Biophysical *Journal*
Biophysical Letter

Biophysical Society

Multiplexed Dynamic Imaging of Genomic Loci by Combined CRISPR Imaging and DNA Sequential FISH

Yodai Takei,[1] Sheel Shah,[1,2] Sho Harvey,[3] Lei S. Qi,[4,5,6] and Long Cai[3,*]
[1]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, California; [2]UCLA-Caltech Medical Scientist Training Program, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California; [3]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California; [4]Department of Bioengineering, [5]Department of Chemical and Systems Biology, and [6]ChEM-H, Stanford University, Stanford, California

ABSTRACT Visualization of chromosome dynamics allows the investigation of spatiotemporal chromatin organization and its role in gene regulation and other cellular processes. However, current approaches to label multiple genomic loci in live cells have a fundamental limitation in the number of loci that can be labeled and uniquely identified. Here we describe an approach we call "track first and identify later" for multiplexed visualization of chromosome dynamics by combining two techniques: CRISPR imaging and DNA sequential fluorescence in situ hybridization. Our approach first labels and tracks chromosomal loci in live cells with the CRISPR-Cas9 system, then barcodes those loci by DNA sequential fluorescence in situ hybridization in fixed cells and resolves their identities. We demonstrate our approach by tracking telomere dynamics, identifying 12 unique subtelomeric regions with variable detection efficiencies, and tracking back the telomere dynamics of respective chromosomes in mouse embryonic stem cells.

The three-dimensional chromatin organization in the nucleus plays an important role in gene regulation and other cellular processes (1,2). Visualizing spatiotemporal chromatin organization helps to interrogate its relationship with biological functions. Recently developed CRISPR imaging techniques can be a powerful and versatile tool to label and track genomic loci in live mammalian cells (3,4), supplementing dynamics to the static information from fluorescence in situ hybridization (FISH) in fixed cells. One of the challenges of live cell imaging of genomic loci is imaging multiple loci simultaneously in individual cells. To overcome this issue and enable multicolor CRISPR imaging, several methods have been developed by using orthogonal CRISPR-Cas9 systems (5,6) or engineered single guide RNA (sgRNA) scaffolds (7–9). However, even these methods only allow the simultaneous imaging of two or three loci. More recently, the color barcoding approach, using engineered sgRNA scaffolds recruiting different combinations of spectrally distinct fluorescent proteins, has demonstrated simultaneous imaging of six chromosomal loci in single cells (10). Although these multicolor approaches have expanded the potential of CRISPR imaging, they have a fundamental bottleneck in multiplexing due to the limited number of available orthogonal CRISPR-Cas9 systems, sgRNA scaffolds, or fluorescent proteins with spectrally distinct fluorophores.

Here we propose, to our knowledge, a new approach to label and distinguish multiple genomic loci using the combination of CRISPR imaging and DNA sequential FISH (DNA seqFISH), which provides large multiplexing capabilities. The principle of our approach is illustrated in Fig. 1. Multiple genomic loci are labeled with the CRISPR-Cas9 system all in a single color, and tracked in individual live cells. At the end of the live recording, cells are fixed and the identity of each locus is resolved by the color barcodes from DNA seqFISH. In this manner, even if the identities of labeled loci are indistinguishable during the live recording, as long as their positions are distinctly tracked in live imaging, these chromosomal loci can be subsequently identified with DNA seqFISH.

This "track first and identify later" approach can circumvent the multiplexing limitations of live cell imaging. As a proof-of-principle, we applied our technique to track telomeric loci in live mouse embryonic stem (mES) cells, and uniquely assigned 12 telomeric loci to particular chromosomes by performing DNA seqFISH of distal subtelomeric regions after the live tracking (Fig. 2 *A*).

