed by mass cytometry were barcoded at this step as described previously[50]. After two washes, cells were incubated with 100 nM insert/backbone oligonucleotides in PBS, 1× SSC, 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, followed by a 2 h (flow cytometry) or 4 h (mass cytometry) incubation 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 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 minutes at 37 °C in PBS, 1× SSC, 0.1% Tween, 40 U/mL RNasin. Two fluorophore-conjugated (Alexa488 and Alexa647) 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 PE-Cy7-streptavidin or an anti-BrdU-PE antibody conjugate as appropriate. For mass cytometry, cells were incubated with metal-conjugated detection oligonucleotides at a concentration of 10 nM for 30 minutes at 37 °C in PBS, 5 mg/mL BSA, 0.02 % sodium azide. After washing, cells were processed immediately on a fluorescence-based flow cytometer or further processed for CyTOF acquisition as described elsewhere[50].

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 water, spun at 14000 g 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 RNeasy Plus Micro Kit (Qiagen), following the manufacturer's instructions. Reverse transcription was performed using SuperScript III First-Strand Synthesis System (Life Technologies), following the manufacturer's instructions. PCR was carried out in a LightCycler 480 II (Roche) using SYBRGreen I Master (Roche).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank L. Lanier (UCSF) for providing the NKL cell line, and A. Trejo and A. Jager for technical assistance. A.P.F. is supported by a Fellowship for Prospective Researchers by the Swiss National Science Foundation, an EMBO Long-Term Fellowship, and a Marie Curie International Outgoing Fellowship. P.F.G. is a Howard Hughes Medical Institute Fellow of the Life Sciences Research Foundation. F.A.B is supported by a Human Frontier Science Program Long-Term Fellowship. This work was further supported by: U19 AI057229, 1U19AI100627, Department of Defense (CDMRP), Northrop-Grumman Corporation, R01CA184968, 1R33CA183654-01, R33CA183692, 1R01GM10983601, 1R21CA183660, 1R01NS08953301, OPP1113682, 5UH2AR067676, 1R01CA19665701, R01HL120724, and the Rachford & Carlotta A. Harris Endowed Chair.

References

1. Jaitin DA, et al. Massively parallel single-cell RNA-seq for marker-free decomposition of tissues into cell types. Science. 2014; 343:776–779. [PubMed: 24531970]
2. Hashimshony T, Wagner F, Sher N, Yanai I. CEL-Seq: single-cell RNA-Seq by multiplexed linear amplification. Cell Rep. 2012; 2:666–673. [PubMed: 22939981]
3. Islam S, et al. Highly multiplexed and strand-specific single-cell RNA 5′ end sequencing. Nat Protoc. 2012; 7:813–828. [PubMed: 22481528]
4. Islam S, et al. Quantitative single-cell RNA-seq with unique molecular identifiers. Nat Methods. 2014; 11:163–166. [PubMed: 24363023]
5. Ramsköld D, et al. Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells. Nat Biotechnol. 2012; 30:777–782. [PubMed: 22820318]
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. 2013; 14:R31. [PubMed: 23594475]
7. Shalek AK, et al. Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. Nature. 2013; 498:236–240. [PubMed: 23685454]
8. Wu AR, et al. Quantitative assessment of single-cell RNA-sequencing methods. Nat Methods. 2014; 11:41–46. [PubMed: 24141493]
9. Deng Q, Ramsköld D, Reinius B, Sandberg R. Single-cell RNA-seq reveals dynamic, random monoallelic gene expression in mammalian cells. Science. 2014; 343:193–196. [PubMed: 24408435]
10. Picelli S, et al. Full-length RNA-seq from single cells using Smart-seq2. Nat Protoc. 2014; 9:171–181. [PubMed: 24385147]
11. Tang F, Lao K, Surani MA. Development and applications of single-cell transcriptome analysis. Nat Methods. 2011; 8:S6–11. [PubMed: 21451510]
12. Fan HC, Fu GK, Fodor SPA. Expression profiling. Combinatorial labeling of single cells for gene expression cytometry. Science. 2015; 347:1258367. [PubMed: 25657253]
13. Dvinge H, et al. Sample processing obscures cancer-specific alterations in leukemic transcriptomes. Proc Natl Acad Sci USA. 2014; 111:16802–16807. [PubMed: 25385641]
14. Grün D, van Oudenaarden A. Design and Analysis of Single-Cell Sequencing Experiments. Cell. 2015; 163:799–810. [PubMed: 26544934]
15. Bauman JG, Bayer JA, van Dekken H. Fluorescent in-situ hybridization to detect cellular RNA by flow cytometry and confocal microscopy. J Microsc. 1990; 157:73–81. [PubMed: 1688948]
16. Patterson BK, et al. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. Science. 1993; 260:976–979. [PubMed: 8493534]
17. Belloc F, Durrieu F. Detection of mRNA species by flow cytometry. Methods Cell Biol. 1994; 42(Pt B):59–69. [PubMed: 7533256]

