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Multiplexed profiling of RNA and protein expression signatures in individual cells using flow or mass cytometry

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Abstract

Advances in single-cell analysis technologies are providing novel insights into phenotypic and functional heterogeneity within seemingly identical cell populations. RNA within single cells can be analyzed using unbiased sequencing protocols or through more targeted approaches using in situ hybridization (ISH). The proximity ligation assay for RNA (PLAYR) approach is a sensitive and high-throughput technique that relies on in situ and proximal ligation to measure at least 27 specific RNAs by flow or mass cytometry. We provide detailed instructions for combining this technique with antibody-based detection of surface/internal protein, allowing simultaneous highly multiplexed profiling of RNA and protein expression at single-cell resolution. PLAYR overcomes limitations on multiplexing seen in previous branching DNA-based RNA detection techniques by integration of a transcript-specific oligonucleotide sequence within a rolling-circle amplification (RCA). This unique transcript-associated sequence can then be detected by heavy metal (for mass cytometry)- or fluorophore (for flow cytometry)-conjugated complementary detection oligonucleotides. Included in this protocol is methodology to label oligonucleotides with lanthanide metals for use in mass cytometry. When analyzed by mass cytometry, up to 40 variables (with scope for future expansion) can be measured simultaneously. We used the described protocol to demonstrate intracolon heterogeneity within primary cells from chronic lymphocytic leukemia patients, but it can be adapted to other primary cells or cell lines in suspension. This robust,

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Author contributions

A.D.D., M.S. and F.Y. performed the experiments. A.D.D. and P.F.G. collaborated on assay development and optimization. A.D.D., P.F.G., G.P.N., J.R.S. and N.K. contributed to critical analysis of data and manuscript preparation.

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Competing interests

G.P.N. had a personal financial interest in Fluidigm, the manufacturer of the mass cytometer used in this study, for the duration of this project. The other authors declare no competing interests.

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reliable and reproducible protocol can be completed in 2–3 d and can be paused at several stages for convenience.

Introduction

Analysis of single cells for their individual complexities in form and function allows greater understanding of biological systems. Through analysis and quantification of gene products at single-cell resolution, it is becoming evident that despite being genetically identical, there are considerable differences in basal and perturbed gene expression, even within populations of cells that would otherwise be considered a homogeneous ‘cell type’¹. This variance is thought to arise due to the stochastic nature of gene expression and differences in the micro-environmental milieu of individual cells. Techniques for profiling and understanding RNA expression at single-cell resolution have rapidly progressed in recent years, and the methods broadly fall into two complementary categories: single-cell RNA-sequencing (scRNA-seq) or ISH.

Over the past 9 years, and since the publication of a landmark article describing an approach for single-cell mRNA-seq², different methodologies for scRNA-seq have evolved. All methods rely on three major steps: (i) unique nucleotide barcoding and reverse transcription of the RNA of each individual cell through either physical isolation^{3,4} or probabilistic labeling⁵; (ii) amplification of the resulting cDNA; and (iii) library preparation for sequencing. Recently, this technology has been combined with oligonucleotide-labeled antibodies to allow simultaneous quantification of both protein and RNA within individual cells by using DNA sequencing^{6,7}. Despite being an extremely promising technology, a number of experimental constraints of scRNA-seq are pertinent: (i) financial considerations may restrict sequencing depth, resulting in a trade-off between the number of cells/samples that are analyzed and the sensitivity for detection of transcripts with lower abundance; (ii) the technique currently relies on reverse transcription using an oligo-dT primer, and therefore analysis is limited to poly-adenylated RNA; (iii) the method can be laborious and complicated, and takes weeks to months to produce informative data. Owing to these limitations, scRNA-seq may not always be practical for clinical applications.

A complementary method to scRNA-seq is detection and quantitation of RNA at single-cell resolution using ISH. This technique measures fewer RNAs that have to be preordained, but the transcripts can be sensitively quantified in a larger number of cells, within a much shorter time frame, and with less experimental complexity. Thus, quantification of the resulting signal by cytometry allows hundreds to thousands of cells to be analyzed per second. The technology is an adaptation of fluorescent ISH and relies on bright signals with high signal-to-noise ratio. Branching-DNA technology also allows measurement of RNA in single cells using flow cytometry⁸, and specificity is increased through probe pairs that are required to bind in close proximity on their target RNA strand. Adjacently bound probes can then be detected using sequential binding of predesigned DNA molecules to form repetitive, expanding structures of nucleotides that amplify the signal. However, there are currently only four non-interfering versions of branching-DNA sequences, meaning opportunities for multiplexing using this method are limited. This restrictive feature of branching DNA is a

major limiting factor for utility in platforms such as mass cytometry, which have higher multiplexing capabilities. An alternative methodology to branching DNA for increasing signal intensity is to use targeted padlock DNA probes and then produce concatenated copies of a resulting circular DNA molecule using RCA catalyzed by Phi29 DNA polymerase⁹. We have recently developed the PLAYR, which provides routine simultaneous measurements for both RNA and proteins at single-cell resolution with enhanced multiplexing capabilities for detection in both flow and mass cytometry platforms¹⁰.

Here we provide a detailed protocol that allows analysis of protein expression using antibodies, and simultaneous detection of up to 27 RNA transcripts (multiplexing can be extended beyond this limit). We have now optimized the antibody staining procedure to allow robust and reproducible characterization of surface and internal antigen expression while maintaining RNA integrity for the PLAYR assay. Although we describe the technique for the analysis of primary peripheral blood mononuclear cells (PBMCs) from chronic lymphocytic leukemia (CLL) patients, it can be applied to other primary cells or cell lines that are in suspension. The protocol described here can potentially be adapted for the simultaneous measurement of RNA and protein in tissue sections, by using imaging technologies such as fluorescent microscopy and imaging mass cytometry.

Overview of the procedure

PLAYR—A schematic overview of the critical steps taken to detect RNA in the PLAYR procedure and the method to combine this with antibody staining is depicted in Fig. 1a. PLAYR specifically detects transcripts of interest by relying on proximal ligation of predesigned and specific PLAYR probe pairs (Fig. 1b). Multiple probe pairs are designed to directly bind along each target RNA transcript to produce a robust signal with a high dynamic range of detection (1,000 to <10 transcripts per cell). Cells are fixed and permeabilized before incubation with PLAYR probe pairs that are designed to bind adjacently on their target transcripts (Steps 1–17). Two functional sections separated by a linker region of ten adenine residues make up each probe. The 5′ end of the probe binds to its cognate target RNA sequence, whereas the 3′ end forms one-half of a template for the binding and circularization of two additional oligonucleotides, termed ‘insert’ and ‘backbone’. Only when both PLAYR probes bind to an RNA transcript in proximity can they generate the whole complementary platform for the formation of the nicked circular insert/backbone DNA molecule, which is ligated in a subsequent step (Steps 18–26) (Fig. 1c). The circular insert/backbone DNA molecule is then amplified, via RCA using Phi29 DNA polymerase, into its complementary concatenated form (Steps 27 and 28), thus yielding multiple copies of the cognate insert sequence that is detected by metal or fluorescently labeled detection oligonucleotides (Steps 29–32). Any off-target binding of single probes is insufficient to generate a complete circular DNA molecule, which is required for RCA. Multiplexing is achieved through use of parallel and unique PLAYR systems (3′ probe sequences, insert oligonucleotide sequences and the detection probe sequences) for each transcript (Fig. 1). Unique insert sequences introduce a nucleotide barcode within the RCA that can be specifically detected for each transcript. Common substrings between PLAYR insert sequences do not exceed 4 bp in length, but they have similar thermodynamic properties and base composition, allowing formation of unique RCA

products with similar efficiencies. Our described insert sequences therefore allow simultaneous quantification of 27 different transcripts. A list of these 27 PLAYR systems including PLAYR probe 3' sequences, inserts, backbone and detection probe sequences are detailed in Table 1.

Combining antibody staining with PLAYR—Antibody staining has been optimized to alleviate effects on RNA copy numbers (through transcription and degradation) while preserving the surface and intracellular protein integrity for quantification. Staining using metal or fluorescently labeled antibodies that target intracellular antigens is performed alongside the incubation with PLAYR detection probes in the final stage of the assay (Steps 29–32). By contrast, surface antigens are stained in ‘unfixed’ cells (Steps 5–7), covalently cross-linked to the cell using bis(sulfosuccinimidyl) suberate (BS3) and then fixed using paraformaldehyde (PFA) (Steps 8–10). If used first, PFA will compete with BS3 for protein amine groups, reducing the efficiency of the cross-linking. As unfixed cells are prone to changes in both transcript levels and protein post-translational modifications, the incubation with antibodies and subsequent BS3 and PFA treatments are performed on ice. We find that signal-to-noise ratios of the staining antibodies in unfixed cells on ice are similar if not better than those achieved using the standard mass cytometry staining protocol on fixed cells at room temperature (RT, 15–30 °C) (Fig. 2). Once the cells are fixed, they can then be permeabilized in methanol (Steps 11 and 12) and the PLAYR assay can be performed with minimal loss of antibody staining. Cells can be stored for several months in methanol at –80 °C (after Step 12) without noticeable effects on RNA integrity or antibody binding.