Submitted January 30, 2017, and accepted for publication March 23, 2017.
*Correspondence: lcai@caltech.edu
Editor: Antoine van Oijen.
http://dx.doi.org/10.1016/j.bpj.2017.03.024

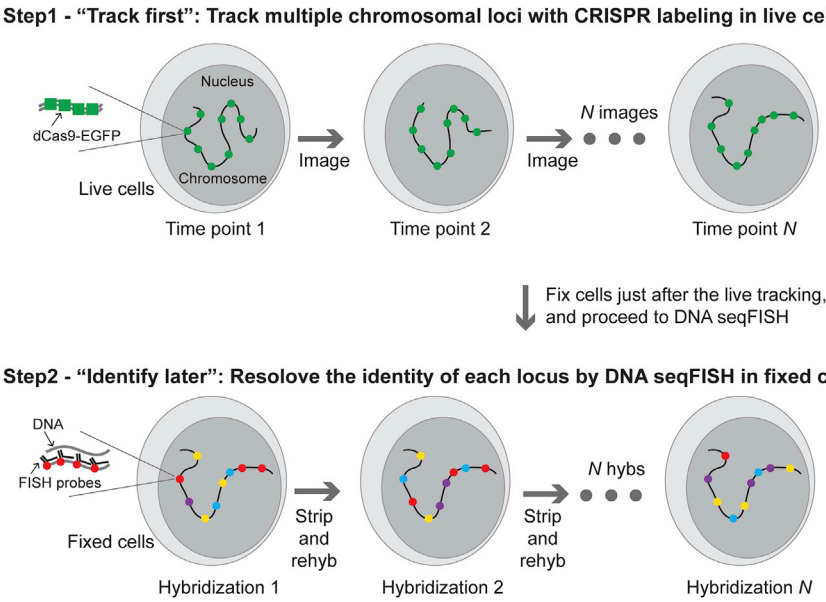

FIGURE 1 Schematic of the "track first and identify later" approach with the combination of the CRISPR labeling and DNA seqFISH techniques. Nine regions in one chromosome are illustrated in this schematic. Each chromosomal position can be identified from the DNA seqFISH step and its motion can be backtracked from the live imaging. To see this figure in color, go online.

To observe the dynamics of telomeric loci in live mES cells, we generated a mES cell line stably expressing *Streptococcus pyogenes* nuclease-deactivated Cas9 (dCas9) fused to EGFP (dCas9-EGFP) and sgRNA targeting telomeric loci by following a previous study (3). The dCas9-EGFP protein carried two nuclear localization signals for proper nuclear import. The mouse telomeric loci are ~20–30 kb with a 6-bp repeat sequence TTAGGG (4), which potentially allows the recruitment of hundreds of dCas9-EGFP proteins per locus with a single 22-nt sgRNA sequence (3). Using the clonal line, we performed live imaging over 6 min (Figs. 2 *B* and S1 and Movie S1), and tracked the dynamics of telomeric loci in three-dimensional space.

Immediately after the live tracking, cells were fixed and processed for DNA seqFISH (Fig. 2, *B–E*). We quantified the number of telomeric dots (Fig. 2 *F*) and observed that on average, 73.0% of telomeric dots at the last frame of the live tracking were uniquely assigned to telomeric dots after the fixation (Fig. 2 *G*), indicating that the majority of the dCas9-EGFP labeled loci do not move significantly before and during fixing. Subtelomeric regions in respective chromosomes were barcoded based on a sequential barcoding method we demonstrated previously with RNA FISH (11,12). With this method, the number of loci that can be distinguished scales as F^N, where F is the number of distinct fluorophores and N is the number of hybridization rounds. Each subtelomeric region was targeted with a set of FISH probes labeled with a single fluorophore during each round of hybridization. Specifically, the primary probes targeting the genomic loci also contain overhang sequences that are unique to each locus. A set of adaptor probes that are dye-labeled are hybridized to the overhang sequences (Fig. S2 *A*). We imaged cells, and then treated them with 70% formamide solution to displace the adaptor probes (Fig. S2). We imaged cells again to confirm the probe displacement, and subsequent rounds of hybridizations were performed (Fig. S2, *B* and *C*). To cover 12 subtelomeric regions (Table S1), we used three dyes and three rounds of hybridizations (Fig. 2 *D*). We also used a fourth round of hybridization to image telomeres with DNA FISH (Fig. 2 *E*), and three different subtelomeric regions independently in a single channel as a control to quantify barcoding efficiency (Figs. S3 and S4 *A*).