18. Borzì RM, et al. A fluorescent in situ hybridization method in flow cytometry to detect HIV-1 specific RNA. J Immunol Methods. 1996; 193:167–176. [PubMed: 8699030]
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. Analytical Biochemistry. 1992; 207:298–303. [PubMed: 1336322]
20. Just T, Burgwald H, Broe MK. Flow cytometric detection of EBV (EBER snRNA) using peptide nucleic acid probes. J Virol Methods. 1998; 73:163–174. [PubMed: 9766887]
21. Larsson C, Grundberg I, Söderberg O, Nilsson M. In situ detection and genotyping of individual mRNA molecules. Nat Methods. 2010; 7:395–397. [PubMed: 20383134]
22. Weibrecht 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. 2013; 8:355–372. [PubMed: 23348363]
23. Player AN, Shen LP, Kenny D, Antao VP, Kolberg JA. Single-copy gene detection using branched DNA (bDNA) in situ hybridization. J Histochem Cytochem. 2001; 49:603–612. [PubMed: 11304798]
24. Porichis F, et al. High-throughput detection of miRNAs and gene-specific mRNA at the single-cell level by flow cytometry. Nature Communications. 2014; 5:5641.
25. Bendall SC, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science. 2011; 332:687–696. [PubMed: 21551058]
26. Lin Y, Sohn CH, Dalal CK, Cai L, Elowitz MB. Combinatorial gene regulation by modulation of relative pulse timing. Nature. 2015; 527:54–58. [PubMed: 26466562]
27. Chubb JR, Trcek T, Shenoy SM, Singer RH. Transcriptional pulsing of a developmental gene. Curr Biol. 2006; 16:1018–1025. [PubMed: 16713960]
28. Cai L, Friedman N, Xie XS. Stochastic protein expression in individual cells at the single molecule level. Nature. 2006; 440:358–362. [PubMed: 16541077]
29. Fredriksson S, et al. Protein detection using proximity-dependent DNA ligation assays. Nat Biotechnol. 2002; 20:473–477. [PubMed: 11981560]
30. Söderberg O, et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat Methods. 2006; 3:995–1000. [PubMed: 17072308]
31. Lizardi PM, et al. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat Genet. 1998; 19:225–232. [PubMed: 9662393]
32. Leuchowius KJ, et al. Parallel visualization of multiple protein complexes in individual cells in tumor tissue. Molecular & Cellular Proteomics. 2013; 12:1563–1571. [PubMed: 23436906]
33. Untergasser A, et al. Primer3–new capabilities and interfaces. Nucleic Acids Res. 2012; 40:e115. [PubMed: 22730293]
34. Jurka J, et al. Repbase Update, a database of eukaryotic repetitive elements. Cytogenet Genome Res. 2005; 110:462–467. [PubMed: 16093699]
35. O'Gorman WE, 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. 2015; doi: 10.1016/j.jaci.2015.04.008
36. Newell EW, Sigal N, Bendall SC, Nolan GP, Davis MM. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8+ T cell phenotypes. Immunity. 2012; 36:142–152. [PubMed: 22265676]
37. Amir EAD, et al. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nat Biotechnol. 2013; doi: 10.1038/nbt.2594
38. DeForge LE, Remick DG. Kinetics of TNF, IL-6, and IL-8 gene expression in LPS-stimulated human whole blood. Biochem Biophys Res Commun. 1991; 174:18–24. [PubMed: 1989598]
39. Agarwal S, Piesco NP, Johns LP, Riccelli AE. 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. 1995; 74:1057–1065. [PubMed: 7782536]
40. Irish JM, et al. Single cell profiling of potentiated phospho-protein networks in cancer cells. Cell. 2004; 118:217–228. [PubMed: 15260991]