Limitations

An inherent drawback of the technique is the paucity of unique heavy metal tags that can be used for detection of specific RNA targets, which currently limits the PLAYR assay to 40 transcripts. This is due to the chemical properties of the polymer that is used to chelate the metal tags. There are, however, efforts underway to expand the panel of unique tags to assay a larger spectrum of parameters¹¹.

A further limitation is the requirement for multiple probe pairs to detect a single transcript. Thus, it can be challenging to distinguish different isoforms if the regions that are specific to each one are not big enough to accommodate multiple probes. In addition, owing to constraints on the number of probe pairs that are able to bind to transcripts of limited length, the detection of small RNAs such as miRNAs is problematic. In the case of miRNAs, the technique may be applicable for detection of longer pre-miRNAs or primary miRNAs as surrogates. More generally, it may be possible to alleviate some of these limitations by using locked nucleic acids (LNAs), which have stronger affinity for their targets^{12,13}, and as such require fewer probe pairs to obtain a robust signal. However, the hybridization conditions for LNAs would have to be modified to achieve good signal-to-noise ratios.

Although the described PLAYR protocol should work well with most antibodies, there remains the possibility that the signal intensity of some antibodies may be compromised. To be certain, we recommend that the signal strength of each antibody be tested with and without the PLAYR procedure. From a practical point of view, the protocol does involve

many more wash steps than a standard antibody staining protocol, which could result in loss of cells available for final cytometric analysis.

In addition, the issues that surround other scRNA technologies (such as specificity, sensitivity and variable modes of expression) are probably also relevant to PLAYR¹⁴. Finally, unlike RNA-seq, which uses next-generation sequencing, the technique is currently not applicable for discrimination between wild-type and mutated RNA transcripts.

Experimental design

An overview of the experimental design workflow is illustrated in Fig. 3.

PLAYR probe design—We recommend the use of four or five probe pairs for each transcript of interest to ensure a robust and specific signal. The signal generated from these probe pairs is dependent upon the RNA transcript copy number per cell, the efficiency of the probe binding sequences (some probes may fail to bind because of RNA secondary structures) and the duration of the RCA during the assay (see ‘Protocol optimization’ below). It is also possible to use different numbers of probe pairs for different transcripts, according to their expected abundance. This does not constitute a problem for quantitative analysis because quantification is only relative (as with any other affinity-based reagent, e.g., antibodies). An example of how the number of probe pairs impacts PLAYR signal is shown in Supplementary Fig. 4a in the article by Frei et al.¹⁰.

PLAYR probes can be designed using our open-source R software package called PLAYRDesign⁹. Detailed instructions on how to download and install PLAYRDesign and its dependencies are available at <https://github.com/nolanlab/PLAYRDesign>. The PLAYRDesign software works by shortlisting candidate probes on the basis of their thermodynamic properties (using Primer3; ref.¹⁵) and displaying them along the transcript in a graphical interface. Additional graphs are aligned to the target transcript to illustrate BLAST-evaluated sequence similarities to the rest of the transcriptome, as well as a database of repetitive sequences. Non-constitutively spliced exons are also displayed, thus aiding the user in manually choosing the best probe pairs. The user can then select the PLAYR system (i.e., the multiplexing sequences) desired for the 3′ end of the transcript probes; the complete probe sequences are generated in a .txt file.

Handling of samples and reagents—All enzymes used during the PLAYR assay should be stored at −20 °C and diluted in reaction buffer immediately before addition to the cells. T4 ligase buffer contains ATP and should therefore be batched to avoid degradation by freeze–thaw cycles.

The procedure involves many more wash steps than a standard flow cytometry staining protocol, and as such, cell loss is a concern. We recommend using polypropylene V-bottom 200-μL PCR strips or, alternatively, 1.5-mL Eppendorf tubes. Avoid regular U-bottom polystyrene FACS tubes, as their use will result in substantial cell loss. Centrifugation of cells should preferably be performed in a swinging-bucket centrifuge, and the supernatant should be ideally removed by aspiration (i.e., not by flicking the tubes). The use of small

pipette tips (e.g., P10) during aspiration will provide greater precision and help to minimize cell loss.

When incubating cells, make sure to use an agitation speed such that the cells will not deposit at the bottom during incubation (this is especially critical during amplification with phi29 polymerase). If using PCR strips, use a shaker with a very small orbit to guarantee mixing of the liquids. Tubes subjected to such vigorous agitation should always be spun down before opening to prevent cell loss, as the cells will be on the sides of the tube and on the cap. As cellular RNA can be prone to degradation by both internal and external RNases, care must be taken to work as efficiently and quickly as possible, particularly when the cells are in any water-based solution after fixation and before probe hybridization (e.g., when the cells are removed from methanol before hybridization). All samples should remain on ice during those critical steps to minimize RNase activity. Once the circular DNA insert/backbone molecule is bound to the probe pairs and ligated, maintaining RNA integrity is less important (unless it is required for other relevant downstream applications).

Controls—The specificity of the PLAYR signal can be verified using cell lines known to reliably express the RNA transcript of interest. Perturbation of target transcripts through cellular stimulation or by using RNA knockdown/knockout followed by correlation of mean PLAYR signals with measurements obtained by qPCR can be used to further validate probe pair specificity (Fig. 4a). Further guidance on examples of controls that may be useful can be found in Supplementary Figs. 3 and 4 of Frei et al.¹⁰.

Each transcript of interest should be examined individually using all the designed probe pairs for the target RNA. The PLAYR probes for multiple transcripts are then pooled to ensure that the detection of each RNA is not compromised by multiplexing (Fig. 4b). Reassuringly, we have never encountered cases of interference.

Validation that each PLAYR system (including detection probe) is working with equal efficiency can also be performed using a suspension cell line of your choice to probe a gene of known high abundance. An example using two to three probe pairs that target GAPDH transcripts in the B-cell line MAVER1 is shown (Fig. 4c).

Staining of proteins should be optimized for each antibody panel. Antibodies should be stained according to the manufacturers' recommendations (e.g., on fixed cells at RT for 30 min) and compared with results obtained using the described PLAYR staining protocol. Verification of similar antibody staining patterns and clustering should be ensured using appropriate software, such as bivariate plots and viSNE analysis in Cytobank.

We provide viability staining procedures for both mass and flow cytometry in this protocol. We advise the inclusion of this procedure because cell viability can greatly affect RNA levels detected within cells. For example, see Fig. 5c, cisplatin-194Pt vs ACTB mRNA; dead cells (highest cisplatin levels) are associated with much lower ACTB mRNA.

Further useful controls and troubleshooting steps are listed in the Troubleshooting section.

Protocol optimization—The duration of the RCA can be altered to optimize signal intensity. We have found that 4 h for mass cytometry and 2 h for flow cytometry yield comparable and strong signal amplifications. Longer incubation times (16 h) further enhance signal levels but may be detrimental to antibody staining and final cell numbers, and will somewhat increase background as well. In our experience, cells that do not generate a detectable signal after 2–4 h tend not to show enhanced signal even after longer RCA times.

The PLAYR protocol can be optimized further to help preserve efficient antibody binding and, for flow cytometry, fluorophore stability. We have found that the methanol permeabilization step can be excluded because the Tween 20 present within the probe hybridization buffer is sufficient to permeabilize the cells for RNA detection (Supplementary Fig. 1). However, exclusion of the methanol step from the protocol means that the cells can no longer be stored at -80°C for a prolonged period of time and, because Tween 20 does not permeabilize the nuclear membrane, RNA residing within this compartment may not be stained during the assay. Lowering of the temperature from 40 to 37°C during the PLAYR probe hybridization and post-hybridization wash steps (Steps 17 and 22) may help further maintain antibody staining. Also, the dilution of the detergent Tween 20 used during the probe hybridization step and the concentration of saline–sodium citrate solution during the PLAYR post-hybridization wash can be modified if needed. Despite the fact that we see no major differences in RNA detection for our transcripts of interest during less stringent conditions (Supplementary Fig. 2), we advise that all changes in the protocol be controlled appropriately for your gene(s) of interest (see ‘Controls’ above).

