SpliceRCA: in Situ Single-Cell Analysis of mRNA Splicing Variants

Xiaojun Ren,†‡ Ruijie Deng,† Kaixiang Zhang,† Yupeng Sun,† Xucong Teng,† and Jinghong Li*‡†‡

†Department of Chemistry, Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology, Tsinghua University, Beijing 100084, China
‡School of Chemistry and Chemical Engineering, Beijing Institute of Technology, Beijing 100081, China

ABSTRACT: Immune cell heterogeneity due to the differential expression of RNA splicing variants still remains unexplored. This is mainly because single-cell imaging technologies of splicing variants with precise sequence or base resolution are now not readily available. Herein, we design a splice-junction anchored padlock-probe-mediated rolling circle amplification assay (SpliceRCA) for single-cell imaging of splice isoforms of essential regulatory immune gene (CD45) upon T-cell activation. Two recognition regions in the padlock probe can target the splice-junction sequence, resulting in a close proximity for triggering in situ one-target-one-amplicon amplification. With the read length of ~30 nucleotides, this method allows discrimination of isoforms with single-base precision and quantification of isoforms with single-molecule resolution. We applied SpliceRCA to single-cell image splice variants of essential regulatory immune gene (CD45) upon T-cell activation. It is found that CD45RO isoform presents a distal nuclear spatial distribution and is coregulated with CD45RB upon activation. Our strategy provides a single-cell analysis platform to investigate the mechanism of complex immune responses and may further guide immunotherapy.

INTRODUCTION

Alternative splicing is a fundamental regulatory process of gene expression that allows generation of multiple mRNA isoforms from single genes,1,2 thereby increasing transcriptome complexity.3–5 Splicing variability among individual cells accounts for a large part of gene expression heterogeneity, which plays a significant role in the immune system for the efficient battling rich variability of pathogens.6–8 Isoforms with diversified functions were evolved in the generation of the immune response.9 CD45, the prototypic receptor-like protein tyrosine phosphatase gene, acts as an essential regulator of signal transduction pathways in immune cells.8 The transition from naïve to activated T cells is marked by CD45 pre-mRNA alternative splicing.10 Abnormal CD45 splice variant expressions are associated with autoimmune and infectious diseases.10,11,12 However, the roles of differential CD45 splicing variants, such as their relative expression level, intracellular localization, expression heterogeneity, and regulation in eliciting immune responses, are underexplored. Thus, visualizing12 intracellular mRNA variants in single cells is essential for understanding differential splicing-mediated immune cell heterogeneity and resolving the complexity and heterogeneity of RNA variant-related immune diseases.

Currently, only a limited number of methods have been explored to detect RNA splicing variants at the single-cell level. Single-molecule fluorescence in situ hybridization (smFISH) is a facile and powerful method to in situ image RNA splice variants in single cells, and brings a significant advance of RNA splicing in single-cell study.13,14 However, the FISH method can only detect RNA sequences with a minimum length of 600 nucleotides (nt) (usually need >30 different hybridization probes with ~20 nt to ensure that the labeling signals are distinguished from the background).15,16 However, the human exon is with the average length of only 320 nt; thus, large amounts of exons can hardly be detected by smFISH.17,18 Recently, a newly proposed plasmonic in situ hybridization (plasmonic ISH) based on gold nanoparticle (AuNP) labeling is masterly adapted to analyze RNA splicing and is able to differentiate splice variants with ~300 nt sequence.19 However, limitations on AuNPs remain currently unsolvable, such as their large size for impeding the delivery efficiency, alteration in a real cell state, and easy aggregation, etc.20,21 The visualization of short exon mRNA variants still remains a challenge. Meanwhile, a large amount of short exon mRNA variants are involved in crucial biological processes,22 like CD45 isoforms, of which the average length of the alternative exon is less than 200 nt.23 There is still an urgent need for developing a versatile RNA imaging method with high sequence resolution capable of in situ analyzing splice isoforms with different lengths.

