Published in final edited form as:

Angew Chem Int Ed Engl. 2016 July 25; 55(31): 8975–8978. doi:10.1002/anie.201603945.

Cross-platform DNA encoding for imaging single-cell gene expression

Dr. Pavel Zrazhevskiy^[a], Dr. Shreeram Akilesh^[b], Dr. Wanyi Tai^[a], Konstantin Queitsch^[c], Prof. Lawrence D. True^[b], Prof. Jonathan Fromm^[d], Prof. David Wu^[d], Prof. Peter Nelson^[e],^[f], Prof. John A. Stamatoyannopoulos^{[c],[e]}, and Prof. Xiaohu Gao^{*,[a]}

^[a] Department of Bioengineering, University of Washington, Seattle, WA, 98195, USA

^[b] Department of Pathology, University of Washington, Seattle, WA, 98195, USA

^[c] Department of Genome Sciences, University of Washington, Seattle, WA, 98195, USA

^[d] Department of Laboratory Medicine, University of Washington, Seattle, WA, 98195, USA

^[e] Department of Medicine, University of Washington, Seattle, WA, 98195, USA

^[f] Fred Hutchinson Cancer Research Center, Seattle, WA, 98109, USA

Abstract

Integration of imaging data across different molecular target types can provide in-depth insights into cell physiology and pathology, but remains challenging due to poor compatibility between target type-specific labeling methodologies. Here, we show that cross-platform imaging analysis can be readily achieved *via* DNA encoding of molecular targets, which translates the molecular identity of various target types into a uniform *in situ* array of ssDNA tags for subsequent labeling with complementary imaging probes. The concept is demonstrated *via* multiplexed imaging of mRNAs and corresponding proteins with multicolor quantum dots, uncovering heterogeneity of cell transfection with siRNA and outlining disparity in RNAi kinetics at the mRNA and downstream protein levels.

Keywords

DNA encoding; gene expression; imaging; quantum dots; single-cell

Gaining access to detailed “molecular portraits” of individual cells within the context of their natural cell culture or tissue microenvironment promises to revolutionize biomedical research and dramatically advance clinical diagnostics and drug discovery.^[1–3] A number of imaging platforms have been developed for comprehensive analysis of individual types of molecular targets, particularly proteins, mRNA, and DNA.^[4–12] However, simultaneous analysis across different target types remains challenging due to poor compatibility between target type-specific labeling methodologies, further restricted by additional requirements imposed by the imaging probe properties.

xgao@uw.edu.

Supporting information for this article is given via a link at the end of the document.

Here we describe a versatile cross-platform single-cell imaging technology that enables simultaneous visualization of multiple molecular targets irrespective of target type and imaging probe used. An essential step to achieving cross-platform compatibility is implementation of a new indirect labeling methodology built on the concepts of (i) decoupling of target binding and labeling steps, (ii) translation of heterogeneous molecular information into an intermediate standardized molecular code amenable to read-out *via* imaging probes, and (iii) employing the enormous encoding capacity and self-assembly capabilities of DNA bonding.^[13,14] Specifically, molecular targets of interest are first encoded with unique ssDNA tags *via* binding by ssDNA-conjugated target-recognition moieties under optimized conditions favoring strong and specific target binding. Individual ssDNA tags are then converted into detectable signals *via* sequence-specific hybridization with complementary ssDNA'-conjugated imaging probes under probe-optimized conditions. Thus, molecular target uniqueness, localization, abundance, and specimen morphology information are preserved through all steps of labeling procedure, producing comprehensive molecular signatures of a physiological or pathological process. It should be emphasized that this methodology is not meant to be applied towards studying DNAs, RNAs, or proteins in a massive parallel fashion. Instead, it aims to enable interrogation of a panel of selected genomic, transcriptomic, and/or proteomic markers in single cells by bringing them onto the same imaging platform.

To demonstrate the DNA encoding concept for imaging molecular targets across different types, we performed a concurrent assessment of molecular expression at mRNA and protein levels (Figure 1), one of the most actively explored methodologies for evaluation of gene regulation, cell signaling, RNA-protein interactions, and many other applications.^[15-19] Fluorescent quantum dot probes (QDots)^[20-22] in combination with fluorescence microscopy and hyperspectral imaging (HSI)^[23,24] were employed for simultaneous visualization of all ssDNA tags following separate encoding of mRNA and protein targets (Figure 1a). Unlike direct labeling procedures performed at a single incubation condition fixed for all targets and probes,^[25-27] DNA encoding enables tuning of conditions to favor recognition of individual target types and hybridization with detection probes in separate steps, offering great flexibility in the choice of specimens, targets, and imaging systems. Conceptually, any combination of target-specific affinity moieties and imaging probes should be amenable to coupling via intermediate DNA tags, rendering this technology useful for broader multi-omics imaging studies.