Considerations specific for use with mass cytometry—Detection probes can be purchased as 5′ C6-thiol-modified oligonucleotides that can then be labeled using commercial Maxpar X8 metal labeling kits (Fluidigm). A detailed protocol for labeling of detection probes is described in Box 1. Metal-labeled detection probes can be validated by diluting the oligonucleotides in water (e.g., to 100 pM–1 nM) and running the samples through the mass cytometer to confirm the presence of the metal ion. Validation of detection probe metal labeling can be further verified by staining for a known gene of high abundance (e.g., GAPDH, Fig. 4c).

We include in this protocol an optional step for mass cytometry, which enables palladium (Pd)-based barcoding of up to 20 samples, so they can be subsequently pooled together and analyzed as one sample¹⁶. The use of Pd barcoding has been optimized to ensure that it does not interfere with the PLAYR assay and should be performed after probe hybridization (Steps 20–22) as described. Pd barcoding is encouraged because it has multiple benefits, including reducing cell loss during processing, which allows the use of a smaller starting number of cells per sample; removing variability in PLAYR and internal antibody staining efficiencies; decreasing reagent use; decreasing instrument measurement time; and reducing sample processing time. In silico separation of the samples is performed after data collection by using an integrated feature on the CyTOF system control software. Further details can be found in the Cell-ID 20-Plex Pd Barcoding Kit user guide on the Fluidigm website.

Considerations for use with flow cytometry—Care must be taken regarding the choice of fluorophores for surface antigen staining. Alongside the usual concerns regarding

spectral overlap, additional attention must be paid to whether fluorophores conjugated to surface staining antibodies will be inactivated during the PLAYR staining procedure. Fluorophores need to withstand chemical treatments (e.g., BS3 cross-linking, PFA treatment and methanol), temperatures and buffers used during the PLAYR assay. Generally, smaller fluorophores are more resistant to these conditions than protein-based fluorophores, which can be denatured by methanol¹⁷. A list of ten fluorophores that we have tested for performance under different PLAYR optimization conditions can be found in Supplementary Table 2. However, inactivation is less of a concern for fluorophores that are employed at the end of the protocol (i.e., for PLAYR detection probes and internal antigen staining). The use of PLAYR with advanced multi-parameter flow cytometry may be limited by the number of channels and suitable fluorophores. See the ‘Protocol optimization’ section above for other potential ways to increase surface antibody staining efficiency and preservation of fluorophores. As with routine flow cytometry, a negative-control sample for PLAYR (e.g., an assay performed with probe 1 or 2 only; see Fig. 5a) is required to correct for background fluorescence. Finally, all incubation steps when using fluorophores should be performed in the dark.

Materials

Biological materials

- Cultured cell lines of interest. We demonstrate our protocol in this manuscript using the B cell lines MEC1 (DSMZ, cat. no. ACC 497) and MAVER1 (ATCC, cat. no. CRL-3008), and the T cell line HUT78 (ATCC, cat. no. TIB-161) that were authenticated by short tandem repeat analysis and tested negative for mycoplasma. **! CAUTION** Regularly check cell lines to ensure they are authentic and are not infected with mycoplasma.
- Primary cells of interest. We have also demonstrated the protocol use on primary CLL PBMC samples from consenting patients. **! CAUTION** All experiments involving animal or human samples should adhere to the relevant governmental and institutional ethics guidelines and regulations. CLL cells used for this protocol were obtained from peripheral blood taken from patients with informed consent and with the approval of the Liverpool Research Ethics Committee. **▲ CRITICAL** Cryo-preserved CLL samples stored in the Liverpool Leukemia Biobank were prepared using standard protocols and stained immediately after thawing.

Reagents

(Optional) Surface and internal antibody staining—▲CRITICAL Staining with antibody is not required for RNA to be detected by the PLAYR assay. Surface and/or intracellular antibodies should be included if analysis of RNA expression is performed in specific cell types or cell phenotypes within mixed populations of cells (e.g., studying B cells within PBMCs).

- Metal- (for mass cytometry; Fluidigm, ref.¹¹) or fluorophore-conjugated (for flow cytometry) antibodies **▲ CRITICAL** Fluorophore-conjugated antibodies

used to stain surface antigen need to withstand conditions used during the remaining steps of the protocol (i.e., PFA fixation, MeOH treatment and PLAYR incubations). See the ‘Considerations for use with flow cytometry’ section for more details. For a list of antibodies used in this protocol, see Supplementary Table 1.

- Bis(sulfosuccinimidyl) suberate (BS3 crosslinker; Sigma-Aldrich, cat. no. S5799)
- Cell staining buffer (Fluidigm, cat. no. 201068)
- PBS (Gibco, cat. no. 10010015)

PLAYR

- 100 μ M PLAYR probes (Integrated DNA Technologies). PLAYR probes can be designed using our open-source R software package called PLAYRDesign⁹. Detailed instructions on how to download and install PLAYRDesign and its dependencies are available at <https://github.com/nolanlab/PLAYRDesign> (see ‘Experimental design’, ‘PLAYR probe design’). See Table 1 for oligonucleotide sequences for 27 PLAYR systems.
- 100 μ M 5’-phosphorylated insert and backbone oligonucleotides (Integrated DNA Technologies). Alternatively, oligonucleotides can be 5’-phosphorylated using T4 polynucleotide kinase (PNK; New England Biolabs, cat. no. M0201S).
- RNasin Plus (Promega, cat. no. N2611/N2615) **▲ CRITICAL** Other RNAsins may result in suboptimal inhibition and compromise RNA detection.
- 25% (vol/vol) Polyvinyl sulfonic acid (PVS; Polysciences, cat. no. 9002-97-5)
- 200 mM Ribonucleoside vanadyl complex (RVC; New England BioLabs, cat. no. S1402S) **▲ CRITICAL** RVC precipitates at low temperatures and oxidizes upon repeat freeze–thaw cycles. Thaw the bottle of RVC, heat it to 65 °C, and produce an even suspension of RVC precipitates by gentle agitation before making aliquots. 100- μ L Aliquots can then be produced; these should be stored at –20 °C for up to 3 years. Similarly, when using an aliquot, heat it to 65 °C with agitation to dissolve any precipitate that has re-formed upon cooling.
- UltraPure Salmon Sperm DNA Solution (Life Technologies, cat. no. 15632011)
- 16% (wt/vol) Paraformaldehyde (Electron Microscopy Sciences, cat. no. 15710) **! CAUTION** Skin contact should be avoided. Wear appropriate personal protective equipment (PPE). **▲ CRITICAL** Keep the ampoule at 4 °C in the dark once opened, and discard it after 1 week.
- Methanol (Fisher Scientific, cat. no. M/4056/15) **! CAUTION** Methanol is toxic. Wear appropriate PPE.
- T4 DNA ligase (Thermo Scientific, cat. no. EL0011)
- Phi29 DNA polymerase (Thermo Scientific, cat. no. EP0091)

- 10 mM dNTPs (Invitrogen, cat. no. 18427013)
- Cell-staining buffer (Fluidigm, cat. no. 201068)
- Tween 20 (Thermo Scientific, cat. no. BP337–100)
- Diethyl pyrocarbonate (DEPC; Sigma Aldrich, cat. no. 40718)
- 10× PBS (pH 7.4; Invitrogen, cat. no. AM9625)
- 20× Saline–sodium citrate solution (20×; Fisher Scientific, cat. no. 10418353)

Additional reagents for mass cytometry

- 1 μ M Thiol-modified C6 S-S detection probe(s) for detection by mass cytometry (see Box 1 for protocol for metal labeling of detection probes; Integrated DNA Technologies). See Table 1 for oligonucleotide sequences for 27 PLAYR systems.
- Maxpar X8 metal labeling kit(s) (Fluidigm)
- Cell-ID Intercalator-Ir (Fluidigm, cat. no. 201192A)
- (Optional) Cell-ID cisplatin (Fluidigm, cat. no. 201064) **▲ CRITICAL** Use cisplatin if determination of cell viability is required.
- (Optional) Cell-ID 20-Plex Pd Barcoding Kit (Fluidigm, cat. no. 201060) **▲ CRITICAL** Pd barcoding allows samples to be pooled together in one tube and provides a number of benefits. See the description of Pd barcoding within the ‘Experimental design’ section for more details.
- Maxpar water (Fluidigm, cat. no. 201069)
- EQ Four Element Calibration Beads (Fluidigm, cat. no. 201078)

Additional reagents for flow cytometry

- 1 μ M Fluorophore-conjugated detection probe(s) (resuspended in ddH₂O) for detection by flow cytometry (Integrated DNA Technologies). See Table 1 for oligonucleotide sequences for 27 PLAYR systems.
- (Optional) Live/Dead Fixable Dead Cell Stain Kits (Invitrogen) **▲ CRITICAL** Use Live/Dead Fixable Dead Cell Stain Kits if determination of cell viability is required.