To address these issues, we develop a high base-resolution strategy termed as splice-junction anchored padlock probe-mediated rolling circle amplification14 (SpliceRCA), enabling single-cell imaging of CD45 splicing variants. Rolling circle amplification (RCA) can achieve localized isothermal amplification, converting the target sequence into a long single-stranded DNA or RNA product with thousands of tandem repeats.24,25 Attribution to its one-target-one-amplicon amplifica-
tion process, RCA can achieve target RNA/DNA detection or imaging at the single-molecule level. Furthermore, the padlock probe-based RCA method has the capability to target short RNAs and discriminate highly similar sequences to genotype RNAs with single-nucleotide variations, which prompted us to explore the potential of RCA in RNA splicing variant detection. In this study, two recognition regions in the padlock probe are specifically hybridized to a newly formed splice-junction sequence, resulting in a close proximity for triggering in situ one-target-one-amplicon amplification, achieving shortening the read length of the imaging method to ∼30 nt. This method allows discrimination of isoforms with single-base precision and quantification of isoforms with single-molecule resolution.

**RESULTS AND DISCUSSION**

**Overview of SpliceRCA.** Scheme 1 illustrates the procedures of the direct visualization of RNA splice variants in single cells by SpliceRCA. Alternative splicing of exon 4 (198 nt), 5 (141 nt), and 6 (144 nt) in CD45 pre-mRNA is strictly regulated in T-cell activation: naïve T cells express various larger isoforms whereas memory T cells tend to produce small isoforms. We chose three critical alternative splicing variants of the CD45 gene involved in the T-cell activation process which include the largest isoform CD45RA (all exons retained), middle isoform CD45RB (exon skipping, exon 4 deleted), and smallest isoform CD45RO (exon skipping, exons 4, 5, and 6 deleted) (Scheme 1A). SpliceRCA is performed with a splice-junction anchored padlock probe composed of four modules: the recognition of exon junction sites (Rx, Ry), universal priming region (P), and tag motif (T) modules (Scheme 1B). The splice junction in the target splice isoforn brings close proximity between Rx and Ry in the padlock probe for circularizing, following primer hybridized with the P, triggering in situ RCA. Upon tuning of the sequence of T corresponding to different fluorophores, the three RNA splicing variants can thus be simultaneously differentiated, and visualized with single-molecule resolution attributed to the in situ one-target-one-amplicon amplification method.

**Multiplex in Situ Imaging of CD45 mRNA Splicing Variants in Single Cells by SpliceRCA.** The specific scheme of in situ RCA is shown in Figure 1A. The splice-junction sequence was specifically recognized by the padlock probes and as the template for ligating the padlock probe. Then, RCA is initiated with the help of an additional DNA primer, resulting in a long DNA amplicon with hundreds of copies of the

---

**Scheme 1. Schematic Diagram of Multiplex Detection of mRNA Variants in Single Cells by SpliceRCA**

*(A) Alternative splicing patterns of CD45 during T-cell activation. Isoforms (CD45RA, CD45RB, CD45RO) with decreasing exon inclusion were expressed upon T-cell activation. (B) The procedures of SpliceRCA for detecting splice variants in single cells. The splice-junction anchored padlock probe is composed of four modules: the recognition of exon junction sites (Rx, Ry), universal priming region (P), and tag motif (T) modules. The newly formed splice junction in the target splice isoform brings close proximity between Rx and Ry in the padlock probe for circularizing, following primer hybridized with the P, triggering in situ RCA. Upon tuning of the sequence of T corresponding to different fluorophores, the three RNA splicing variants can thus be simultaneously differentiated, and visualized with single-molecule resolution attributed to the in situ one-target-one-amplicon amplification method.*
The RCA amplicon forms as a nanoclew that can be visible as a diffusion-limited fluorescent spot upon hybridization with different detection probes. To demonstrate the feasibility of this principle for detection of RNA splice isoforms, we first performed the assay in vitro to amplify the target synthetic sequence of CD45RO, CD45RA, and CD45RB (Table S1, Supporting Information, SI). This method can effectively discriminate isoforms and exhibit a high-sensitivity performance for in vitro detection (Figures S1 and S2).