To implement and systematically characterize the model QDot-based cross-platform imaging technology, a set of 6 unique 16bp ssDNA/ssDNA' linkers has been designed for encoding of up to 6 different molecular targets (Table S1) along with a library of complementary 6-color QDot-ssDNA probes (Figure S1) and a control set of 6 secondary antibody-ssDNA (2'Ab-ssDNA) bioconjugates (Figure S2). Indirect labeling of β -tubulin in HeLa cells via a 3-step procedure involving incubation with unmodified primary antibodies, 2'Ab-ssDNA bioconjugates, and complementary QDot-ssDNA' probes demonstrated preserved antigen-recognition functionality of ssDNA-modified antibodies and high specificity of QDot staining via DNA hybridization (Figure 1b).

Multiplexed protein immuno-labeling was realized through the preparation of a library of primary antibody-ssDNA (1'Ab-ssDNA) bioconjugates (Figures S3 and S4). Characterization of such bioconjugates with PAGE and cell staining confirmed preserved stability and antigen-binding functionality of antibodies, specificity of target staining with QDots in a 2-step procedure, and consistent target identification with different QDot colors in a multiplexed imaging format (Figure S4b-e). Nuclear envelope protein Lamin A, microtubule β -tubulin, and cytoplasmic proteins HSP90-alpha and GAPDH were labeled as model target molecules with distinct characteristic intracellular localization.

Labeling of model GAPDH and HSP90-alpha mRNA molecules via an indirect *in situ* hybridization (ISH) procedure was done with modified mRNA ISH oligonucleotide probes featuring 5' 20nt mRNA-recognition portion and a 3' 16nt QDot-binding tag separated through a single-stranded AAAAA spacer (Tables S2 and S3). Hybridization of oligonucleotide probes under optimized ISH conditions yielded labeling of each mRNA molecule with multiple ssDNA tags (up to 36 for GAPDH and 48 for HSP90-alpha), producing distinct 'spots' upon staining with complementary QDot-ssDNA probes (Figure S5).

Separation of target-recognition and QDot-labeling events via DNA encoding enabled straightforward implementation of a model cross-platform imaging protocol, with both mRNA and protein targets being robustly labeled by respective QDot probes and accurately identified through hyperspectral imaging and analysis. For example, GAPDH and HSP90-alpha mRNA molecules and their respective product proteins could be readily labeled with 4-color QDots to highlight relative intracellular distribution and abundance of the two target types at a single-cell level (Figure 1c), corroborating broad applicability of the DNA encoding strategy for simultaneous detection and imaging of various types of targets within the same specimen.

Cross-platform imaging methodology was then applied to study gene knock-down via RNAi at a single-cell level. HeLa cells were transfected with GAPDH-targeting siRNA (as well as non-targeting siRNA for control) for 24 Hrs, and GAPDH mRNA abundance was assessed with RT-PCR and QDot-based imaging. Interestingly, single-cell imaging revealed complete degradation of GAPDH mRNA (Figure 2a), whereas bulk GAPDH mRNA measurement by RT-PCR indicated silencing efficiency of only 78% with forward transfection and 95% with reverse transfection. Imaging at a cell population level explained this discrepancy through revealing heterogeneity in RNAi, likely resulting from heterogeneous cell transfection with siRNA throughout different regions of cell culture (Figure 2b,c). For example, forward transfection failed to achieve efficient GAPDH mRNA degradation in dense cell populations, yielding areas of completely silenced cells along with patches of cells with normal GAPDH mRNA expression levels (Figure 2b). In contrast, reverse transfection achieved a more uniform cell transfection in suspension, producing a greater proportion of silenced cells with only a few wild-type clones (Figure 2c).

Selectivity of GAPDH RNAi was confirmed by performing dual-target imaging of GAPDH mRNA and HSP90-alpha mRNA. Target-selective siRNA should trigger degradation of only its complementary target mRNA, having no immediate effect on non-targeted mRNA

molecules. This was indeed observed with GAPDH RNAi studies (Figure 2d, Figure S6). Indirect dual-target ISH produced robust staining of both mRNA species in reference HeLa cells grown in culture medium. Similarly, cell transfection with non-targeting control siRNA failed to produce any effect on mRNA expression. Transfection with GAPDH-targeting siRNA, however, triggered rapid degradation of GAPDH mRNA within 24 Hrs post-transfection, while leaving non-targeted HSP90-alpha mRNA intact. Notably, a single non-transfected cell within the field of view featured intact expression of both GAPDH and HSP90 mRNA, suggesting an all-on/all-off mode of GAPDH RNAi.

