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ARTICLE TYPE

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

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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.[1] Although DRAQ5 is a deep red fluorescent nucleus-specific probe,[3] it shows high cytotoxicity.[4e] 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,[4] which include, for example, a green fluorescent dye C61[4e] and a red fluorescent dye DEAB-TO-3.[4g] 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.[1,5] 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

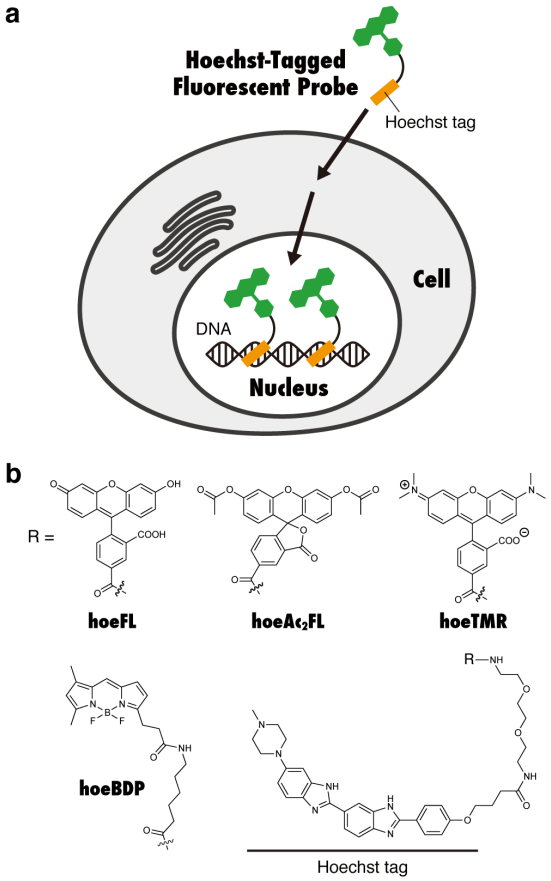

Fig. 1 (a) Schematic illustration of the Hoechst tagging strategy for creating DNA-binding fluorescent dyes. (b) Chemical structures of Hoechst-tagged fluorescent probes used in this study.

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

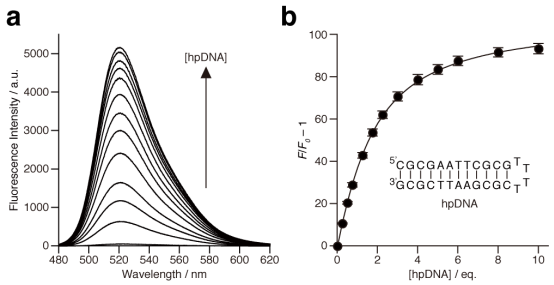

Fig. 2 (a) Fluorescence spectral changes of **hoeFL** (2 μM) upon addition of hpDNA (0–20 μM). Measurement conditions: 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, 20 °C, λ_{ex} = 460 nm. (b) Curve-fitting analysis of the fluorescence change at 520 nm. The inset shows the structure of hpDNA. Data are represented as mean ± s.d. of three independent experiments.

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.[9] 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 (Φ_{hoeFL}) was < 0.01] (Fig. 2a, Table 1). However, the FL emission significantly increased (by 94-fold) upon addition of hpDNA with a typical saturation behavior (the maximum $\Phi_{hoeFL\text{-}hpDNA}$ was 0.44) (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.[10] 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).[11] 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 **1**-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).[11c] 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 **hoeAc$_2$FL** using 5-carboxyfluorescein diacetate for cell experiments (Fig. 1b).[12] As a control experiment, we first stained HeLa cells with

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

Probes	K_d for hpDNA[a] (M)	F/F_0[ab]	Φ_{probe}[c]	$\Phi_{probe\text{-}hpDNA}$[d]
hoeFL	2.5×10^{-6}	94	0.0072	0.44
hoeBDP	2.8×10^{-8}	4.9	0.024	0.23
hoeTMR	1.8×10^{-6}	27	0.0078	0.089