We quantified 12 regions that were detected robustly in most cells with a mean of 1.9 ± 0.5 dots (±SD) per cell (Fig. S5 and Supporting Materials and Methods). Consistent with our targeting of 12 distal subtelomeric regions out of a total of 40 distal and proximal subtelomeric regions, we observed that 22.9% of the dCas9-EGFP-labeled telomere spots corresponded to subtelomeric regions barcoded by DNA seqFISH (Fig. 2 *G*). Similarly, we observed 20.0% of telomere DNA FISH spots corresponded to subtelomere DNA seqFISH spots (Fig. S4 *B*). We note that we do not expect the telomeres and subtelomeres to colocalize perfectly because they can be genomically distant (Fig. S4 *A*; Table S1). We quantified the distribution of the distance between aligned telomeric and subtelomeric spots (Fig. S4 *C*).

From the barcode uniquely assigned to each subtelomeric region, we assigned a unique identity to each tracked region in the live recording. To document the differences of telomeric dynamics from each chromosome, we then analyzed the movements of telomeres assigned to each chromosome (Fig. 2 *H*) and quantified their cumulative square displacements of adjacent time frames as a function of time (Fig. 2 *I*). We also provided multiple quantified traces from additional single cells (Fig. S6).

Based on a calculation of the optical space available in a mammalian nucleus, the single color method could in principle track and identify a larger number of loci