Next, we explored the potential of the SpliceRCA method for imaging CD45 RNA splicing variants in Jurkat T cells. As illustrated in Figure 1B, the generated superbright dots amplified from the target splice isoform could be clearly distinguished from the background inside cell. These bright dot signals were generated from one-target-one-amplicon in situ amplification, thus presumably corresponding to separated mRNAs. Characterization of RCA amplicons by dynamic light scattering (DLS) and transmitted electron microscopy (TEM) demonstrated that the RCA amplicons are monodisperse particles with a size of ∼300 nm (Figure 1C and Figure S3). The size of the RCA amplicon is larger than the resolution of optical imaging, thus conferring the single-molecular RCA amplicons resolvable by confocal microscopy.16,34 The signal intensities of the RCA amplicon amplified from individual splice isoforms were much larger than the background (Figure 1D). Next, for verification that signals were amplified from the target, several experiments were performed as control. As shown in Figure S4, no bright spot was observed when no padlock probe or trigger primer was used. There was only a rare fluorescence signal when random probes were added. Additionally, to confirm that the signal resulted from the target sequence, we blocked the binding sites with an unlabeled complementary probe before carrying out SpliceRCA. Only less than 0.2 amplicons per cell could be seen, thus suggesting that the bright spots came from the amplification of the target RNA sequence. Furthermore, we also performed a siRNA knockdown experiment to suppress the expression of CD45; the signals of CD45RO, CD45RA, and CD45RB isoforms decreased 24.59%, 31.27%, and 27.01%, respectively, with the knockdown of CD45 (Table S3). The knockdown efficiency was verified by RT-qPCR assay (Figure S5 and Table S4). All of these results suggest that the bright dot signals come from target mRNA splicing isoforms.

As shown in Figure 1E, CD45 isoforms were presented at different levels. The average numbers of amplicons for CD45RO, CD45RA, and CD45RB were 3.70, 5.98, and 11.07 per cell. The in situ detection efficiency of mRNA splice isoform-initiated RCA was estimated to be 10−20% on the basis of a comparison to RT-qPCR data (Table S5 and discussed in the Supporting Information). The obvious
variability in the copy numbers of CD45 isoforms indicates that significant cell-to-cell variation in isoform expression would be exhibited even in the same batch cells. In addition, SpliceRCA has been successfully applied to image splicing isoforms of BRCA1, breast cancer susceptibility gene 1.19,35 The alternative splicing of BRCA1 was closely related with the transformation of malignant breast cancer (Figure S6).36,37 As a validation, we performed the reverse transcription quantitative PCR (RT-qPCR) assay for expression comparison. The results of SpliceRCA are in good accordance with the RT-qPCR results in general (Figures S7 and S8). The single-molecule resolution of the SpliceRCA method allows precise quantification of isoform abundances.

Moreover, the assurance of the discrimination among the various splicing variants, especially which have sharing sequences in splice junction, highly depends on the stringent specificity of the RNA profiling method. To assess the accuracy of SpliceRCA for profiling RNA splicing variants inside cells, we compared the isoform expression level measured by SpliceRCA in simultaneous and separate detection. The quantification of the three isoforms by simultaneous and separate detection shows negligible difference (Figure 1F and Figure S9, P < 0.001), indicating minimal crosstalk among the targets simultaneously detected. Thus, SpliceRCA confers low crosstalk for multiplex in situ mRNA isoforms detection. Further, we test the base precision of the SpliceRCA method, in which the splice-junction padlock probe used for imaging CD45RB was altered by one or two bases. The copy number of amplicons per cell decreased sharply after the introduction of a one- or two-base mismatch (Figure S10). The average numbers of amplicons when using one-base mismatched (Mis-1) and two-base mismatched (Mis-2) padlock probes were 0.88 per cell and 0.20 per cell, respectively, much less than the 11.02 per cell measured with the matched probe (Mis-0) (Figure 1G). Thus, the SpliceRCA presents high specificity conferring single-nucleotide resolution, ensuring the precise recognition of mRNA isoforms in situ.