Imaging of mRNA unambiguously demonstrated heterogeneity in RNAi likely stemming from incomplete cell transfection with siRNA. However, such heterogeneity could not be detected at the protein level, as GAPDH protein remained unperturbed 24 Hrs post-transfection in both transfected and non-transfected cells, as was evident from dual labeling of GAPDH mRNA and protein (Figure S7). To further investigate the disparity between RNAi effect at mRNA and protein levels, HeLa cells were reverse transfected with GAPDH-targeting siRNA for 24 and 48 Hrs and processed for multiplexed imaging of GAPDH and HSP90-alpha mRNA and their respective protein products. Consistent with studies discussed earlier, 24 Hrs post-transfection a complete degradation of GAPDH mRNA was observed, whereas GAPDH protein level remained nearly unperturbed (Figure 3a). In contrast, 48 Hrs post-transfection a substantial reduction of GAPDH protein level could be observed, with GAPDH mRNA remaining below the detection limit (Figure 3b). HSP90 mRNA and protein levels remained unperturbed through 48 hours, confirming selectivity of GAPDH silencing. Further, all molecular targets exhibited consistent unperturbed levels in cells transfected with non-targeting control siRNA (Figure 3c,d) throughout the study, confirming that the observed GAPDH knock-down indeed resulted from RNAi mechanism.

Delay in RNAi effect at the protein level is not surprising, as proteins are typically degraded and cleared slower in comparison to siRNA-mediated mRNA degradation. However, in light of imaging results presented here, heterogeneity in cell transfection needs to be taken into consideration when assessing RNAi efficiency with bulk RT-PCR measurements and especially downstream phenotypic and molecular signaling analysis. Non-transfected cells might gain growth advantage and achieve substantial clonal expansion during the time it takes for higher-level manifestations of RNAi to occur, thus distorting observed RNAi effect at a population level. Imaging-based analysis at a single-cell level should, therefore, offer a more accurate insight into cellular response to perturbation.

In conclusion, cross-platform imaging technology presented here enables simultaneous *in situ* detection and visualization of multiple types of molecular targets at a single-cell level. Decoupling of target recognition and labeling events and conversion of heterogeneous molecular information into a uniform intermediate ssDNA array amenable to read-out via variety of imaging probes represents a key technological advance towards gaining access to single-cell molecular profiles via multi-omics imaging, which might be challenging, if not impossible, to achieve via a simple combination of conventional labeling methodologies (e.g., immunofluorescence and *in situ* hybridization). Importantly, DNA code can be readily converted into digital read-out via simple hybridization to multiplexed imaging probes, offering a universal link between multitudes of available selective target-recognition

moieties and emerging powerful multiplexed imaging platforms. Thus, cross-platform DNA encoding technology is expected to facilitate the development of novel single-cell imaging methodologies and implementation of molecular profiling studies across different target types in biomedical research, drug development, and clinical diagnostics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by NIH (R01CA131797, R21CA192985) and the Department of Bioengineering at the University of Washington. S.A. is a Damon Runyon Fellow supported by the Damon Runyon Cancer Research Foundation (DRG-114-13). P.Z. thanks the National Cancer Institute for a T32 fellowship (T32CA138312).