41. Gaudillière B, et al. Clinical recovery from surgery correlates with single-cell immune signatures. Sci Transl Med. 2014; 6:255ra131.
42. Jaye DL, Bray RA, Gebel HM, Harris WAC, Waller EK. Translational applications of flow cytometry in clinical practice. J Immunol. 2012; 188:4715–4719. [PubMed: 22556132]
43. Kaleem Z, et al. Flow cytometric analysis of acute leukemias. Diagnostic utility and critical analysis of data. Arch Pathol Lab Med. 2003; 127:42–48. [PubMed: 12521365]
44. Picelli S, et al. Smart-seq2 for sensitive full-length transcriptome profiling in single cells. Nat Methods. 2013; 10:1096–1098. [PubMed: 24056875]
45. Schulte R, et al. Index sorting resolves heterogeneous murine hematopoietic stem cell populations. Exp Hematol. 2015; 43:803–811. [PubMed: 26051918]
46. Wilson NK, et al. Combined Single-Cell Functional and Gene Expression Analysis Resolves Heterogeneity within Stem Cell Populations. Cell Stem Cell. 2015; 16:712–724. [PubMed: 26004780]
47. Angelo M, et al. Multiplexed ion beam imaging of human breast tumors. Nat Med. 2014; 20:436–442. [PubMed: 24584119]
48. Wernig M, et al. A drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. Nat Biotechnol. 2008; 26:916–924. [PubMed: 18594521]
49. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. Cytometry A. 2003; 55A:61–70. [PubMed: 14505311]
50. Zunder ER, et al. Palladium-based mass tag cell barcoding with a doublet-filtering scheme and single-cell deconvolution algorithm. Nat Protoc. 2015; 10:316–333. [PubMed: 25612231]