Quantiﬁcation of Isoform Variability in Jurkat Cells upon T-Cell Activation. Determining the alternative splicing changes induced by T-cell activation is crucial for understanding the cellular outcomes of the antigen challenge. To characterize the extent of expression variability on CD45 mRNA isoforms and decipher its functional implications, we applied SpliceRCA to profile the isoform expression in the Jurkat T-cell response to phorbol-12-myristate13-acetate (PMA) stimulation. Immunofluorescence imaging of HnRNPLL, one marker of T-cell maturation,38,39 shows a skewed increase in expression level after PMA stimulation (Figure S11), indicating that Jurkat T cells were efficiently stimulated. Fluorescent images reveal a different isoform pattern after stimulation, and the copy numbers of three mRNA isoforms all present a shift upon T-cell activation (Figure 2A,B). Moreover, the expressions of CD45RO and CD45RB isoforms show 70% and 32% increase (Figure 2C). Then, RT-qPCR analysis further conﬁrms the isoform expression patterns and the splicing shift in PMA stimulation (Figure S12). These results indicate that T-cell activation leads to the skipping of three variable exons in CD45 pre-mRNA (Figure 2D). The skipping process would reduce the phosphatase activity of the CD45 molecule and preserve the normal status to prevent autoimmune disease.30,40 Interestingly, cell-to-cell variability in isoform ratios differed between the two cell states before and after PMA stimulation. After stimulus, the three isoforms showed subtle lower cell-to-cell variability [coefficients of variation (CV, SD/mean) of CD45RO, CD45RA, and CD45RB were 0.328, 0.361, and 0.283, respectively].

Figure 2. Cell-to-cell variation in isoform expression upon PMA stimulation. (A) Fluorescent images of three CD45 splicing isoforms visualized by SpliceRCA before and after PMA stimulation. (B) Histograms of the copy numbers of three mRNA isoforms in Jurkat T cells measured by SpliceRCA before and after stimulus. The arrow shows the expression shift; arrow to the right (left) means increased (decreased) expression upon PMA stimulation. (C) Quantification of average expression for three CD45 isoforms before and after stimulus. (D) Schematic diagram of the alternative splicing upon T-cell activation.
respectively] than resting T cells (isoform CV > 0.4) (Table S6). The variability in single-cell isoform expression may reflect functional differences in the stimulated Jurkat T cell population.

Mapping the Spatial Distribution of CD45 mRNA Variants in Single Cells. The location distribution of splicing variants from the same gene has not been well-characterized in mammalian cells, let alone suspension cells such as T cells. We then exploit the in situ RNA visualization ability of SpliceRCA to investigate the spatial distributions of multiple splice variants simultaneously. From the visual inspection of each mRNA variant in resting Jurkat T cells, isoform CD45RO appeared enriched near the cell periphery, whereas CD45RA and CD45RB appeared in a random distribution throughout the cell (Figure 3A). Quantitative analysis of the distances between each mRNA splicing variant and the cell nucleus or the cell periphery further confirmed the visual impression (Figure 3B). The average distances to the nucleus for the mRNA variants CD45RO, CD45RA, and CD45RB were 3.25, 3.32, and 4.15 μm, respectively. The average distances to cell edge for mRNA variants CD45RA, CD45RB, and CD45RO were 2.30, 3.24, and 2.47 μm, respectively. Furthermore, the spatial distribution of splice isoforms after T-cell activation was investigated. As shown in Figure 3C, the average distances to the nucleus for CD45RO, CD45RA, and CD45RB increased by 0.07, 0.08, and 0.67 μm, respectively, after T-cell activation. The average distances to the cell edge for CD45RO, CD45RA, and CD45RB increased by 0.07, 0.08, and 0.67 μm, respectively, after T-cell activation.
CD45RB decreased by 0.02, 0.08, and 0.54 μm, respectively, after T-cell activation. The results indicate that CD45RO and CD45RA showed a modest change in the distance to the nucleus and cell edge upon T-cell activation, while the CD45RB presented an obvious change to be more likely expressed in the cell periphery after T-cell activation. The different spatial patterns may result from the expression of some RNA binding proteins as splice mRNA localization is closely related with regulation of many interaction proteins.\(^{41,42}\) The ability for spatial mapping of splice isoforms in single cells may offer us new ways to predict and study the rich diversity in splicing functions.