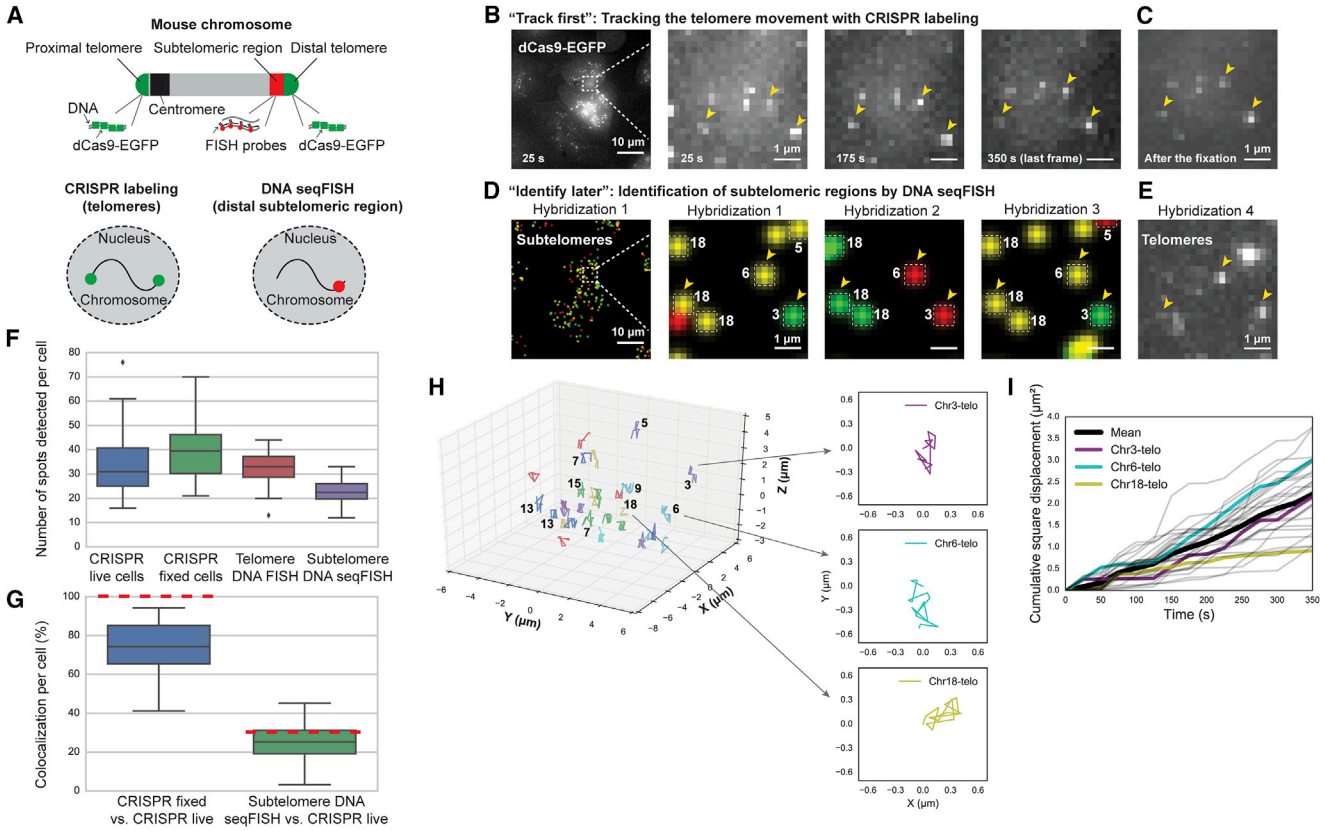

FIGURE 2 **Multiplexed telomere tracking and identification of chromosomes with the "track first and identify later" approach in mES cells. (*A*) Schematic of the approach applied to telomere in a mouse chromosome. Proximal and distal telomere were labeled by the CRISPR-Cas9 system whereas only the distal subtelomeric region was labeled by DNA seqFISH. In total, 12 distal subtelomeric regions in 12 chromosomes were robustly read out by DNA seqFISH. (*B* and *C*) Here, we show one-color telomere imaging in live cells at different time points (*B*) and after fixing cells (*C*), using the constructed mES cell line. (*D* and *E*) Composite digitized three-color (Alexa 647: *red*, Alexa 594: *green* and Cy3B: *yellow*) DNA seqFISH data for three rounds of hybridizations targeting subtelomeric regions (*D*), and one-color (Cy7) data for the fourth hybridization targeting telomeres (*E*) is given. Based on the barcode identities, chromosome numbers are assigned to each of the subtelomeric spots (*D*). Note that DNA seqFISH spots do not perfectly colocalize with CRISPR imaging spots because they target adjacent regions in the genome. Dots without colocalization between hybridizations are due to nonspecific binding of probes or mishybridization in the cells. Images are maximum intensity projections of a z-stack of fluorescence images and the boxed region of the cell is magnified (*B*–*E*). (*F*) Here, we compare the number of telomeric or subtelomeric spots detected per cell with the CRISPR labeling and DNA seqFISH methods. In total, 938 CRISPR spots in live cells (last frame of the movie), 1138 CRISPR spots in fixed cells, 909 telomeric spots by DNA FISH, and 628 subtelomeric spots by DNA seqFISH in 28 cells were analyzed. (*G*) Here, we compare colocalization percentage of spots detected per cell. (*Red dashed lines*) Expected colocalization percentage per cell is given. (*H*) Trajectories of telomeric loci in the magnified cell are shown. In this cell, 30 telomeric trajectories were detected from CRISPR imaging and 10 of these trajectories were uniquely assigned to particular chromosomes based on the subtelomere color barcodes. Trajectories of three loci in the magnified images (*B*)–(*E*) were also highlighted as *xy* projections (*inset*). Projected trajectories start from (0.0, 0.0). (*I*) Cumulative square displacement traces ($n = 30$) calculated with two adjacent frames as a function of time from the magnified cell are shown. Traces of three loci in the magnified images (*B*)–(*E*) were shown as colored traces. To see this figure in color, go online.**

(Supporting Materials and Methods) to provide a valuable global view of the chromosomes in single cells.