[a] Measurement conditions: 2 μM **hoeFL**, 0.1 μM **hoeBDP**, or 2 μM **hoeTMR** in 50 mM Tris-HCl, 100 mM NaCl, pH 7.4, 20 °C. λ_{ex} = 460 nm for **hoeFL** and **hoeBDP**, and 500 nm for **hoeTMR**. [b] F/F_0 indicates the relative fluorescence intensities (F) at 520 nm for **hoeFL**, 514 nm for **hoeBDP**, 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). [c] Φ_{probe} indicates the fluorescence quantum yields of the probes in the absence of hpDNA. [d] $\Phi_{probe\text{-}hpDNA}$ indicates the fluorescence quantum yields of the probes in the complexed state with hpDNA.

fluorescein diacetate and observed the cells by confocal laser scanning 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 **hoeAc$_2$FL** (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 **hoeAc$_2$FL** 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).[13] In addition, the **hoeAc$_2$FL**-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, **hoeBDP** was generated simply by replacing the fluorescein unit in **hoeAc$_2$FL** 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**, **hoeBDP** 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).[14] The K_D values to hpDNA were estimated to be 28 nM and 1.8 μM for **hoeBDP** and **hoeTMR**, respectively.[10] We then carried out live-cell imaging using these probes. After incubating HeLa cells with either **hoeBDP** or **hoeTMR**, the cells were washed and observed by CLSM. As shown in Fig. S9a and S10, both probes clearly stained the nucleus.[15] These results unambiguously show the generality of the Hoechst tagging approach. Owing to the high cell-permeability and nucleus-binding capability, **hoeBDP** was also applicable to visualize the cellular nucleus without a washing procedure (Fig. S9b). Finally,

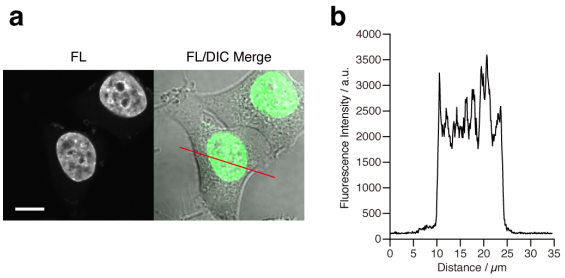

Fig. 3 (a) CLSM images of HeLa cells stained with **hoeAc$_2$FL**. Cells were incubated with **hoeAc$_2$FL** (5 μM) for 15 min, washed, and then observed by CLSM. Left, FL fluorescence image; right, DIC image merged with the fluorescence image. Scale bar, 10 μm. (b) Fluorescence intensity profile across the red line shown in the merge image in (a).

it should be noted that no significant cytotoxity 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.[16] 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.[17] Our research is now ongoing along this line.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [Figures S1–12 and experimental details]. See DOI: 10.1039/b000000x/

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

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10 The K_D values of **hoeFL** and **hoeTMR** to hpDNA are nearly three orders of magnitude larger than that of the parental Hoechst 33258 to hpDNA (K_D = 2 nM).[9] Nevertheless, as shown in this work, these probes can clearly visualize the nucleus of living cells.

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12 Fluorescein diacetate is non-fluorescent, but recovers its fluorescence in cells by the hydrolysis of the acetyl group by intracellular esterases. See: B. Rotman and B. W. Papermaster, *Proc. Natl. Acad. Sci. U. S. A.*, 1966, **55**, 134.

13 Non-acetylated **hoeFL** was also found to be cell-permeable and visualize the nucleus, although in this case a longer incubation time and a washing procedure are required (Fig. S4b).

14 The turn-on fluorescence mechanism of **hoeBDP** and **hoeTMR** should be similar with that described for **hoeFL**. UV-visible absorption spectra of these probes in the absence and presence of hpDNA are shown in Fig. S7c 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 **hoeAc$_2$FL**, 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 now shown). However, the nuclear fluorescence remained observable for at least 6 h after the staining and washing procedure.

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TOC

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