**Analysis of Expression Covariation among Different Isoforms.** We next examined the expression covariation among different isoforms that might arise from differential activity of immune activation. Pairwise correlation coefficients for the RNA species were calculated as shown in Figure 4A. Upon T-cell activation, the correlation coefficients of CD45RO and CD45RB presented a pronounced increase whereas pair correlations of CD45RO and CD45RA, and CD45RA and CD45RB, presented a modest change. The single-cell correlation coefficients of CD45RO and CD45RB vary from 0.281 to 0.740 upon stimulus (Figure 4B). Covariation across different isoforms may not be associated with the complex interplay of regulators that may lead to the simultaneous up-regulation of CD45RO and CD45RB, presented a modest change. The single-cell RNA species were calculated as shown in Figure 4A. Upon T-cell activation, the correlation coefficients of CD45RO and CD45RB encode the transcript mRNA which both skip exon 4. T-cell activation would lead to the up-regulation of some regulators, such as HnRNP LL, SR proteins, or other RNA binding protein which can alter alternative splice sites by binding to different exons.\(^{43}\) We suggest that the expression of some RNA binding proteins involved in exon 4 skipping was up-regulated with T-cell activation, which thus may lead to the simultaneous up-regulation of CD45RO and CD45RB, resulting in the increase of their correlation coefficients.\(^{44}\) The normal functioning of the immune system may be associated with the complex interplay of regulators that mediate the appropriate splicing of CD45 exon 4. Analysis of covariations in the expression levels of different isoforms on the single-cell level could reveal coregulated isoforms and help to elucidate splicing regulatory circuits in the T-cell maturation.

**MATERIALS AND METHODS**

**Oligonucleotide Sequences.** The DNA sequences (Tables S1 and S2 in the Supporting Information) were purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The sequences modified with Alexa488, Alexa555, and Cy5 were purchased from Thermo Fisher Scientific (Beijing, China), and were purified by HPLC. RNA sequences were created by in vitro T7 transcription reactions with ordering single-stranded DNA used as templates.

**Cell Culture and Stimulations.** The Jurkat cells were maintained in a standard RPMI 1640 medium supplemented with 15% fetal bovine serum, 100 units mL\(^{-1}\) of penicillin and 100 μg mL\(^{-1}\) of streptomycin. The cells were grown at 37 °C, 5% CO\(_2\), and 95% air humidity. For stimulations, cells were diluted to 3 × 106 cells mL\(^{-1}\) and incubated in medium with PMA plus ionomycin (100 ng mL\(^{-1}\) and 1 μM, respectively).

**Cell Fixation and Permeabilization.** Cells were fixed at a proper density on a 22 mm × 22 mm gelatin-coated cover glass (VWR, Radnor, PA) enclosed by a PDMS with a chamber (5 mm in diameter) by being maintained in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature (20–25 °C), washed twice with 1× DEPC-treated PBS (DEPC-PBS). Then, the cells were permeabilized for 5 min with 0.5% v/v Triton-X100 in 1× PBS at room temperature, and washed twice with DEPC-PBS.