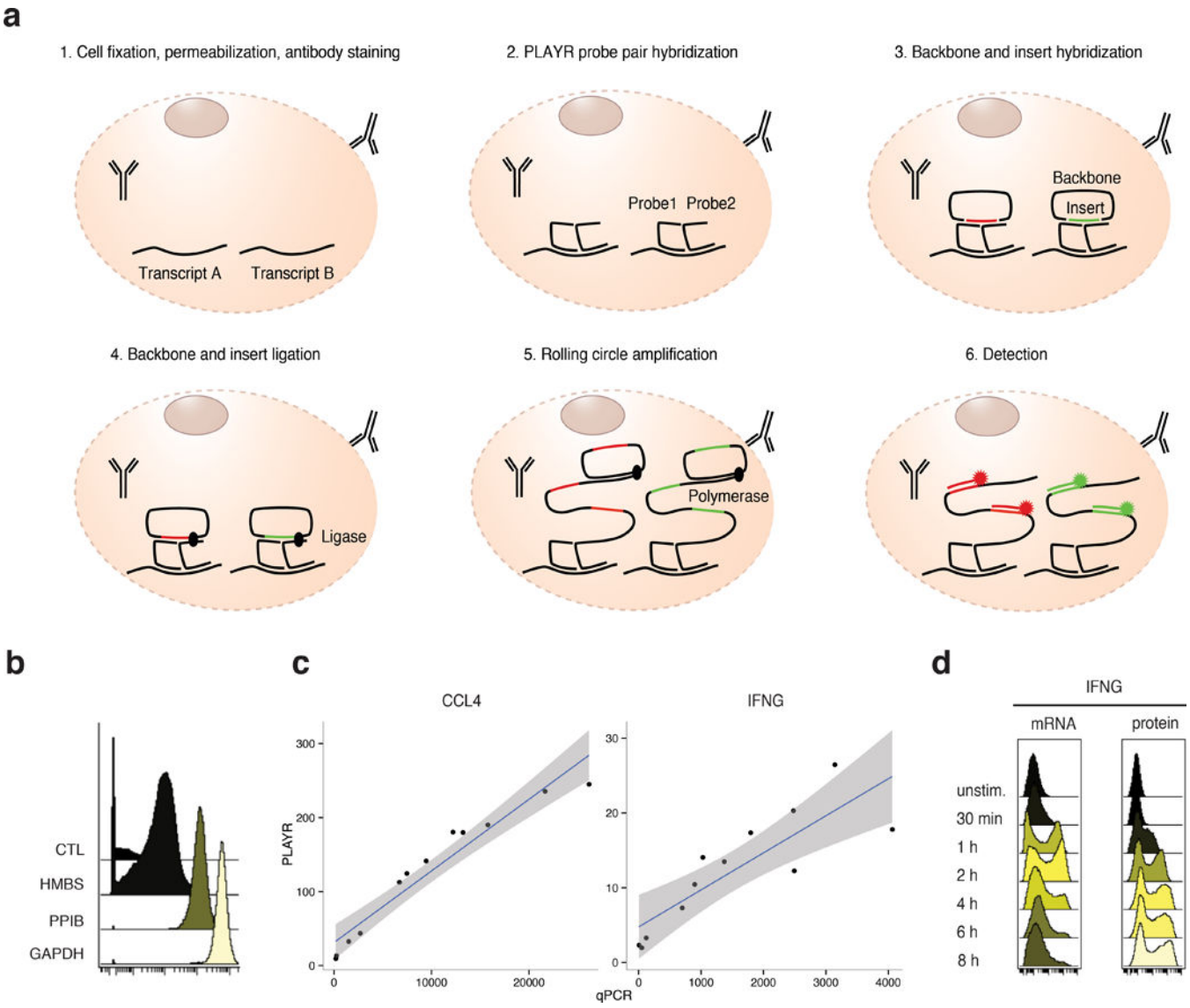

Figure 1. PLAYR enables the simultaneous quantification of specific transcripts and proteins in single cells

a) Main steps of the PLAYR protocol: 1) Fixation of cells captures their native state and permeabilization enables intracellular antibody staining and blocking of endogenous RNAses with inhibitors. 2) PLAYR probe pairs are added for proximal hybridization to target transcripts. 3) Backbone and insert oligonucleotides are added and form a circle if hybridized to PLAYR probes that are in close proximity (bound to a transcript). Insert sequences serve as cognate barcodes for targeted transcripts. 4) Backbone and insert oligonucleotides are ligated into a single-stranded DNA circle by T4 DNA ligase. 5) Rolling circle amplification of the DNA circle by phy29 polymerase. 6) Detection of rolling circle amplicons with suitably labeled oligonucleotides that bind to the insert regions. **b**) Detection of transcripts for three housekeeping genes that span a wide abundance range in U937 cells by mass cytometry. **c**) Quantification of *CCL4* and *IFNG* mRNA by PLAYR and qPCR in NKL cells after stimulation with PMA-ionomycin. Measurements were performed at 4 time

points in 3 replicates. **d**) Simultaneous IFNG mRNA and protein quantification by mass cytometry in NKL cells at indicated time points after stimulation with PMA-ionomycin.

