Hoechst tagging: a modular strategy to design synthetic fluorescent probes for live-cell nucleus imaging

<table>
<thead>
<tr>
<th>Journal:</th>
<th>ChemComm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>CC-COM-03-2014-001753.R1</td>
</tr>
<tr>
<td>Article Type:</td>
<td>Communication</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>08-Mar-2014</td>
</tr>
</tbody>
</table>
| Complete List of Authors: | Nakamura, Akinobu; Nagaoka University of Technology, Department of Electrical Engineering  
                       | Takigawa, Kazumasa; Nagaoka University of Technology, Department of Bioengineering  
                       | Yasutaka, Kurishita; Kyoto University, Department of Synthetic Chemistry and Biological Chemistry  
                       | Kuwata, Keiko; Nagoya University, Institute of Transformative Biomolecules  
                       | Ishida, Manabu; Nagaoka University of Technology, Top Runner Incubation Center for Academia-Industry Fusion  
                       | Shimoda, Yasushi; Nagaoka University of Technology, Department of Bioengineering  
                       | Hamachi, Itaru; Kyoto University, Department of Synthetic Chemistry and Biological Chemistry  
                       | Tsukiji, Shinya; Nagaoka University of Technology, Top Runner Incubation Center for Academia-Industry Fusion |
Hoechst tagging: a modular strategy to design synthetic fluorescent probes for live-cell nucleus imaging†‡

Akinobu Nakamura, a,b Kazumasa Takigawa, c Yasutaka Kurishita, d Keiko Kuwata, f Manabu Ishida, a Yasushi Shimoda, b Itaru Hamachi a,c,d and Shinya Tsukiji a,b

Received (in XXX, XXX) Xth XXXXXXXXXX 20XX, Accepted Xth XXXXXXXXXX 20XX
DOI: 10.1039/b000000x

We report a general strategy to create small-molecule fluorescent probes for the nucleus in living cells. Our strategy is based on the attachment of the DNA-binding Hoechst compound to a fluorophore of interest. Using this approach, simple fluorescein, BODIPY, and rhodamine dyes were readily converted to novel turn-on fluorescent nucleus-imaging probes.

Visualizing the nucleus (chromosomes) in living mammalian cells using synthetic fluorescent DNA probes is an important technique in many areas of biological science and biomedical research. DAPI (4',6-diamidino-2-phenylindole) and Hoechst dyes have been most commonly used for this purpose, because they are cell-permeable and DNA-specific.1,2 However, they emit only blue fluorescence, and require UV excitation, which can lead to cell damage and significant cellular auto-fluorescence. SYTO probes are visible light-excitable dyes, but they stain DNA and RNA in cells.3 Although DRAQ5 is a deep red fluorescent nucleus-specific probe,4 it shows high cytotoxicity.5 Therefore, there is still a need for the development of more fluorescent nucleus stains. In particular, with the growing importance of live-cell multicolor imaging, it is highly desired to assort cell-permeable DNA-specific probes with various emission colors. To this end, several researchers have developed new fluorescent DNA probes,6 which include, for example, a green fluorescent dye C616 and a red fluorescent dye DEAB-TO-3.6 Nevertheless, the development of fluorescent nucleus stains is not an easy task, requiring tremendous efforts to create even a single new probe. This is clearly because the current probe design in this area has focused on integrating DNA-binding and fluorescent properties simultaneously into a small molecule structure.

Here we introduce a novel modular strategy to design fluorescent nucleus-imaging probes. Over the past decades, a diverse array of small-molecule fluorophores with various excitation/emission properties have been developed.7,8 If we are able to convert these fluorescent dyes into DNA-binding probes by a simple modification, it will facilitate the development of various new fluorescent probes for live-cell nucleus imaging. Recently, we reported a small-molecule trimethoprim (TMP) ligand possessing the ability to localize in the nucleus of living cells.6 The nucleus-localizing TMP was generated by linking TMP and the DNA-binding Hoechst compound via a flexible spacer. Since the Hoechst dye is a well-established DNA-binding molecule, the Hoechst-tagged TMP efficiently and spontaneously localized in the nucleus through its binding to the genomic DNA.