Equipment

General equipment

- Filter tips
- Polypropylene V-bottom 200- μ L PCR strips (Fisher Scientific, cat. no. 11849241) or, alternatively, 1.5-mL Eppendorf tubes (Starlab, cat. no. S1615–5500)
- Refrigerated microcentrifuge

- Mass cytometer or flow cytometer; we use the Helios (Fluidigm) and Attune NxT (Invitrogen), respectively.

Antibody staining

- Weighing scale capable of four decimal places or more for the weighing of BS3 (Fisherbrand, cat. no. 15315113). Alternatively, pre-weighed BS3 can be purchased (Thermo Scientific, cat. no. 21585).

PLAYR

- Thermal mixer (Eppendorf ThermoMixer C, cat. no. 5382000031)

Software

- Fluidigm CyTOF system control software for mass cytometry (<https://www.fluidigm.com/software>)
- NxT software (Thermo Fisher; cat. no. A25554) for the Attune flow cytometer (or equivalent)
- Software appropriate for analysis of multiparametric mass cytometry data files (e.g., Cytobank (<https://www.cytobank.org/>) and Cytosplore (<https://www.cytosplore.org/>))
- R software (<https://www.r-project.org/>) for PLAYR probe design
- PLAYRDesign software to design PLAYR probes for the RNA of interest is available at <https://github.com/nolanlab/PLAYRDesign>

Reagent setup

10% (vol/vol) Tween 20—Dissolve one part Tween 20 in nine parts DEPC-treated ddH₂O. Store at RT for up to 6 months.

PLAYR oligonucleotides—PLAYR probes, inserts and backbone oligonucleotides are delivered dry, having been purified by standard desalting. Resuspend the oligonucleotides in DEPC-treated ddH₂O at a concentration of 100 μ M, and store them at -20°C for up to 3 years. To increase the speed of the protocol, it is recommended that all oligonucleotides be divided into aliquots before storing at -20°C . PLAYR probes with the same insert system that target an individual RNA transcript can be mixed together in equal quantities before dividing them into aliquots and freezing at -20°C . For example, if four probe pairs target a unique transcript, then these eight probes can be mixed together in equal quantities and stored as 8- μ L aliquots. Each aliquot is enough to make 1 mL of hybridization buffer, and each aliquot can then be pooled together before heating (see PLAYR probe hybridization buffer below).

PLAYR dilution buffer (PBST)—Take a 9-mL aliquot of DEPC-treated ddH₂O and add 1 mL of DEPC-treated 10 $\times$ PBS. Add 100 μ L of 10% (vol/vol) Tween 20 and place on ice. Prepare this buffer fresh at the start of the PLAYR assay (Step 13).

PLAYR wash buffer (PBSTR)—Add RNasin Plus to PBST at a final concentration of 4 U/mL. Prepare this buffer fresh at the start of the PLAYR assay (Step 13).

PLAYR wash buffer with extra RNasin (PBSTR+)—Add RNasin Plus to PBST to a final concentration of 40 U/mL. Prepare this buffer fresh at the start of the PLAYR assay (Step 13).

PLAYR probe hybridization buffer—To simplify and speed up the procedure for preparation of this buffer, we recommend dividing the PLAYR probes into aliquots, so that each aliquot is enough for 1 mL of hybridization buffer (see Reagent setup of ‘PLAYR oligonucleotides’ above). We make this buffer at a minimum volume of 1 mL, which therefore uses one aliquot of each transcript probe set, and one aliquot of RVC (enough for 10 samples). If >10 samples are being stained, then 2 mL of hybridization buffer would be made from two aliquots of PLAYR probes and RVC, and so on. Prepare this buffer fresh at the start of the PLAYR assay (Step 14), using the following procedure:

1. Thaw and pool together the required PLAYR probes and leave them at RT. For 1 mL of hybridization buffer, initially heat an aliquot of RVC to 65 °C, using a thermal mixer with vigorous agitation (we use 1,400 r.p.m.) for 25 min to dissolve any precipitation (two aliquots are required for 2 mL, and so on).
2. While the RVC is being incubated at 65 °C, mix together the following at RT for 1 mL of hybridization buffer:

Reagent	Volume	Final concentration
DEPC-treated ddH ₂ O	x μ L	—
DEPC-treated 20 $\times$ saline-sodium citrate solution	50 μ L	1 $\times$
10% (vol/vol) Tween 20	100 μ L	1% (vol/vol)
25% (vol/vol) PVS	100 μ L	2.5% (vol/vol)
Salmon sperm DNA	10 μ L	100 μ g/mL

The volume x μ L of DEPC-treated ddH₂O is calculated as the remaining volume required to produce 1 mL of solution after addition of all other reagents, including RVC, RNasin, and probes (see Step 5 below).

3. Remove the RVC from the thermal mixer and leave at RT to cool while preparing the cells (Step 15 of the main Procedure). **▲ CRITICAL STEP** Do not cool the RVC on ice, as this will cause it to precipitate.
4. 10 min before addition of the PLAYR hybridization buffer to the cells, heat the probe mixture for 5 min at 90 °C and then immediately place on ice for 5 min to avoid denaturing the RNasin.
5. Immediately before addition of the PLAYR hybridization buffer to the cells, add the following to complete its constituents:

Reagent	Volume	Final concentration
200 mM Pre-heated RVC	100 μ L	20 mM
RNasin Plus	1 μ L	40 U/mL
Pre-heated PLAYR probes	1 μ L of each	100 nM of each

PLAYR post-hybridization wash buffer—Dilute DEPC-treated 20 $\times$ saline–sodium citrate solution to 4 $\times$ saline–sodium citrate solution (1:5 (vol/vol) dilution) in PBST and add RNasin Plus to a final concentration of 40 U/mL. Prepare fresh just before use (Step 21).

PLAYR backbone/insert hybridization mix—Using PBST as the diluent, prepare a solution containing 1 $\times$ DEPC-treated saline–sodium citrate solution, 40 U/mL RNasin Plus, 100 nM backbone oligonucleotide, and 100 nM of each insert oligonucleotide. Prepare fresh just before use (Step 23).

PLAYR ligation mix—Using DEPC-treated ddH₂O as the diluent, prepare a solution containing 1 $\times$ T4 ligase buffer (as supplied by manufacturer), 5 U/mL T4 ligase and 40 U/mL RNasin. Prepare fresh just before use (Step 25).

PLAYR Phi29 amplification mix—Using DEPC-treated ddH₂O as diluent, prepare a solution containing 1 $\times$ Phi29 buffer (as supplied by manufacturer), 5 U/mL Phi29 DNA polymerase and 200 μ M dNTP. Prepare fresh just before use (Step 27).

PLAYR mass cytometry detection probe buffer—Use this buffer when performing mass cytometry. Using cell staining buffer as a diluent, prepare a solution containing 1 nM of each metal-conjugated detection probe and, if required, an appropriate concentration of metal-labeled antibodies against internal antigens. Prepare fresh just before use (Step 29).

PLAYR flow cytometry detection probe buffer—Use this buffer when performing flow cytometry. Using PBST as a diluent, prepare a solution containing 1 nM of each fluorophore-conjugated detection probe and, if required, an appropriate concentration of fluorophore-labeled antibodies directed against internal antigens. Prepare fresh just before use (Step 29). For a list of antibodies used in this protocol, see Supplementary Table 1.

Cell-ID Intercalator-Ir buffer—Make a 1:5 dilution of 8% (vol/vol) PFA with PBS to generate a 1.6% (vol/vol) solution and add Cell-ID Intercalator-Ir to a final concentration of 62.5 nM (i.e., 1:8,000 (vol/vol) of 500 μ M Cell-ID Intercalator-Ir). Intercalator-Ir buffer should be kept at 4 $^{\circ}$ C in the dark and can be stored for up to 1 week.

Procedure

▲ **CRITICAL** See Fig. 1c for an overview of the workflow.

Cell preparation ● Timing 6 min

1. Count the cells and place 3×10^6 cells into an Eppendorf tube on ice. In the example described in this protocol, we use PBMCs, but the approach can be used with other primary or cultured cell lines of interest.

▲ **CRITICAL STEP** We encourage a minimum of 3×10^6 cells per sample, because of cell loss throughout the assay. This yields approximately (depending on pipetting efficiency) 1.5×10^6 cells at the final wash step to analyze by mass/flow cytometry. Fewer cells per sample can be used for mass cytometry if the experimental plan includes Pd barcoding and pooling of the samples (performed during Steps 20–22) (see ‘Experimental design’, ‘Considerations specific for use with mass cytometry’).