**In Situ Visualization of Splicing Variants by SpliceRCA.** The hybridization of the exon-junction padlock probe with the target mRNA splicing variants was conducted in a volume of 20 μL containing 2 μL 20× saline-sodium citrate buffer (SSC) (Ambion, AM9763), 2 μL of each phosphorylated padlock probe (10 μM), 1 μL of DT T (100 mM), 2 μL of yeast tRNA (10 mg mL\(^{-1}\)), and 0.5 μL of RiboLock RNase inhibitor (40 U μL\(^{-1}\)) overnight at 37 °C. The sample was then washed twice using PBS-T (DEPC-PBS with 0.05% Tween-20) for 3 min at room temperature. The ligation reaction was carried out in 10 μL of ligation reaction mixture containing [1 μL 10X ligase reaction buffer, 1 μL of 20× SSC, 1 μL of T4 DNA ligase (5 U μL\(^{-1}\)), 0.25 μL of RiboLock RNase inhibitor (40 U μL\(^{-1}\)), 6.75 μL of RiboLock RNase-free water] at 37 °C for 2 h. After ligation, a 20 μL mixture containing 2 μL of 20× SSC, 1 μL of each primer (4 μM), 2 μL of formamide, 1 μL of DTT (100 mM), 13.5 μL of RNase-free water, and 0.5 μL of RiboLock RNase inhibitor (40 U μL\(^{-1}\)) were added to the sample and incubated for 60 min at 37 °C, following a wash using PBS-T. RCA was then conducted with a 10 μL mixture containing 1 μL of 10X phi29 DNA polymerase reaction buffer, 0.5 μL of phi29 DNA polymerase, 3 μL of dNTPs (10 mM for each of dATP, dGTP, dCTP, and dTTP), 5.25 μL of RNase-free water, and 0.25 μL of RiboLock RNase inhibitor (40 U μL\(^{-1}\)) for 120 min at 37 °C. The incubation was followed by a wash in PBS-T. Then, the hybridization of amplicons with detection probes was conducted in a 20 μL mixture of 100 nM fluorophore-labeled detection probes, 2× SSC, 15% formamide, and 10 ng μL\(^{-1}\) salmon sperm DNA for 30 min at 37 °C, following two washes using PBS-T. After mounting with Fluoromount-G.
mRNA using the 2 were evaluated by referring to the expression of GAPDH mRNA splicing variants CD45RO, CD45RA, and CD45RB were performed in triplicate. The copy numbers of target were converted into absolute GAPDH copy numbers using a setting the threshold value. For determination of the copy number of amplicons per cell, the number of bright pixels was counted by particle analysis in ImageJ software.

Real-Time Quantitative PCR (RT-qPCR) Analysis of RNA Splice Variants inside Cells. Total RNA from Jurkat cells was harvested by using TransZol according to the included protocol. The reverse transcription was performed using TransScript one-step gDNA removal and cDNA synthesis kit. In brief, a total volume of 20 μL of mixture containing 10 μL of 2X TS reaction mixture, 2 μL of the total RNA (from 50 ng to 5 μg), 1 μL of RT primer, 1 μL of gDNA remover, and 5 μL of RNase-free water was incubated at 42 °C for 15 min, followed by heat inactivation of reverse transcriptase at 85 °C for 5 s. The produced cDNA samples were then stored at −80 °C for future use. Upstream primers used for the reverse transcription reaction are listed in Table S1.

For qPCR analysis, following the manufacturer’s instructions on a Bio-Rad C1000TM (Bio-Rad) instrument, the 20 μL reaction solution contained 10 μL of SYBR Select master mix, 2 μL of cDNA sample, 2 μL of forward primer (5 μM), 2 μL of reverse primer (5 μM), and 4 μL of RNase-free water. The RT-qPCR was done by staying at 50 °C for 2 min for the hot start, annealing at 95 °C for 2 min, then followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C, and 5 min at 60 °C. Ct values were converted into absolute GAPDH copy numbers using a standard curve from a control RNA (human GAPDH mRNA in RevertAid First Strand cDNA synthesis kit). The experiments were performed in triplicate. The copy numbers of target mRNA splicing variants CD45RO, CD45RA, and CD45RB were evaluated by referring to the expression of GAPDH mRNA using the 2−ΔΔCt method. Calculations of mRNA copy numbers were based on the number of counted cells at harvest. No unexpected or unusually high safety hazards were encountered in the experiments.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00081. Additional experimental details including fluorescence analysis of RCA products; TEM image; additional confocal microscopy analysis; siRNA knockdown data; discussion of detection efficiency; immunofluorescence analysis; and RT-qPCR data (PDF)

AUTHOR INFORMATION

Corresponding Author
*E-mail: jhli@mail.tsinghua.edu.cn.

ORCID

Jinghong Li: 0000-0002-0750-7352

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was financially supported by National Natural Science Foundation of China (21621003, 21235004, 21327806), and Tsinghua University Initiative Scientific Research Program.