Based on this observation, we reasoned that by linking the Hoechst tag to a fluorescent dye of interest, the resultant molecule would function as a DNA-binding fluorescent probe that can visualize the nucleus in living cells (Fig. 1a).7,8 To test this idea, we first chose fluorescein, one of the most classical and versatile organic fluorophore platforms, as a model fluorescent dye. We designed and synthesized hoeFL by attaching the Hoechst tag to 5-carboxyfluorescein through an
As a control experiment, we first stained HeLa cells with membrane eye (Fig. S1) of the fluorescence (Fig. S1). Using human epithelial HeLa cells. Because the cell permeability of fluorescein is known to be very low, the hairpin DNA (hpDNA) shown in the inset of Fig. 2b was used. The hpDNA contains the AATT sequence, a Hoechst recognition motif, in the 12-base-pair stem and is reported to bind with the Hoechst compound at a 1:1 stoichiometry. Fluorescence measurement with fluorescein (FL) excitation at 460 nm revealed that the FL emission was very weak when the Hoechst compound at a 1:1 stoichiometry. However, the FL emission significantly increased (by 94%) (Fig. 2a, Table 1). Based on the titration curve, the dissociation constant \( K_d \) of Hoechst to hpDNA was estimated to be 2.5 µM. To investigate this fluorescence switching mechanism, we also measured UV-visible absorption spectra of Hoechst and its control compounds 1 and 2 (Fig. S1a). The absorption maximum of the fluorescein moiety in Hoechst (alone) was 501 nm (Fig. S1c), which was red-shifted relative to that of the control fluorescein derivative 2 (495 nm) (Fig. S1d). This result suggests the ground-state interaction (dye-to-dye contact) of the Hoechst and fluorescein moieties in Hoechst, which can lead to fluorescence quenching (Fig. S1e). On the other hand, the absorption maxima of the Hoechst and fluorescein moieties in Hoechst moved to 351 nm (from 338 nm) and 495 nm, respectively, upon the binding of Hoechst to hpDNA (Fig. S1c). These absorption maxima were identical to those of the I-DNA complex (351 nm) and 2 (495 nm) (Fig. S1d). Therefore, the interaction of the Hoechst and fluorescein moieties in Hoechst is suppressed when Hoechst binds to hpDNA via the Hoechst group, leading to an enhancement (recovery) of the fluorescence (Fig. S1e). The turn-on fluorescence response was also clearly detectable by the naked eye (Fig. S1f).

Next we moved on to evaluate the nucleus imaging ability of Hoechst using human epithelial HeLa cells. Because the cell membrane permeability of fluorescein is known to be very low due to its anionic nature, we newly synthesized HoeAcFL using 5-carboxyfluorescein diacetate for cell experiments (Fig. 1b). As a control experiment, we first stained HeLa cells with ethylene glycol-based linker (Fig. 1b; for synthesis, see ESI†).

Prior to cell-based experiments, we evaluated the DNA-binding and fluorescent properties of HoeFL in vitro. For this purpose, the hairpin DNA (hpDNA) shown in the inset of Fig. 2b was used. The hpDNA contains the AATT sequence, a Hoechst recognition motif, in the 12-base-pair stem and is reported to bind with the Hoechst compound at a 1:1 stoichiometry. Fluorescence measurement with fluorescein (FL) excitation at 460 nm revealed that the FL emission was very weak when HoeFL alone was dissolved in aqueous buffer [the fluorescence quantum yield \( \Phi_{\text{HoeFL}} \) was < 0.01] (Fig. 2a, Table 1). However, the FL emission significantly increased (by 94%) (Fig. 2a, Table 1). No fluorescence increase was observed when we performed the same experiment in the presence of excess Hoechst 33342 (Fig. S1b), demonstrating that HoeFL binds to the same binding site with the original Hoechst compound. Based on the titration curve, the dissociation constant \( K_d \) of HoeFL to hpDNA was estimated to be 2.5 µM. To investigate this fluorescence switching mechanism, we also measured UV-visible absorption spectra of HoeFL and its control compounds 1 and 2 (Fig. S1a). The absorption maximum of the fluorescein moiety in HoeFL (alone) was 501 nm (Fig. S1c), which was red-shifted relative to that of the control fluorescein derivative 2 (495 nm) (Fig. S1d). This result suggests the ground-state interaction (dye-to-dye contact) of the Hoechst and fluorescein moieties in HoeFL, which can lead to fluorescence quenching (Fig. S1e). On the other hand, the absorption maxima of the Hoechst and fluorescein moieties in HoeFL moved to 351 nm (from 338 nm) and 495 nm, respectively, upon the binding of HoeFL to hpDNA (Fig. S1c). These absorption maxima were identical to those of the I-DNA complex (351 nm) and 2 (495 nm) (Fig. S1d). Therefore, the interaction of the Hoechst and fluorescein moieties in HoeFL is suppressed when HoeFL binds to hpDNA via the Hoechst group, leading to an enhancement (recovery) of the fluorescence (Fig. S1e). The turn-on fluorescence response was also clearly detectable by the naked eye (Fig. S1f).

Next we moved on to evaluate the nucleus imaging ability of HoeFL using human epithelial HeLa cells. Because the cell membrane permeability of fluorescein is known to be very low due to its anionic nature, we newly synthesized HoeAcFL using 5-carboxyfluorescein diacetate for cell experiments (Fig. 1b). As a control experiment, we first stained HeLa cells with ethylene glycol-based linker (Fig. 1b; for synthesis, see ESI†).