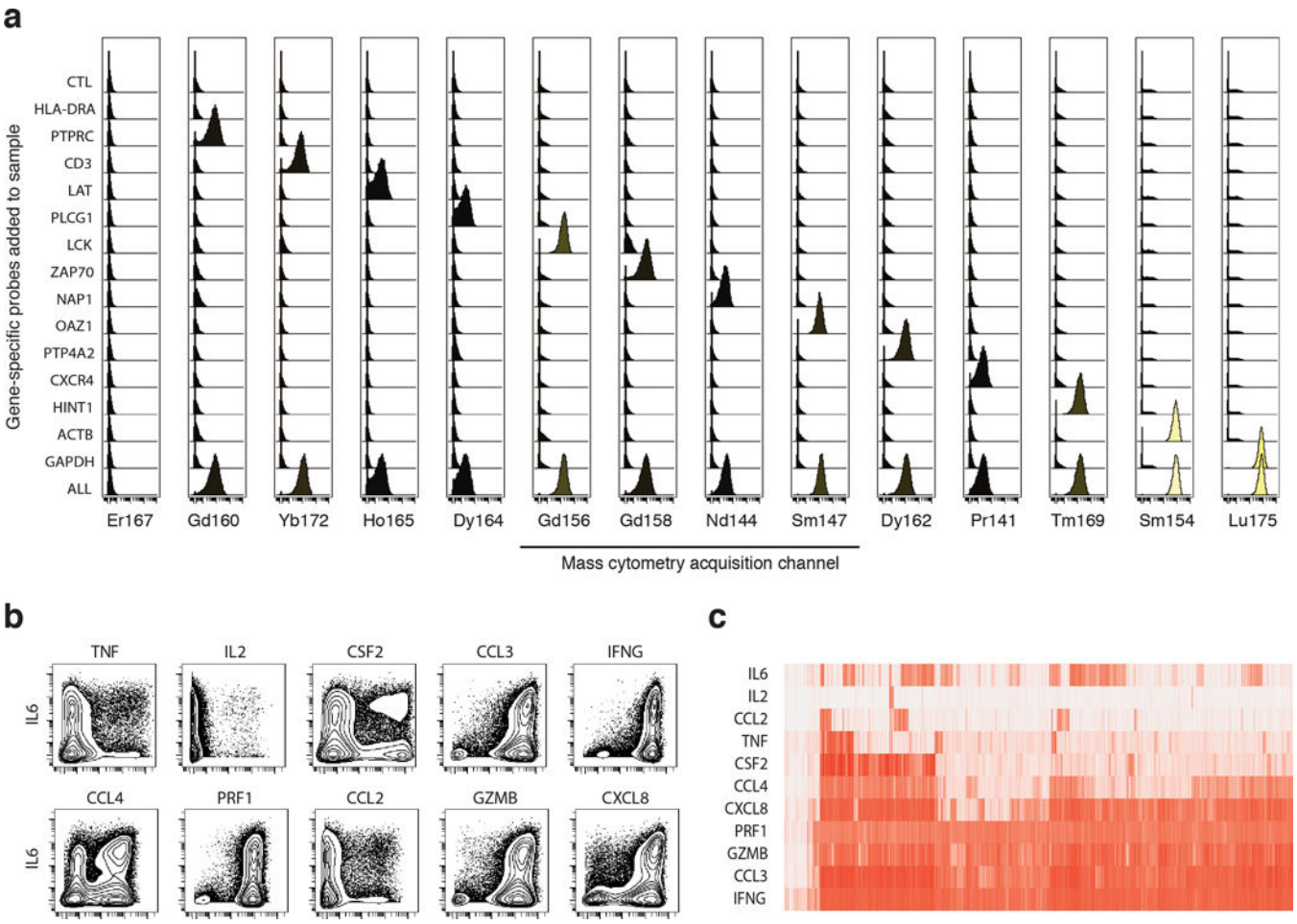

Figure 2. Highly multiplexed measurement of different transcripts in single cells

a) Detection of 14 different transcripts in Jurkat cells by mass cytometry. PLAYR probes to transcripts not expressed in T cells (*HLA-DRA*) or to those encoding T cell surface markers, T cell signaling molecules, and housekeeping proteins of different abundance levels were used. Each row represents a sample to which probe pairs for one gene only or all genes simultaneously (bottom row) were added. Each column represents a mass cytometry acquisition channel that monitors a metal reporter used to detect transcripts of a given gene. Non-cognate probes that are using the same insert system but bind to different target transcripts were included as an additional control (CTL). **b**) NKL cells were primed with IL2, IL12 and IL18 and stimulated with PMA-ionomycin for 3 hours. Contour plots display co-expression of NKL effector transcripts as measured by mass cytometry. **c**) 10000 cells were randomly sampled from the data in (b) and transcript expression was represented in heat map format. Each column corresponds to a single cell and rows denote different effector transcripts. Rows and columns of the heat map were clustered for visual clarity (dendrograms not shown). Experiments were run multiple times and representative examples are shown.