(Optional) Staining to identify dead cells ● Timing 15 min

▲ **CRITICAL** Use viability staining if identification of cell death is required. See ‘Experimental design’, ‘General considerations’ section for more details.

▲ **CRITICAL** If staining to identify dead cells is not required, proceed to surface antibody staining.

2. Pellet the cells by centrifugation (500g, 3 min, 4 °C) and remove the supernatant. Wash the cells once by adding 200 μ L of RT PBS, followed by centrifugation (500g, 3 min, 4 °C) and removal of the supernatant.
3. Resuspend the cells in either 200 μ L of a 1:1,000 (vol/vol) dilution of Cell-ID Cisplatin (for mass cytometry) or Live/Dead viability dye (for flow cytometry) in RT PBS and incubate for 5 min (cisplatin) or 10 min (Live/Dead dye) at RT.

▲ **CRITICAL STEP** Incubation of the cells with viability dye should be done at RT, because cold conditions cause non-specific staining of live cells.

Surface antibody staining ● Timing 1 h 15 min

▲ **CRITICAL** Surface antibody staining can be omitted; this will not compromise the PLAYR assay.

If surface antibody staining is not required, perform only the PBS cell wash in Step 7 and proceed to Step 10.

4. Wash the cells (500g, 3 min, 4 °C) once in 200 μ L of ice-cold cell staining buffer.
5. Resuspend the cells in ice-cold antibody cocktail (for a list of antibodies used in this protocol, see Supplementary Table 1) in cell staining buffer (100 μ L/ 3×10^6 cells). Leave on ice for 30 min, agitating the cells every 10 min.

▲ **CRITICAL STEP** The cells must remain on ice at all times to avoid further transcription and RNA changes in live cells that may result from binding of surface antibodies.

6. Wash the cells (500g, 3 min, 4 °C) in 200 μ L of ice-cold cell staining buffer.

7. Wash the cells (500g, 3 min, 4 °C) in 200 µL of ice-cold PBS.
8. Resuspend the cells in 50 µL of 5 mM BS3 crosslinker in ice-cold PBS; mix immediately and leave on ice for 30 min, agitating the cells every 10 min.
▲ CRITICAL STEP The NHS-ester moiety within BS3 readily hydrolyzes upon contact with water. Warm the vial to RT to avoid moisture formation upon opening and weigh fresh powder for each experiment/time point. Dissolve the powder immediately before addition to the cells to avoid loss of reactivity.
9. Pellet cells (500g, 3 min, 4 °C) and remove the supernatant.

Cell fixation and permeabilization ● Timing 25 min

10. Resuspend the cells in ice-cold 1.6% (wt/vol) PFA at a maximum cell concentration of 2×10^6 cells/mL. Leave on ice for 10 min.
▲ CRITICAL STEP PFA fixation should be performed at RT if no surface antibody staining and BS3 cross-linking was performed.
11. Spin down the cells (500g, 3 min, 4 °C), remove the supernatant, loosen the pellet by flicking the tube and add ice-cold methanol dropwise, while vortexing (300 µL/ 3×10^6 cells).
▲ CRITICAL STEP Methanol can cause clumping of cells if they are not in a single-cell suspension.
12. Incubate for 10 min on ice.
▲ CRITICAL STEP Cells in methanol should always be kept on ice during handling.
■ PAUSE POINT Once placed in methanol, the cells can be stored at –80 °C for an extended period of time (several months) without RNA degradation.

PLAYR and intracellular antibody staining ● Timing 7–8 h for flow cytometry or 9–10 h for mass cytometry, with frequent incubation steps

13. Prepare PBST, PBSTR and PBSTR+ buffers (Reagent setup) and place on ice.
14. Prepare PLAYR probe hybridization buffer (refer to ‘Reagent setup’ for a detailed guide).
15. Spin down the cells (800g, 3 min, 4 °C) that are suspended in methanol and wash (800g, 3 min, 4 °C) with 200 µL of ice-cold PBSTR+ buffer.
▲ CRITICAL STEP Methanol has a higher density at –80 °C, and pelleting of cells by centrifugation may be inhibited; warm the tube on ice for 5 min if stored at –80 °C before centrifugation.
▲ CRITICAL STEP Once cells are removed from the ice-cold methanol, RNA degradation can set in and hence speed in sample preparation during this stage is essential to maintaining RNA integrity and PLAYR signal.

16. Resuspend the cells immediately in 100 μ L of RT PLAYR probe hybridization buffer and place on the thermal mixer at 40 °C.
▲ CRITICAL STEP Due to the small volumes used, failure to remove all PBSTR+ wash supernatant before addition of probe hybridization buffer can affect assay efficiency.
17. Incubate the cells for 1 h (this can be extended if the signal is weak) at 40 °C with vigorous agitation (we use 1,400 r.p.m.).
18. (Optional) When analyzing using mass cytometry, remove an aliquot of PBST from the ice while incubating the cells, and place at RT to perform Pd barcoding of cells (Step 20).
▲ CRITICAL STEP Perform this step only if you are Pd barcoding for mass cytometry.
19. Wash the cells once (800g, 3 min, 4 °C) with ice-cold PBSTR.²⁰ (Optional) When analyzing using mass cytometry, perform Pd barcoding at RT in PBSTR+ buffer (add RNAsin Plus to RT PBST from Step 16). We recommend resuspending the cells with 99 μ L of RT PBSTR+, adding 1 μ L of the appropriate Pd barcode and then incubating for 10 min.
▲ CRITICAL STEP Perform this step only if you are using Pd barcoding for mass cytometry. Pd barcoding allows samples to be pooled together in one tube, which provides a number of benefits. See ‘Experimental design’, ‘General considerations’ for more details.
20. Wash the cells twice (800g, 3 min, 4 °C) in 200 μ L of ice-cold PBSTR, prepare PLAYR post-hybridization wash buffer (‘Reagent setup’), and place on ice.
21. Resuspend the cells in 100 μ L of ice-cold post-hybridization wash buffer and incubate for 20 min at 40 °C with vigorous agitation (we use 1,400 r.p.m.). Pd-barcode cells for mass cytometry can be pooled together at this stage.
▲ CRITICAL STEP Remember to increase the volume of buffer if using an increased number of cells per sample.
22. Wash cells twice (800g, 3 min, 4 °C) in 200 μ L of ice-cold PBSTR and prepare PLAYR backbone/insert hybridization mix (‘Reagent setup’) on ice.
23. Resuspend the cells in 100 μ L of ice-cold PLAYR backbone/insert hybridization mix and incubate at 37 °C for 30 min under agitation (we use 1,100 r.p.m.).
24. Wash the cells twice (800g, 3 min, 4 °C) in 200 μ L of ice-cold PBSTR and prepare PLAYR ligation mix (‘Reagent setup’) on ice.
25. Resuspend the cells in 50 μ L of ice-cold PLAYR ligation mix and incubate at 21 °C for 30 min with agitation (we use 1,100 r.p.m.).
26. Wash the cells twice (800g, 3 min, 4 °C) in 200 μ L of ice-cold PBSTR and prepare PLAYR Phi29 amplification mix (‘Reagent setup’) on ice.

27. Resuspend the cells in ice-cold PLAYR Phi29 amplification mix and incubate at 30 °C with agitation for 2 h (flow cytometry) or 4 h (mass cytometry). Longer incubations (up to overnight) may increase signal intensity. We typically agitate the cells at 1,100 r.p.m. In any case, make sure that you use a speed such that the cells do not settle at the bottom of the tubes, as this will decrease the amplification efficiency).
28. Wash the cells twice (800g, 3 min, 4 °C) in 200 µL of ice-cold cell staining buffer (mass cytometry) or ice-cold PBSTR (flow cytometry), and prepare PLAYR mass cytometry detection probe buffer or PLAYR flow cytometry detection probe buffer on ice. Intracellular antibodies can also be added to this buffer as described in the 'Reagent setup' section.
29. Resuspend cells in 100 µL of the appropriate ice-cold detection probe buffer and incubate at 37 °C for 30 min with agitation (we use 1,100 r.p.m.).
30. Wash the cells twice (800g, 3 min, 4 °C) in ice-cold cell staining buffer (for mass cytometry) or twice in ice-cold PBSTR (for flow cytometry).
▲ CRITICAL STEP Do not use PBSTR to wash cells for mass cytometry after detection probe hybridization, as this will lead to signal loss.
31. For flow cytometry, resuspend the cells in ice-cold PBS and analyze immediately, using laboratory-specific protocols. For mass cytometry, resuspend the cells in Cell-ID Intercalator-Ir buffer, incubate for 1 h at RT or for up to 2 weeks at 4 °C and proceed to Step 33.