Preparative fluorescence microscopy (CLSM). As well known, the FL fluorescence was distributed over the cell (Fig. S2), indicating that non-tagged fluorescein has no specific subcellular localization property. In contrast, when HeLa cells were treated with HoeAcFL (5 µM) for 15 min, the FL fluorescence was observed specifically in the nucleus (Fig. 3a). The nuclear staining was confirmed by co-staining with Hoechst 33342 (Fig. S3). Quantitative line scan analysis of the fluorescence image also verified the highly selective staining of the nucleus over the cytoplasm (Fig. 3b). Taken together, these data indicate that HoeAcFL is cell-permeable, converted intracellularly to HoeFL, and then binds to and visualize the live-cell nucleus with high efficiency. Nuclear fluorescence was observable with minimum background staining even under no-wash conditions (Fig. S4a).

In addition, the HoeAcFL-mediated nucleus imaging was applicable to various other cell lines, such as mouse fibroblast NIH3T3, human embryonic kidney (HEK) 293, and T-lymphocyte Jurkat cells (Fig. S5), and even to live rat hippocampal tissue slices (Fig. S6).

Having succeeded in creating the nucleus-staining fluorescein, we next attempted to apply the Hoechst tagging strategy to different fluorescent dyes. Here we targeted two other fluorophore platforms: BODIPY and rhodamine. By taking advantage of the modular design, HoeBD was generated simply by replacing the fluorescein unit in HoeAcFL with the BODIPY dye (Fig. 1b). HoeTMR was also prepared in the same manner using tetramethylrhodamine (Fig. 1b). We first characterized the DNA-binding and photophysical properties of these molecules in vitro as described above. Interestingly, as is the case for HoeFL, HoeBD and HoeTMR both showed the turn-on fluorescence response upon binding to hpDNA, although their fluorescence enhancement ratios were smaller than that of HoeFL (Fig. S7 and S8, Table 1).

The \( K_d \) values to hpDNA were estimated to be 28 nM and 1.8 µM for HoeBD and HoeTMR, respectively. We then carried out live-cell imaging using these probes. After incubating HeLa cells with either HoeBD or HoeTMR, the cells were washed and observed by CLSM. As shown in Fig. S9a and S10, both probes clearly stained the nucleus. These results unambiguously show the generality of the Hoechst tagging approach. Owing to the high cell-permeability and nucleus-binding capability, HoeBD was also applicable to visualize the cellular nucleus without a washing procedure (Fig. S9b). Finally,

### Table 1 Summary of the dissociation constants of Hoechst-tagged fluorescent probes to hpDNA and their photophysical properties

<table>
<thead>
<tr>
<th>Probes</th>
<th>( K_d ) for hpDNA (M)</th>
<th>( F/F_0 )</th>
<th>( \Phi_{\text{probe}} )</th>
<th>( \Phi_{\text{probe-hpDNA}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>HoeFL</td>
<td>2.5 \times 10^{-8}</td>
<td>94</td>
<td>0.0072</td>
<td>0.44</td>
</tr>
<tr>
<td>HoeBD</td>
<td>2.8 \times 10^{-8}</td>
<td>4.9</td>
<td>0.028</td>
<td>0.23</td>
</tr>
<tr>
<td>HoeTMR</td>
<td>1.8 \times 10^{-8}</td>
<td>27</td>
<td>0.0078</td>
<td>0.089</td>
</tr>
</tbody>
</table>

\( K_d \) measurement conditions: 2 µM HoeFL, 0.1 µM HoeBD, or 2 µM HoeTMR in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, 20 °C, \( \lambda_{em} = 460 \) nm for HoeFL and HoeBD, and 500 nm for HoeTMR. \( F/F_0 \) indicates the relative fluorescence intensities (F) at 520 nm for HoeFL, 514 nm for HoeBD, and 580 nm for HoeTMR in the presence of hpDNA at the highest concentration used in the titration assay against those of the initial state \( (F_0) \). \( \Phi_{\text{probe}} \) indicates the fluorescence quantum yields of the probes in the absence of hpDNA. \( \Phi_{\text{probe-hpDNA}} \) indicates the fluorescence quantum yields of the probes in the complexed state with hpDNA.
Fig. 3 (a) CLSM images of HeLa cells stained with hoeAcFL. Cells were incubated with hoeAcFL (5 μM) for 15 min, washed, and then observed by CLSM. Left, FL fluorescence image; right, DIC image merged with the fluorescence image. Scale bar, 10 μm. (b) Fluorescence intensity profile across the red line shown in the merge image in (a).

It should be noted that no significant cytotoxicity was observed for all the Hoechst-tagged probes reported herein under the conditions used, whereas the traditional Hoechst 33342 showed obvious cytotoxicity (Fig. S11).