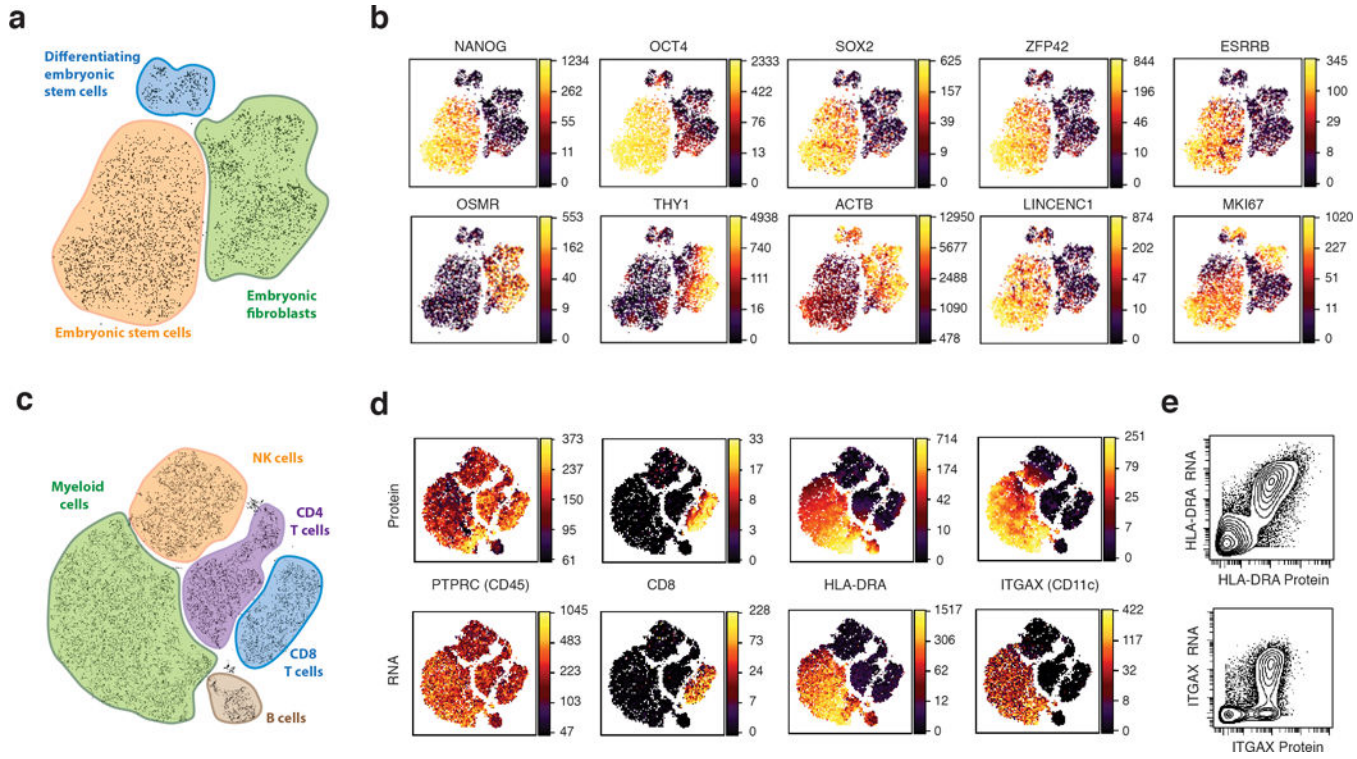

Figure 3. Highly multiplexed measurement of transcripts within cell types defined by other transcripts or protein epitopes

a) viSNE analysis of embryonic stem cells, differentiating embryonic stem cells, and embryonic fibroblasts of mice based on expression of 15 transcripts (*Cd44, Mki67, Cdh1, Cd47, Klf4, Esrrb, Actb, Sox2, Lincenc1, Zfp42, Sall4, Cd9, Pou5f1 (Oct4), Thy1, Nanog*) with overlays showing the location of the three cell populations. **b**) Color-coded expression levels of selected transcripts used to construct the viSNE map. **c**) viSNE analysis of PBMCs based on expression of 10 surface protein markers (CD19, CD4, CD8, CD20, PTPRC (CD45), PTPRCRA (CD45RA), CD33, ITGAX (CD11c), CD3, HLA-DRA) with overlays showing the location of major cell populations. **d**) Expression of selected proteins and the corresponding transcripts was overlaid in the viSNE map shown in (c) and color-coded by signal intensity. **e**) Contour plots displaying correlations of protein and transcript levels for HLA-DRA and ITGAX in individual PBMCs. Experiments were run multiple times and representative examples are shown.