However, there are a few key technological bottlenecks preventing large numbers of loci to be imaged in this fashion. Firstly, targeting nonrepetitive regions requires the delivery of a substantial number of distinct sgRNAs to cells. Future work will be focused on ameliorating this limitation as recently demonstrated with a single chromosome painting in live cells by targeting nonrepetitive regions (13). As an alternative to reduce the number of sgRNAs, sets of sgRNAs targeting region-specific repetitive DNAs (10) can be used, while adjacent nonrepetitive unique regions or repeat regions themselves can be targeted by DNA seqFISH. In addition, engineering cell lines, which contain multiple target sites randomly integrated in the genome (14), can be an alternative approach to label a large number of genomic regions with a small number of sgRNAs in live cells. The integrated regions can be sequenced (14), targeted, and distinguished by DNA seqFISH. This approach is also applicable to other labeling methods such as the LacI-LacO system. Secondly, physical interactions of distinct loci during the live tracking can prevent accurate position tracking and thus reduce the number

of uniquely tracked loci per cell, which can be minimized by using multicolor CRISPR imaging (5–10). However, long-term tracking (i.e., beyond a cell-cycle) can be difficult due to the large-scale rearrangement and crossovers of chromosomes during mitosis. Lastly, DNA FISH signals can be improved with a robust signal amplification method such as single molecule hybridization chain reaction (12,15) or alternative DNA FISH methods such as CASFISH (16) to increase the detection efficiency.

The key idea in our work is separating the tasks of dynamic tracking of chromosomal loci and the unique identification of these loci. Previous works in multiplexed CRISPR imaging tried to accomplish both goals at the same time, which requires orthogonal Cas9 systems and multiple fluorophores for live imaging. In our approach, we use a single color channel to first track the motion of the chromosomal loci and then use highly multiplexed DNA seqFISH to identify the loci. In addition to the original seqFISH implementation (11), this strategy is another manifestation of the "noncommutative" approach (17,18) to experimental design that breaks experimental goals into distinct tasks and combines them to accomplish what cannot be easily achieved in a single experimental step. Our method combines advantages of CRISPR labeling and seqFISH for multiplexed live cell detection of genomic loci. During preparation of this article, a similar strategy was described by Guan et al. (19). Finally, we note that our method can also be combined with sequential RNA FISH (11,12,18,20) and immunofluorescence to correlate transcriptional and epigenetic states of individual cells with spatiotemporal chromosomal organization in a highly multiplexed manner.

SUPPORTING MATERIAL

Supporting Materials and Methods, six figures, one table, and one movie are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(17)30343-0.

AUTHOR CONTRIBUTIONS

All authors reviewed and contributed to the writing of the manuscript. L.C., L.S.Q., and Y.T. designed the project. Y.T. and S.H. performed experiments. S.S. wrote analysis codes. Y.T. and S.S. performed data analysis. L.C. supervised the project.

ACKNOWLEDGMENTS

We thank James Linton for kindly providing cell line and plasmids, and Eric Lubeck for help with probe designing.

Y.T. is supported by a Graduate Fellowship from the Nakajima Foundation. L.C. is supported by the Allen Distinguished Investigator Award, and NIH grant No. U01-EB 021240-01.

SUPPORTING CITATIONS

Reference (21,22) appear in the Supporting Material.