References

- [1]. Burrell RA, McGranahan N, Bartek J, Swanton C. *Nature*. 2013; 501:338. [PubMed: 24048066]
- [2]. Bedard PL, Hansen AR, Ratain MJ, Siu LL. *Nature*. 2013; 501:355. [PubMed: 24048068]
- [3]. Krebs MG, Metcalf RL, Carter L, Brady G, Blackhall FH, Dive C. *Nat Rev Clin Oncol*. 2014; 11:129. [PubMed: 24445517]
- [4]. Angelo M, Bendall SC, Finck R, Hale MB, Hitzman C, Borowsky AD, Levenson RM, Lowe JB, Liu SD, Zhao S, Natkunam Y, Nolan GP. *Nat. Med*. 2014; 20:436. [PubMed: 24584119]
- [5]. Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. *Science*. 2015; 348:aaa6090. [PubMed: 25858977]
- [6]. Jungmann R, Avendano MS, Woehrstein JB, Dai M, Shih WM, Yin P. *Nat Methods*. 2014; 11:313. [PubMed: 24487583]
- [7]. Porichis F, Hart MG, Griesbeck M, Everett HL, Hassan M, Baxter AE, Lindqvist M, Miller SM, Soghoian DZ, Kavanagh DG, Reynolds S, Norris B, Mordecai SK, Nguyen Q, Lai C, Kaufmann DE. *Nat Commun*. 2014; 5:5641. [PubMed: 25472703]
- [8]. Stack EC, Wang C, Roman KA, Hoyt CC. *Methods*. 2014
- [9]. Zrazhevskiy P, Gao X. *Nat Commun*. 2013; 4:1619. [PubMed: 23511483]
- [10]. Giesen C, Wang HA, Schapiro D, Zivanovic N, Jacobs A, Hattendorf B, Schuffler PJ, Grolimund D, Buhmann JM, Brandt S, Varga Z, Wild PJ, Gunther D, Bodenmiller B. *Nat Methods*. 2014; 11:417. [PubMed: 24584193]
- [11]. Choi HM, Chang JY, Trinh le A, Padilla JE, Fraser SE, Pierce NA. *Nat Biotechnol*. 2010; 28:1208. [PubMed: 21037591]
- [12]. Gerdes MJ, Sevinsky CJ, Sood A, Adak S, Bello MO, Bordwell A, Can A, Corwin A, Dinn S, Filkins RJ, Hollman D, Kamath V, Kaanumalle S, Kenny K, Larsen M, Lazare M, Li Q, Lowes C, McCulloch CC, McDonough E, Montalto MC, Pang Z, Rittscher J, Santamaria-Pang A, Sarachan BD, Seel ML, Seppo A, Shaikh K, Sui Y, Zhang J, Ginty F. *Proc Natl Acad Sci U S A*. 2013; 110:11982. [PubMed: 23818604]
- [13]. Jones MR, Seeman NC, Mirkin CA. *Science*. 2015; 347:1260901. [PubMed: 25700524]
- [14]. Tjong V, Tang L, Zauscher S, Chilkoti A. *Chem Soc Rev*. 2014; 43:1612. [PubMed: 24352168]
- [15]. Frei AP, Bava FA, Zunder ER, Hsieh EW, Chen SY, Nolan GP, Gherardini PF. *Nat Methods*. 2016
- [16]. Weibrecht I, Lundin E, Kiflemariam S, Mignardi M, Grundberg I, Larsson C, Koos B, Nilsson M, Soderberg O. *Nat Protoc*. 2013; 8:355. [PubMed: 23348363]
- [17]. Xiu J, Zhang Q, Zhou T, Zhou TT, Chen Y, Hu H. *Nat. Neurosci*. 2014; 17:1552. [PubMed: 25242305]
- [18]. Xu H, Sepulveda LA, Figard L, Sokac AM, Golding I. *Nat Methods*. 2015; 12:739. [PubMed: 26098021]

- [19]. Darmanis S, Gallant CJ, Marinescu VD, Niklasson M, Segerman A, Flamourakis G, Fredriksson S, Assarsson E, Lundberg M, Nelander S, Westermark B, Landegren U. *Cell Rep.* 2016; 14:380. [PubMed: 26748716]
- [20]. Petryayeva E, Algar WR, Medintz IL. *Appl Spectrosc.* 2013; 67:215. [PubMed: 23452487]
- [21]. Wegner KD, Hildebrandt N. *Chem Soc Rev.* 2015; 44:4792. [PubMed: 25777768]
- [22]. Zrazhevskiy P, Sena M, Gao X. *Chem Soc Rev.* 2010; 39:4326. [PubMed: 20697629]
- [23]. Levenson RM, Fornari A, Loda M. *Expert Opin Med Diagn.* 2008; 2:1067. [PubMed: 23495926]
- [24]. True LD, Gao X. *J Mol Diagn.* 2007; 9:7. [PubMed: 17251330]
- [25]. de Planell-Saguer M, Rodicio MC, Mourelatos Z. *Nat Protoc.* 2010; 5:1061. [PubMed: 20539282]
- [26]. Gasnier M, Dennis C, Vaurs-Barriere C, Chazaud C. *Nat Protoc.* 2013; 8:2538. [PubMed: 24263093]
- [27]. Zimmerman SG, Peters NC, Altaras AE, Berg CA. *Nat Protoc.* 2013; 8:2158. [PubMed: 24113787]

Figure 1.