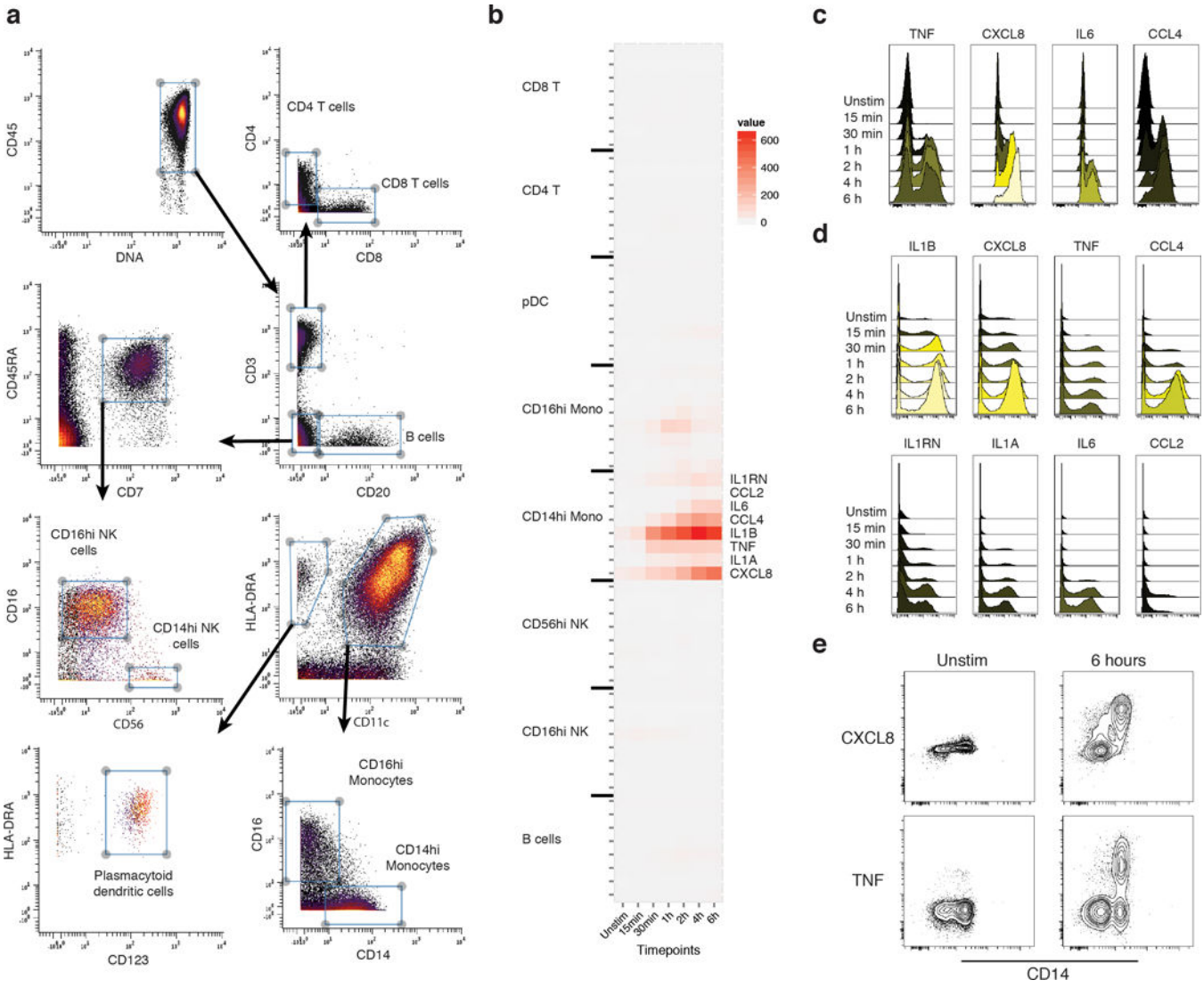

Figure 4. Measurements of cytokine transcript induction in human PBMCs by mass cytometry and fluorescence flow cytometry

a) Mass cytometry gating strategy for human PBMCs. **b**) Heat map representing the mean expression values of cytokine transcripts at different time points after stimulation with LPS in different cellular populations defined by protein surface markers. **c**) Cytokine expression in the $CD14^+$ monocyte population as measured by fluorescence flow cytometry. **d**) Cytokine transcript expression in the $CD14^+$ monocyte population as measured by mass cytometry. **e**) Contour plots showing interleukin 8 *(CXCL8)* and tumor necrosis factor alpha (*TNF)* transcript expression in $CD14^+$ monocytes as measured by fluorescence flow cytometry. Experiments were run multiple times and representative examples are shown.