? TROUBLESHOOTING

■ PAUSE POINT Samples for mass cytometry can be stored at 4 °C for up to 2 weeks in Cell-ID Intercalator-Ir buffer with minimal loss of signal.

(Optional) Preparation of samples for mass cytometry analysis ● Timing 20 min

▲ CRITICAL Steps 33–37 describe how to prepare the samples for mass cytometry.

32. To prepare the sample for the mass cytometer, wash the cells once (800g, 3 min, 4 °C) with 1 mL of cell staining buffer, then once (800g, 3 min, 4 °C) with 1 mL of Maxpar water.
33. Resuspend the cells in 1 mL of Maxpar water, filter the cells through a 30-µm filter and count the cells.
34. Adjust the concentration of cells to a maximum of 5×10^5 cells/mL in Maxpar water.
35. Add EQ Four Element Calibration Beads 1:10 (vol/vol), mix by vortexing and then analyze, using the mass cytometer. Details on instrument and panel-template setup can be found at <http://www.fluidigm.com/productsupport/cytof-helios-support-hub>.

Analysis of mass cytometry data ● Timing 2–6 h

36. Analyze the data, using linked software (e.g., Cytobank).

? TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 2.

Timing

Step 1, cell preparation: 6 min

(Optional) Steps 2 and 3, staining to identify dead cells: 15 min

Steps 4–9, surface antibody staining: 1 h 15 min

Steps 10–12, cell fixation and permeabilization: 25 min

Steps 13–32, PLAYR and internal antibody staining: 7–8 h for flow cytometry or 9–10 h for mass cytometry

(Optional) Steps 33–36, preparation of samples for mass cytometry: 20 min

Step 37, analysis of mass cytometry data: 2–6 h

Box 1, metal labeling of detection probe oligonucleotides: 23 h, including an overnight incubation step

Anticipated results

This protocol provides a detailed and step-by-step guide to multiplexed and simultaneous detection of RNA and antigen expression using the PLAYR technique combined with antibody labeling for up to 40 variables. The methodology described allows the use of the technique for either flow or mass cytometric applications.

Assay optimization

We recommend optimizing PLAYR and antibody assays separately before the two procedures are combined (Fig. 3). We have optimized the PLAYR assay by studying control genes with high copy numbers in a cell line to confirm assay efficiency. See Fig. 5a for an example comparing signal intensity achieved in primary CLL cells vs the MEC1 cell line. It is important to understand that expression of ‘classic’ control genes may not be uniform within cell populations, particularly in primary cells. For example, we observed high unimodal expression of ACTB, RPL27 and GAPDH in cell lines, but GAPDH exhibited a multimodal pattern of expression in primary CLL cells (Fig. 5a, top right panel). Upon confirming that each PLAYR detection probe was correctly labeled and working with similar efficiencies on our positive-control gene (Fig. 4c), we applied the assay to other transcripts of interest. To confirm that PLAYR signals were specific for each transcript, we performed a

time course analysis of RNA expression changes known to occur upon in vitro PMA and ionomycin stimulation of MAVER1 cells and correlated PLAYR mean signal intensities with qPCR quantification (Fig. 4a and Supplementary Fig. 2). When optimized, the pattern of expression of each individual transcript—examined on its own or when multiplexed—should be consistent (Fig. 4b).

As demonstrated in Fig. 2, classification of cell identities through detection of antigen should not be compromised by additional steps required for the PLAYR assay (second row of each set of 18 panels in Fig. 2) when compared with standard antibody staining of cells (top row of each set of 18 panels). Similarly, the additional steps for antibody staining of cells should not affect the performance of the PLAYR assay.

Insights into genotypic and phenotypic heterogeneity at single-cell resolution

The main advantage of the combined analysis of antigen and RNA expression using the technique is the potential to address genotypic and phenotypic variations at single-cell resolution. Our study of primary CLL cells (Fig. 5) is an example of how the technique can provide novel insights into intraclonal heterogeneity. CLL cells from a single patient were resolved into newly emerged vs old quiescent cells (Fig. 5d) on the basis of the intensity of CXCR4 and CD5 surface antigen expression¹⁸. It is clear that there is considerable variation of Ki67 expression (Fig. 5e) and CD83, JUND and TXNIP mRNAs as measured by PLAYR (Fig. 5f). This was not apparent in previously reported studies performed on whole-cell populations^{17,19–21}. Additional computational analyses allow clustering of seemingly identical neoplastic CLL cells into distinct phenotypic and genotypic subpopulations (Fig. 5g,h) with a potential for variant behavior and therapeutic responses. Hence, the combined and multiplexed analysis of antigen and RNA is a useful tool for monitoring and tracking CLL disease evolution, biology and heterogeneity. Relevant questions regarding heterogeneity in other cellular contexts can be addressed using our protocol.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary.

Data availability and patient-derived material

The data that support the findings of this study are available from the authors on reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1 |**Metal labeling of detection probe oligonucleotides ● Timing 23 h, including overnight incubation step****Reagents**

- Ice-cold 100% (vol/vol) ethanol
- Ice-cold 75% (vol/vol) ethanol
- 3 M Sodium acetate (pH 5.2; Thermo Scientific, cat. no. R1181)
- Bond-Breaker TCEP solution (neutral pH; Thermo Scientific, cat. no. 77720)
- Maxpar X8 Antibody Labeling Kit (Fluidigm)

▲ **CRITICAL** The choice of metal for each PLAYR detection probe depends upon your panel design and the PLAYR insert system used.

- Detection probe carrying a 5'-thiol-modifier C6 S-S (IDT)

Equipment

- NanoDrop spectrophotometer (Thermo Scientific, cat. no. ND-2000)
- Centrifugal filter with 30 kDa MWCO (EMD Millipore, cat. no. UFC503024)
- Centrifugal filter with 3 kDa MWCO (EMD Millipore, cat. no. UFC500396)

Procedure

1. Resuspend the detection probes carrying a 5'-thiol-modifier C6 S-S at 250 μ M in ddH₂O. Calculate or make a note of the starting number of nmoles for each detection probe, as this will be required during calculations in step 15.
2. Add TCEP to a final concentration of 50 mM and incubate at RT for 30 min.
3. Increase the volume, if required, to a minimum of 200 μ L using ddH₂O and add 0.1 volumes of 3 M sodium acetate and 3 volumes of pure ethanol. Vortex to mix. Precipitate the detection probe at -20 °C overnight.

■ **PAUSE POINT** Alternatively, PLAYR DNA detection probes can be stored at -80 °C in 75% (vol/vol) ethanol for up to 6 months without degradation before being processed.

4. From the Maxpar X8 Antibody Labeling Kit, spin polymer tube (4,000g/30 s/RT) to ensure all reagents are at the bottom.
5. Resuspend the polymer in 95 μ L of L-Buffer and then add 5 μ L of lanthanide metal solution, and mix by pipetting.
6. Incubate the polymer-metal mix at 37 °C for 30 min.
7. While the polymer-metal mix is incubating, centrifuge the detection probe oligonucleotide from step 3 at 14,000g, 4 °C, 30 min.
8. Add 200 μ L of L-Buffer to a 3-kDa filter.

9. After incubation, add the metal-loaded polymer from step 6 to the filter containing the L-Buffer.
10. Centrifuge metal-conjugated polymer at 12,000g, RT, 25 min.
11. Wash the detection probe pellet from the centrifugation in step 7 twice in ice-cold 75% (vol/vol) ethanol, centrifuging at 14,000g, 4 °C, 10 min.
12. Add 300 µL of C-Buffer to the 3-kDa filter from step 10 and centrifuge at 12,000g, RT, 30 min; leave at RT while completing detection probe purification.
13. After the final wash of the detection probe in step 11, remove as much ethanol as possible (a brief centrifuge step (14,000g, 4 °C, 30 s) may help to pool residual ethanol for removal) and air-dry the pellet (the tube can be placed at 40 °C on a heat block to decrease drying time).