REFERENCES

1. Gorkin, D. U., D. Leung, and B. Ren. 2014. The 3D genome in transcriptional regulation and pluripotency. *Cell Stem Cell.* 14:762–775.
2. Bustin, M., and T. Misteli. 2016. Nongenetic functions of the genome. *Science.* 352:aad6933.
3. Chen, B., L. A. Gilbert, …, B. Huang. 2013. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell.* 155:1479–1491.
4. Anton, T., S. Bultmann, …, Y. Markaki. 2014. Visualization of specific DNA sequences in living mouse embryonic stem cells with a programmable fluorescent CRISPR/Cas system. *Nucleus.* 5:163–172.
5. Ma, H., A. Naseri, …, T. Pederson. 2015. Multicolor CRISPR labeling of chromosomal loci in human cells. *Proc. Natl. Acad. Sci. USA.* 112:3002–3007.
6. Chen, B., J. Hu, …, B. Huang. 2016. Expanding the CRISPR imaging toolset with *Staphylococcus aureus* Cas9 for simultaneous imaging of multiple genomic loci. *Nucleic Acids Res.* 44:e75.
7. Shao, S., W. Zhang, …, Y. Sun. 2016. Long-term dual-color tracking of genomic loci by modified sgRNAs of the CRISPR/Cas9 system. *Nucleic Acids Res.* 44:e86.
8. Fu, Y., P. P. Rocha, …, J. A. Skok. 2016. CRISPR-dCas9 and sgRNA scaffolds enable dual-colour live imaging of satellite sequences and repeat-enriched individual loci. *Nat. Commun.* 7:11707.
9. Wang, S., J. H. Su, …, X. Zhuang. 2016. An RNA-aptamer-based two-color CRISPR labeling system. *Sci. Rep.* 6:26857.
10. Ma, H., L. C. Tu, …, T. Pederson. 2016. Multiplexed labeling of genomic loci with dCas9 and engineered sgRNAs using CRISPRainbow. *Nat. Biotechnol.* 34:528–530.
11. Lubeck, E., A. F. Coskun, …, L. Cai. 2014. Single-cell in situ RNA profiling by sequential hybridization. *Nat. Methods.* 11:360–361.
12. Shah, S., E. Lubeck, …, L. Cai. 2016. In situ transcription profiling of single cells reveals spatial organization of cells in the mouse hippocampus. *Neuron.* 92:342–357.
13. Zhou, Y., P. Wang, …, X. S. Xie. 2017. Painting a specific chromosome with CRISPR/Cas9 for live-cell imaging. *Cell Res.* 27:298–301.
14. Akhtar, W., J. de Jong, …, B. van Steensel. 2013. Chromatin position effects assayed by thousands of reporters integrated in parallel. *Cell.* 154:914–927.
15. Shah, S., E. Lubeck, …, L. Cai. 2016. Single-molecule RNA detection at depth by hybridization chain reaction and tissue hydrogel embedding and clearing. *Development.* 143:2862–2867.
16. Deng, W., X. Shi, …, R. H. Singer. 2015. CASFISH: CRISPR/Cas9-mediated in situ labeling of genomic loci in fixed cells. *Proc. Natl. Acad. Sci. USA.* 112:11870–11875.
17. Letsou, W., and L. Cai. 2016. Noncommutative biology: sequential regulation of complex networks. *PLOS Comput. Biol.* 12:e1005089.
18. Frieda, K. L., J. M. Linton, …, L. Cai. 2017. Synthetic recording and in situ readout of lineage information in single cells. *Nature.* 541:107–111.
19. Guan, J., H. Liu, …, B. Huang. 2017. Tracking multiple genomic elements using correlative CRISPR imaging and sequential DNA FISH. *Biophys. J.* 112:1077–1084.
20. Chen, K. H., A. N. Boettiger, …, X. Zhuang. 2015. RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. *Science.* 348:aaa6090.
21. Singer, Z. S., J. Yong, …, M. B. Elowitz. 2014. Dynamic heterogeneity and DNA methylation in embryonic stem cells. *Mol. Cell.* 55:319–331.
22. Li, V. C., A. Ballabeni, and M. W. Kirschner. 2012. Gap 1 phase length and mouse embryonic stem cell self-renewal. *Proc. Natl. Acad. Sci. USA.* 109:12550–12555.