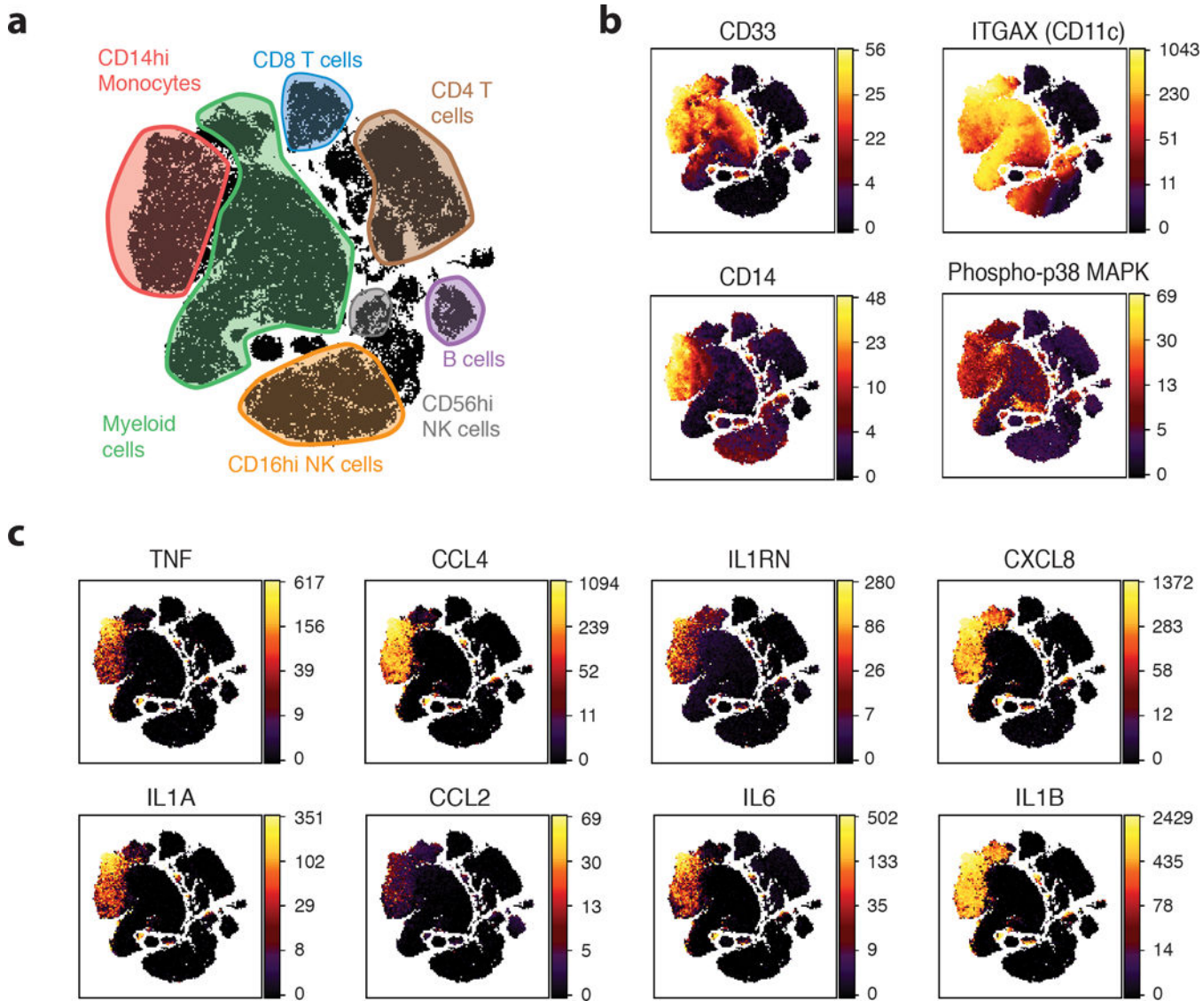

Figure 5. Single-cell resolution map of cytokine induction in human PBMCs

PBMCs were stimulated with LPS and analyzed after 4 hours. Cells were analyzed with antibodies against cell surface proteins (CD19, CD38, CD4, CD8, CD7, CD14, IL3RA (CD123), PTPRC (CD45), PTPRCRA (CD45RA), CD33, ITGAX (CD11c), FCGR3A (CD16), CD3, CD20, HLA-DRA, NCAM1 (CD56) and phosphorylation sites pP38 MAPK (pT180/pY182), pERK1/2 (pT202/pY204). **a**) viSNE map based on cell surface marker expression with overlays showing the location of major cell populations. **b**) Selected protein markers used to define myeloid cell populations and MAPK signaling were color-coded by expression level. **c**) Measurements for 8 different cytokine transcripts were overlaid and color-coded by expression level. The experiment was run multiple times and a representative example is shown.