▲ **CRITICAL STEP** Failure to fully dry the pellet and remove all ethanol can affect downstream applications. The pellet is dry when it turns from opaque/white to bright white in color.
14. Assuming a yield of 80%, resuspend the dried pellet in C buffer (supplied in the Maxpar X8 Antibody Labeling Kit) to a concentration of 1 nmol/25 µL. For example, a starting quantity of 10 nmoles would give an assumed yield of 8 nmoles and would therefore be resuspended in 200 µL of C buffer.
15. Measure the reduced detection probe concentration from step 14 by absorption at 260 nm on a NanoDrop spectrophotometer (use C buffer as a blank).
16. Using the concentration from step 15, calculate the volume required for 2 nmol of detection oligonucleotide (MW = 6,789 g/mol) and transfer this amount to a fresh Eppendorf tube.
17. Transfer the entire metal-loaded polymer from the 3-kDa filter to the Eppendorf tube containing the 2-nmol oligonucleotide.
18. Increase the volume of the mixture to 100 µL using C-Buffer and mix by pipetting.
19. Incubate at RT for 2 h.
20. Add TCEP to a final concentration of 5 mM, immediately mix by vortexing and incubate at RT for 30 min.
21. Add 400 µL of ddH₂O to a 30-kDa filter.
22. Transfer the metal-polymer-detection probe mixture to the 30-kDa filter containing the ddH₂O and centrifuge at 14,000g, RT, 12 min.
23. Discard the flow-through, add 500 µL of ddH₂O to the filter and centrifuge at 14,000g, RT, 12 min.
24. Repeat step 23.

25. Invert the filter into a clean collection tube and centrifuge the filter and tube at 1,000g, RT, 2 min.
26. Wash the filter with 250 μ L of ddH₂O and elute into the same collection tube by inverting the filter and centrifuging the filter and tube at 1,000g, RT, 2 min.
27. Measure the oligonucleotide concentration by NanoDrop spectrophotometer, using ddH₂O as a blank.
28. Adjust the volume of ddH₂O to obtain a final concentration of 1 μ M metal-labeled detection probe (assuming 6,789 ng/mL = 1 μ M).
29. Prepare aliquots and store the metal-labeled detection probe at -20°C for up to 3 years.

▲ CRITICAL STEP Confirm efficient detection probe labeling by targeting a control gene that is known to have high copy number. For example, we have confirmed efficient detection probe labeling by using the cell line MAVER1 stained with PLAYR probe pairs containing the appropriate 3' sequences all targeting the same loci on GAPDH mRNA (Fig. 4c).

? TROUBLESHOOTING

Fig. 1 |. Schematic representation of the PLAYR assay.

a. Schematic overview of PLAYR (stages 1–8) for simultaneous staining of surface/intracellular proteins and mRNAs. The steps in the procedure are indicated for each stage. Initial staining of surface proteins is performed on ice in unfixed cells (stage 1). Bound antibodies are covalently cross-linked to the cells using the chemical agent BS3 (stage 2), and cells can then be fixed with paraformaldehyde and permeabilized using methanol (stage 3). Once suspended in methanol, cells can be stored at -80°C for up to several months without loss of RNA integrity or surface-antibody staining (Pause point). Stages 4–8 illustrate the strategy for RNA detection using PLAYR. Intracellular antibody staining is performed during incubation of the PLAYR detection probes in stage 8. **b.** Overview of the PLAYR probe design, with different sequence components color-coded: RNA-binding sequence (purple); linker region (green); insert-binding sequence (red); backbone-binding sequence (blue). ‘N’ represents nucleotides that vary depending upon the target (RNA-binding sequence) and the PLAYR system (insert-binding sequence) used. **c.** Diagram illustrates the interaction of different oligonucleotide sequences during the PLAYR assay. (Top) 5′ probe sequences bind to their target RNA. (Bottom) The insert and backbone oligonucleotides subsequently bind to the 3′ end of probes 1 and 2. Color-coding of probe components is the same as in **b**.

Fig. 2 | Comparison of surface-antigen staining in the PLAYR assay to that of a standard protocol for a panel of 27 antibodies.

Bivariate dot plots, colored for event density, showing peripheral blood mononuclear cells simultaneously analyzed for 27 surface proteins and measured by mass cytometry. Cells were either fixed first and then stained with antibody at room temperature (standard staining protocol; top row in each set of 18 panels) or stained with antibody for use with PLAYR, which includes BS3 cross-linking, fixation and permeabilization steps (staining for PLAYR; bottom row in each set of 18 panels). For the list of antibodies, see Supplementary Table 1.

Fig. 3 |. Schematic of experimental design workflow for PLAYR.

Workflow showing in silico experimental design (orange); acquisition of labeled detection probes for either mass (blue) or flow (green) cytometry; control experiments for PLAYR RNA detection and antibody staining (pink); data collection and analysis of single-cell RNA expression in samples of interest (purple).

Fig. 4 |. Examples of control experiments for PLAYR.

a, The B-cell line MAVER1, treated for up to 6 h with 100 nM phorbol 12-myristate 13-acetate (PMA) + 1 μ M ionomycin, was examined by PLAYR (mass cytometry) and qPCR for expression changes in nine RNAs. Each dot represents the expression of the transcript at specific time points measured by qPCR (x axis) and PLAYR (y axis); linear regression is represented by the blue lines, with gray shading indicating 95% confidence intervals. **b**, Nine variably expressed mRNAs were examined (individually (rows 1–9) or in multiplex (row 10)) using the PLAYR assay in primary leukemic cells from a CLL patient that were labeled with transcript-specific detection probes (for mass cytometry). **c**, Validation of PLAYR system efficiency (including detection probe labeling) using three (top row) or two (bottom row) probe pairs targeting GAPDH mRNA in the MAVER1 B-cell line. INS, PLAYR insert system.

Fig. 5 |. Use of PLAYR for analysis of surface/internal protein and RNA expression in primary CLL cells.

a, Quantification of three 'housekeeping' mRNAs using PLAYR analyzed by flow cytometry in primary peripheral blood mononuclear cells of CLL patients or the MEC1 cell line. Histograms are representative of three CLL samples and three MEC1 biological replicates (for definition of probes 1 and 2, see Fig. 1). **b**, Flow cytometric quantification of ACTB mRNA (using PLAYR) in a mixed sample containing the HUT78 T-cell line and the MAVER1 B-cell line. Singlet cells are gated using FSC and SSC (top two panels); the cell type was determined using CD5 and CD19 surface antigen expression (bottom left panel; purple gate, CD19⁻CD5⁺⁺ HUT78; blue gate, CD19⁺CD5⁺ MAVER1); the shaded histograms in the bottom right panel show ACTB mRNA expression within the gated HUT78 and MAVER1 cells. Unshaded histograms depict 'probe 2 only' negative controls. **c**,

Gating strategy for mass cytometric detection of live singlet CLL cells and T-cells in human peripheral blood samples. **d**, Mass cytometry data showing gating strategy to identify cells that have remained in the blood for an extended period of time, termed old quiescent (OQ; CD5^{dim}CXCR4^{bright}), and cells that have recently exited the tissue microenvironment, termed newly emerged (NE; CD5^{bright}CXCR4^{dim})¹⁸. **e**, Percentages of CLL cells expressing the proliferative marker Ki67 within the OQ and NE subpopulations of 18 CLL samples. Results are comparable to previous findings that used flow cytometry¹⁸. Error bars represent 1 s.d. from the mean. **f**, Characterization of intraclonal heterogeneity in expression of prognostic mRNAs¹⁹ (top row, CD83, JUND and TXNIP) and surface antigens (bottom row, CD21, CD22 and CD27) within cells resolved for CD5 and CXCR4 surface expression in a representative CLL sample. **g**, viSNE analysis of the data in **f** on the basis of eight CLL surface antigens (CD5, CD19, CD21, CD22, CD27, CXCR4, IgD and IgM). Top panel indicates event density colored by temperature (high = red; low = blue) and the corresponding bottom panel highlights Ki67⁺ cells (red dots) within the viSNE contour plot. **h**, viSNE plot from **g** with additional color-coding for either mRNA (top row) or surface antigen expression (bottom row) to resolve expression for multiple parameters. ATTO647, Alexa Fluor 647 equivalent from IDT; FSC-A, forward scatter area; SSC-A, side scatter area; SSC-W, side scatter width.