In conclusion, we have shown that simple fluorescent dyes can be converted to nucleus-selective imaging probes by conjugating them with the DNA-binding Hoechst compound. The general utility of this Hoechst tagging strategy was demonstrated using three representative organic fluorophore platforms. The Hoechst-tagged fluorophores developed in this work have excitation and emission wavelengths in the visible range, detect DNA with turn-on fluorescence signals, and allow rapid (and even no-wash) staining of the nucleus in various living cells and tissues. These features make them attractive as novel practical nucleus-staining probes for (multicolor) bioimaging. Moreover, taking advantage of its modular nature, the Hoechst tagging strategy should be applicable to more diverse fluorescent dyes with unique photochemical properties. By applying the approach to small-molecule fluorescent sensors, it may also become possible to generate new imaging tools that allow selective detection of specific biomolecules or metal ions in the nucleus of living mammalian cells. Our research is now ongoing along this line.

This work was supported in part by grants from the Nakajima Foundation and the Naito Foundation (to S.T.). Y.K. acknowledges the JSPS Research Fellowships for Young Scientists.

Notes and references

* Top Runner Incubation Center for Academia-Industry Fusion, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan. E-mail: tsukiji@vos.nagaukant.ac.jp; Fax: +81-258-47-9400. Tel: +81-258-47-9400
* Department of Biotechnology, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan
* Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Kyoto 615-8510, Japan. E-mail: tsukiji@ktech.kyoto-u.ac.jp; Fax: +81-7-383-2759; Tel: +81-7-383-2754
* Department of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan
* Department of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan
* Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Kyoto 615-8510, Japan. E-mail: tsukiji@ktech.kyoto-u.ac.jp; Fax: +81-7-383-2759; Tel: +81-7-383-2754

† Dedicated to Professor Seiji Shinkai on the occasion of his 70th birthday.

7. Murthy and coworkers have previously reported a similar approach, in which the Hoechst compound was attached to a near-infrared fluorescent dye. However, their conjugate was cell-impermeable and used to detect extracellular DNA released from necrotic cells. See: (a) M. Disarzi, S. Lee, J. S. Dy, D. Kim, S. Lee, M. Brown, M. Davis and N. Murthy, Org. Lett., 2010, 12, 3300.
8. As a relevant work, Dervan and coworkers have demonstrated that several fluorophores could be localized to the nucleus by conjugating them with DNA--binding pyrrole-imidazole polyamides. The polyamides are quite large (M.W. > 1,000) compared to the Hoechst tag (M.W. of Hoechst 33342 = 453), and a long incubation time (>10 h) was required for introducing the fluorophore-polyamide conjugates into cells. See: (a) T. P. Best, B. S. Edelson, N. G. Nickols and P. G. Dervan, Proc. Natl. Acad. Sci. U. S. A., 2003, 100, 12063; (b) B. S. Edelson, T. P. Best, B. Olenyuk, N. G. Nickols, R. M. Doss, S. Foister, A. Heckel and P. B. Dervan, Nucl. Acids Res., 2004, 32, 2802.
10. The K0 values of HoeFL and HoeTMR to hpdNA are nearly three orders of magnitude larger than that of the parental Hoechst 33258 to hpdNA (K0 = 2 nM). Nevertheless, as shown in this work, these probes can clearly visualize the nucleus of living cells.
13. Non-acetylated HoeFL was also found to be cell-permeable and visualize the nucleus, although in this case a longer incubation time and washing procedure are required (Fig. S4b).
14. The turn-on fluorescence mechanism of hoEBDP and HoeTMR should be similar to that described for HoeFL, UV-visible absorption spectra of these probes in the absence and presence of hpDNA are shown in Fig. S5c and S8c.
15. In the case of HoeTMR, a long incubation time (1 h) was required due to the relatively low cell-permeability of the probe. This
procedure causes slight staining of the lysosomal compartment (Fig. S10).

16 As far as we tested, nuclei were clearly visualized by incubating cells with 0.1–20 µM hoeAc2FL, 0.5–20 µM hoeBDP, and 0.5–20 µM hoeTMR (Fig. S12). In addition, for all the probes, the fluorescence intensity was gradually decreased by incubating the stained cells in a fresh medium (data not shown). However, the nuclear fluorescence remained observable for at least 6 h after the staining and washing procedure.

17 There have been only a few examples of synthetic small-molecule sensors that localize in the nucleus of living cells. See: (a) M. Ikeda, H. Nakagawa, S. Ban, H. Tsumoto, T. Suzuki and N. Miyata, Free Rad. Biol. Med., 2010, 49, 1792; (b) B. C. Dickinson, Y. Tang, Z. Chang and C. J. Chang, Chem. Biol., 2011, 18, 943.

TOC

Attachment of the Hoechst tag to fluorescent molecules allows the creation of (turn-on) fluorescent nucleus-imaging probes.