Cross-platform DNA encoding of molecular information with ssDNA tags for imaging single-cell gene expression. a) Schematic of simultaneous labeling and multiplexed imaging of different molecular targets with multicolor QDots via DNA encoding. In general, each molecular target is encoded by target-specific ssDNA-tagged affinity molecule (e.g., an antibody, aptamer, oligonucleotide, etc.). Resulting array of target-bound ssDNA tags can be sequentially or simultaneously labeled by complementary imaging probes, enabling multiplexed imaging of all targets of interest (e.g., via fluorescence microscopy with hyperspectral imaging, HSI). b) Staining of β -tubulin with a panel of 6-color QDot-ssDNA probes. Target is first encoded through anti- β -tubulin primary antibody and ssDNA-conjugated rabbit anti-mouse secondary antibody followed by hybridization with complementary QDot-ssDNA probes to achieve characteristic microtubule staining pattern. Insets: control experiments skipping incubation with primary and secondary antibodies show a lack of non-specific QDot binding. True-color images for target staining vs. control are obtained at consistent exposure time for each QDot color. c) Multiplexed labeling of GAPDH and HSP90- α mRNA and corresponding proteins (with QDots 605, 655, 585, and 545, respectively). DNA encoding methodology enables ssDNA tagging of mRNA targets via *in situ* hybridization and protein targets via immunorecognition by antibody-ssDNA bioconjugates. All ssDNA tags are simultaneously converted into distinctive optical signals via hybridization with complementary QDot-ssDNA' probes. Fluorescence microscopy with HSI is employed for cell imaging and unmixing of 4 individual QDot channels. Individual grayscale channels are false-colored and merged into a composite 4-color image. Scale bar, 50 μ m.

Figure 2.

Assessment of heterogeneity in cell transfection with siRNA. a) Complete degradation of GAPDH mRNA is observed at a single-cell level after a 24-hour treatment with GAPDH-targeting siRNA (left panel), whereas non-targeting control siRNA had no effect on GAPDH mRNA abundance (right panel). Insets: control experiment with mismatched QDot605-ssDNA probes establish non-specific labeling background. Scale bar, 50 μm . b,c) Heterogeneity of GAPDH silencing is observed in different areas of the culture well (center vs. edge) at a cell population level with forward (on-surface) transfection, but not with reverse (in-suspension) transfection (labeling with QDot605 probes). Such heterogeneity might result from forward transfection failure at the densely populated well edges, and a more uniform reverse transfection achieved in suspension. Interestingly, occasional colonies with full GAPDH expression can be observed with both transfection methods. Scale bar, 250 μm . d) Dual-labeling of GAPDH and HSP90- α mRNA with QDot565 and QDot585, respectively, enables direct visualization of RNAi on different targets. As expected, after a 24-hour treatment with GAPDH siRNA, majority of cells have GAPDH mRNA completely degraded (right panel, green), but retain unperturbed HSP90- α mRNA not targeted by RNAi machinery (right panel, red). Interestingly, a single cell in the field of view has apparently failed to transfect with GAPDH siRNA, featuring mRNA expression pattern consistent with cells treated with control siRNA (middle panel) and reference cells not transfected with siRNA (left panel). Dual-color images are obtained with HSI, unmixed into QDot channels, and false-colored. Scale bar, 50 μm .

Figure 3.

Assessment of disparity in RNAi kinetics at mRNA and protein levels. HeLa cells are transfected with (a,b) GAPDH siRNA vs. (c,d) control siRNA for 24 hours (a,c) or 48 hours (b,d). GAPDH and HSP90- α mRNA along with corresponding proteins are simultaneously assessed with QDot-based cross-platform imaging methodology (labeling with QDots 605, 655, 585, and 545, respectively). a) Consistent with mRNA-only analysis, cross-platform imaging highlights complete and selective degradation of GAPDH mRNA 24 hours post-transfection, whereas GAPDH protein level remains nearly unperturbed. b) Lagging mRNA knock-down, 48 hours post-transfection selective degradation of GAPDH protein can be observed. c,d) Consistent with expected lack of RNAi with control (non-targeting) siRNA, GAPDH and HSP90 expression remains constant through 48 Hrs of incubation, as evident from consistent intensity of mRNA and protein labeling. Multiplexed images are obtained with HSI, and individual channels are normalized to HSP90 protein channel for direct comparison of signal intensity. Scale bar, 50 μ m.