REFERENCES

1. Maniatis, T.; Tasic, B. Alternative pre-mRNA splicing and proteome expansion in metazoans. Nature 2002, 418 (6904), 236–243.
2. Hemphill, J.; Liu, Q.; Uprety, R.; Samanta, S.; Tsang, M.; Juliano, R. L.; Deiters, A. Conditional control of alternative splicing through light-triggered splice-switching oligonucleotides. J. Am. Chem. Soc. 2015, 137 (10), 3656–3662.
3. Chen, M.; Manley, J. L. Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. Nat. Rev. Mol. Cell Biol. 2009, 10 (11), 714–754.
4. Levesque, M. J.; Raj, A. Single-chromosome transcriptional profiling reveals chromosomal gene expression regulation. Nat. Methods 2013, 10 (3), 246–248.
5. Singh, R. K.; Cooper, T. A. Pre-mRNA splicing in disease and therapeutics. Trends Mol. Med. 2012, 18 (8), 472–482.
6. Chattopadhyay, P. K.; Gierzynski, T. M.; Roederer, M.; Love, J. C. Single-cell technologies for monitoring immune systems. Nat. Immunol. 2014, 15 (2), 128–135.
7. Shalek, A. K.; Satija, R.; Shuga, J.; Trombetta, J. T.; Gennert, D.; Lu, D.; Chen, P.; Gertner, R. S.; Gablehomme, J. T.; Yosef, N.; Schwartz, S.; Fowlke, B.; Weaver, S.; Wang, J.; Wang, X.; Ding, R.; Raychowdhury, R.; Friedman, N.; Haochen, N.; Park, H.; May, A. P.; Regan, A. Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. Nature 2014, 510 (7505), 363–369.
8. Hermiston, M. L.; Xu, Z.; Weiss, A. CD45: a critical regulator of signaling thresholds in immune cells. Annu. Rev. Immunol. 2003, 21, 107–137.
9. Machura, E.; Mazur, B.; Pieniazek, W.; Karczewska, K. Expression of naive/memory (CD45RA/CD45RO) markers by peripheral blood CD4+ and CD8+ T cells in children with asthma. Arch. Immunol. Ther. Exp. 2008, 56 (1), 55–62.
10. Tchilian, E. Z.; Beverley, P. C. Altered CD45 expression and disease. Trends Immunol. 2006, 27 (3), 146–153.
11. Zhu, J.; Yamane, H.; Paul, W. E. Differentiation of effector CD4 T cell populations (*). Annu. Rev. Immunol. 2010, 28, 445–489.
12. Chen, T.; Wu, C. S.; Jimenez, E.; Zhu, Z.; Dajac, J. G.; You, M.; Han, D.; Zhang, X.; Tan, W. DNA micelle flares for intracellular mRNA imaging and gene therapy. Angew. Chem., Int. Ed. 2013, 52 (7), 2012–2016.
13. Vargas, D. Y.; Shah, K.; Batish, M.; Levandoski, M.; Sinha, S.; Marras, S. A.; Schell, P.; Tyagi, S. Single-molecule imaging of transcriptionally coupled and uncoupled splicing. Cell 2011, 147 (5), 1054–1065.
14. Waks, Z.; Klein, A. M.; Silver, P. A. Cell-to-cell variability of alternative RNA splicing. Mol. Syst. Biol. 2011, 7, 506.
15. Lubeck, E.; Cai, L. Single-cell systems biology by super-resolution imaging and combinatorial labeling. Nat. Methods 2012, 9 (7), 743–748.

DOI: 10.1021/acscentsci.8b00081
ACS Cent. Sci. 2018, 4, 680–687
(16) Chen, K. H.; Boettiger, A. N.; Moffitt, J. R.; Wang, S.; Zhuang, X. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science* 2015, 348 (6233), aa6090.

(17) Cui, Y.; Liu, J.; Irudayaraj, J. Beyond quantification: in situ analysis of transcriptome and pre-mRNA alternative splicing at the nanoscale. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 2017, 9 (4), e1443.

(18) Long, X.; Colonell, J.; Wong, A. M.; Singer, R. H.; Lionnet, T. Quantitative mRNA imaging throughout the entire Drosophila brain. *Nat. Methods* 2017, 14 (7), 703–706.

(19) Lee, K.; Cui, Y.; Lee, L. P.; Irudayaraj, J. Quantitative imaging of single mRNA splice variants in living cells. *Nat. Nanotechnol.* 2014, 9 (6), 474–480.