Table 1 |

Oligonucleotide sequences for 27 PLAYR systems

System	3' Probe 1	3' Probe 2	Insert	Detection probe
1	AAAAAAAAAACTCAGTCGTGACACTCTT	AAAAAAAAAAAGACGCTAATATCGTGACC	P-ACGACTGAGTTTGGTCACGAT	Z-ACGACTGAGTTTGGTCACGAT
2	AAAAAAAAAAATATCGTCCGGACACTCTT	AAAAAAAAAAAGACGCTAATCTTCGAGAC	P-CGGACGATATTTGTCTCGAAG	Z-CGGACGATATTTGTCTCGAAG
3	AAAAAAAAAAAGATTCTCTCGGACACTCTT	AAAAAAAAAAAGACGCTAATCTGCCAATG	P-CGAGGAATCTTTCATTTGGCAG	Z-CGAGGAATCTTTCATTTGGCAG
4	AAAAAAAAAAACTACCTTGGGACACTCTT	AAAAAAAAAAAGACGCTAATCAGGCTACT	P-CCAAAGGTAGTTTAGTAGCCCTG	Z-CCAAGGTAGTTTAGTAGCCCTG
5	AAAAAAAAAAACTCTTCGAGGACACTCTT	AAAAAAAAAAAGACGCTAATCACCAGTTG	P-CTCGAAGAGTTTCAACTGGTG	Z-CTCGAAGAGTTTCAACTGGTG
6	AAAAAAAAAAACTTAGCCTGGACACTCTT	AAAAAAAAAAAGACGCTAATCCAGACTGT	P-CAGGCTAAGTTTACAGTCTGG	Z-CAGGCTAAGTTTACAGTCTGG
7	AAAAAAAAAAACCGCTTATGGACACTCTT	AAAAAAAAAAAGACGCTAATCTACATGGC	P-CATAAGCGGTTTGGCCATGTAG	Z-CATAAGCGGTTTGGCCATGTAG
8	AAAAAAAAAAACTCGATCTGGACACTCTT	AAAAAAAAAAAGACGCTAATCAACCTGGT	P-CAGATCGAGTTTACCAGGTTG	Z-CAGATCGAGTTTACCAGGTTG
9	AAAAAAAAAAATACGTTCCGGACACTCTT	AAAAAAAAAAAGACGCTAATCATCTCTGAG	P-CGGAACGTATTTCTCAGGATG	Z-CGGAACGTATTTCTCAGGATG
10	AAAAAAAAAAACTGCTCATGGACACTCTT	AAAAAAAAAAAGACGCTAATCGCAAGTCT	P-CATGAGCAGTTTAGACTTTGCG	Z-CATGAGCAGTTTAGACTTTGCG
11	AAAAAAAAAAATGACTCTCGGACACTCTT	AAAAAAAAAAAGACGCTAATCTCGGAATC	P-CGAGAGTCATTTGATTTCCGAG	Z-CGAGAGTCATTTGATTTCCGAG
12	AAAAAAAAAAACTGTCTACGGACACTCTT	AAAAAAAAAAAGACGCTAATCACAGTGTG	P-CGTAGACAGTTTGCACACTGTG	Z-CGTAGACAGTTTGCACACTGTG
13	AAAAAAAAAAATCTCCAGGGACACTCTT	AAAAAAAAAAAGACGCTAATCTCAATCGG	P-CTGGAGAAATTTCCGATTTGAG	Z-CCTGGAGAAATTTCCGATTTGAG
14	AAAAAAAAAAACACTTGTCCGACACTCTT	AAAAAAAAAAAGACGCTAATCAGATGCCT	P-CGACAAAGTTTGTAGGCCATCTG	Z-CGACAAAGTTTGTAGGCCATCTG
15	AAAAAAAAAAACTTCTGCAGGACACTCTT	AAAAAAAAAAAGACGCTAATCCAGGATCT	P-CTGCAGAAAGTTTAGATCTCTGG	Z-CTGCAGAAAGTTTAGATCTCTGG
16	AAAAAAAAAAATCTATCCGGACACTCTT	AAAAAAAAAAAGACGCTAATCTGTAGACC	P-CCGGATAGATTTGGTCTACAG	Z-CCGGATAGATTTGGTCTACAG
17	AAAAAAAAAAAGGCATCTTGGACACTCTT	AAAAAAAAAAAGACGCTAATCTGGCACAT	P-CAAGATGCGTTTATGTGCCAG	Z-CAAGATGCGTTTATGTGCCAG
18	AAAAAAAAAAATCTCACGTGGACACTCTT	AAAAAAAAAAAGACGCTAATCTCTCGAATG	P-CACGTGAGATTTTCATTCGAGG	Z-CACGTGAGATTTTCATTCGAGG
19	AAAAAAAAAAATCGCTACTGGACACTCTT	AAAAAAAAAAAGACGCTAATCGCCATGAT	P-CAGTAGCGATTTATCATGCGG	Z-CAGTAGCGATTTATCATGCGG
20	AAAAAAAAAAATACGCTCTGGACACTCTT	AAAAAAAAAAAGACGCTAATCACACTTGG	P-CAGAGCGTATTTCCAAAGTTG	Z-CAGAGCGTATTTCCAAAGTTG
21	AAAAAAAAAAACGTCTTACGGACACTCTT	AAAAAAAAAAAGACGCTAATCATGCGACT	P-CGTAAGACGTTTAGTCGCAATG	Z-CGTAAGACGTTTAGTCGCAATG
22	AAAAAAAAAAACCATTCGTGGACACTCTT	AAAAAAAAAAAGACGCTAATCATCAGCGT	P-CACGAATGGTTTACGCTGATG	Z-CACGAATGGTTTACGCTGATG
23	AAAAAAAAAAACCTAGTTCCGACACTCTT	AAAAAAAAAAAGACGCTAATCTACTAGG	P-CGAACCTAGGTTTCTCTAGGTAG	Z-CGAACCTAGGTTTCTCTAGGTAG
24	AAAAAAAAAAACTCCGATTGGACACTCTT	AAAAAAAAAAAGACGCTAATCAACGCGTT	P-CAATCGGAGTTTAAACGCGTTG	Z-CAATCGGAGTTTAAACGCGTTG
25	AAAAAAAAAAATTCGCACCTGGACACTCTT	AAAAAAAAAAAGACGCTAATCAATTCGCG	P-CAGTGCGAATTTCCGGAATG	Z-CAGTGCGAATTTCCGGAATG
26	AAAAAAAAAAATCTTCAGGGACACTCTT	AAAAAAAAAAAGACGCTAATCCGCTAAGT	P-CCTGAAGGATTTACTTAGCGG	Z-CCTGAAGGATTTACTTAGCGG
27	AAAAAAAAAAACGTTTACTCTGGACACTCTT	AAAAAAAAAAAGACGCTAATCTTAAAGCGC	P-CGAGTAAAGTTTGGCGTTAAG	Z-CGAGTAAAGTTTGGCGTTAAG
Universal backbone: P-ATTAGCGTCCAGTGAATGCGAGTCCGTCTAGGAGAGTAGTACAGACGCCGTCAGAGTGTG				

P, 5' -phosphorylated; Z, 5' -C6-thiol modification for mass cytometry or fluorophore modification for flow cytometry.

Table 2 |

Troubleshooting table

Step	Problem	Possible reason	Solution
32, 37 and Box 1, step 29	Little or no PLAYR signal	RNA is low in cells of interest	Use appropriate positive-control cell line Increase number of probe pairs Increase RCA time in Step 28
		RNA degradation by release of internal RNases	Confirm correct production of probe hybridization buffer Work quickly after cell permeabilization with MeOH Keep cells cold at all times during wash steps
		Detection probe is not labeled efficiently	Run titrated detection probe through the mass cytometer to confirm metal labeling
		Insert or backbone oligonucleotides are not 5'-phosphorylated	Phosphorylate oligonucleotides with PNK
		Inefficient ligase or Phi29 enzyme activity	Remove all wash buffer from the sample before addition of the enzyme reaction solution Confirm efficiency of the PLAYR system, using a highly expressed RNA transcript Use a new batch of enzymes
	Weak antibody staining	Antigen expression is low in target cells	Confirm staining in positive-control cells
		Low antibody concentrations	Increase antibody concentrations
		BS3 crosslinker was hydrolyzed before contact with cells	Use fresh BS3 crosslinker dissolved just before use Warm the tube containing BS3 to RT before opening to avoid condensation
		Weak antibody cross-linking or PLAYR conditions are too harsh for the antibody clone	Increase BS3 concentration Wash cells in ice-cold PBS to remove all non-cellular proteins from solution Reduce the stringency of the PLAYR assay (see 'Protocol optimization')
	High background PLAYR signals	Non-specific probes	Confirm specificity of the probe pairs in silico Test each probe pair individually Increase the stringency of the PLAYR assay (e.g., temperature, post-hybridization wash buffer)
		Concentration of detection probe is too high	Confirm low background staining of the detection probe, using a negative-control sample such as performing the assay when no ligase enzyme is added to the ligase reaction Titrate detection probe
		Antibody concentrations are too high	Titrate antibody concentrations
	Not enough cells for analysis	Not enough cells at the start of the assay	Use a minimum of 3×10^6 primary leukocytes per sample and increase if necessary
		Loss of cells during assay	Improve pipetting efficiency

Step	Problem	Possible reason	Solution
			During repeat washes, leave small quantities of supernatant to avoid the pellet
			Use swinging-bucket rotors if not already doing so