(20) Liu, G. L.; Yin, Y.; Kunchakarra, S.; Mukherjee, B.; Gerion, D.; Jett, S. D.; Bear, D. G.; Gray, J. W.; Alvisatos, A. P.; Lee, L. P.; Chen, F. A. A nanoplasmonic molecular ruler for measuring nuclease activity and DNA footprinting. *Nat. Nanotechnol.* 2006, 1 (1), 47–52.

(21) Zhang, Y.; Wang, Y.; Wang, H.; Jiang, J. H.; Shen, G. L.; Yu, R. Q.; Li, J. Electrochemical DNA biosensor based on the proximity-dependent surface hybridization assay. *Anal. Chem.* 2009, 81 (5), 1982–1987.

(22) Ankenbruck, N.; Courtney, T.; Naro, Y.; Deiters, A. Optochemical Control of Biological Processes in Cells and Animals. *Angew. Chem., Int. Ed.* 2018, 57 (11), 2768–2798.

(23) Lynch, K. W.; Weiss, A. A model system for activation-induced alternative splicing of CD45 pre-mRNA in T cells implicates protein kinase C and Ras. *Mol. Cell. Biol.* 2000, 20 (1), 70–80.

(24) Ali, M. M.; Li, F.; Zhang, Z.; Zhang, K.; Kang, D. K.; Ankrum, J. A.; Le, X. C.; Zhao, W. Rolling circle amplification: a versatile tool for oligonucleotides for localized DNA detection. *Chen, K. H.; Boettiger, A. N.; Moffitt, J. R.; Wang, S.; Zhuang, X.* *Science* 2015, 348 (6233), aa6090.

(25) Nilsson, M.; Malmgren, H.; Samiotaki, M.; Kwiatkowski, M.; Larsson, C.; Grundberg, I.; Soderberg, O.; Nilsson, M. In situ detection and genotyping of individual mRNA molecules. *Nat. Methods* 2010, 7 (5), 395–397.

(26) Lizardi, P. M.; Huang, X.; Zhu, Z.; Bray-Ward, P.; Thomas, D. C.; Ward, D. C. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat. Genet.* 1998, 19 (3), 225–232.

(27) Ren, X.; Deng, R.; Wang, L.; Zhang, K.; Li, J. RNA splicing process analysis for identifying antisense oligonucleotide inhibitors with padlock probe-based isothermal amplification. *Chem. Sci.* 2017, 8 (8), 5692–5698.

(28) Sun, L.; Gao, Y.; Xu, Y.; Chao, J.; Liu, H.; Wang, L.; Li, D.; Fan, C. Real-Time Imaging of Single-Molecule Enzyme Cascade Using a DNA Origami Raft. *J. Am. Chem. Soc.* 2017, 139 (48), 17525–17532.

(29) Zikherman, J.; Weiss, A. Alternative splicing of CD45: the tip of the iceberg. *Immunity* 2008, 29 (6), 839–841.

(30) Deng, R.; Zhang, K.; Li, J. Isothermal Amplification for MicroRNA Detection: From the Test Tube to the Cell. *Acc. Chem. Res.* 2017, 50, 1059–1068.

(31) Hu, R.; Zhang, X.; Zhao, Z.; Zhu, G.; Chen, T.; Fu, T.; Tan, W. DNA nanoflowers for multiplexed cellular imaging and traceable targeted drug delivery. *Angew. Chem., Int. Ed.* 2014, 53 (23), 5821–5826.

(32) Chen, K. H.; Boettiger, A. N.; Moffitt, J. R.; Wang, S.; Zhuang, X. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science* 2015, 348 (6233), aa6090.

(33) Cui, Y.; Liu, J.; Irudayaraj, J. Beyond quantification: in situ analysis of transcriptome and pre-mRNA alternative splicing at the nanoscale. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 2017, 9 (4), e1443.

(34) Ke, R.; Mignardi, M.; Pacuraru, A.; Svedlund, J.; Botling, J.; Wahlby, C.; Nilsson, M. In situ sequencing for RNA analysis in preserved tissue and cells. *Nat. Methods* 2013, 10 (9), 857–860.

(35) Battich, N.; Stoeger, T.; Pelkmans, L. Image-based transcriptomics in thousands of single human cells at single-molecule resolution. *Nat. Methods* 2013, 10 (11), 1127–1133.