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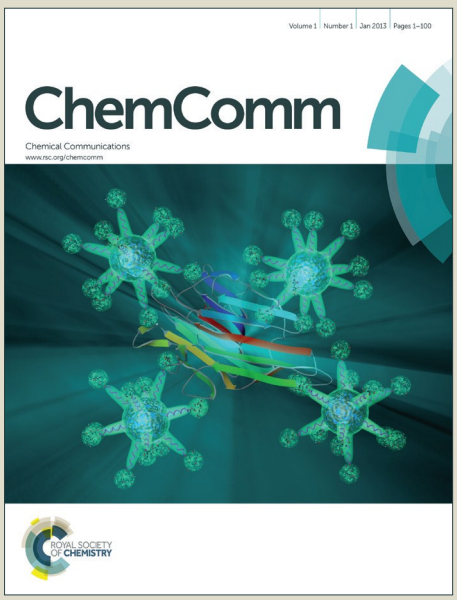

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Development of nucleus staining fluorescent probe for dynamic mitosis imaging in live cells

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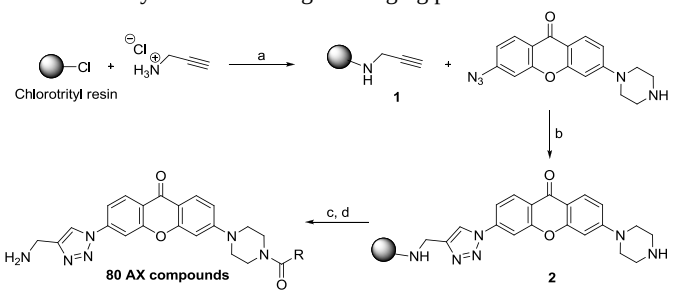
A low-toxicity nucleus staining fluorescent probe, CDb12, was developed for real time mitosis imaging in live cells. CDb12 was identified by unbiased high-throughput imaging-based screening of new xanthone library (AX). Unlike the conventional Hoechst dye, the low toxicity of CDb12 allows a long term monitoring of cell division over more than one cell cycle.

Recently, considerable advancement of imaging techniques allow scientists to visualize characteristic cellular phenomena in living cells.[1] Among them, fluorescence-based biosensors are most widely used tools for real-time analysis of biological processes, but it has been remained challenging to monitor the cell-cycle progression in live cells. The commonly used techniques for cell-cycle progression monitoring are incorporation of bromodeoxyuridine (BrdU) or immunostaining with cell-cycle specific antibodies.[2,3] However, these techniques need cell fixation and permeabilization before the analysis. Hence, they are not useful for monitoring the dynamics of cell-cycle in live cells. In order to resolve this problem, several biological methods have been established using fluorescently tagged cell-cycle reporters including Fucci reporter,[2] eGFP-anillin,[4] and GFP-histone H2B.[5] Although these methods could track the cell-cycle event in live cells, it involves genetic engineering of cells, which is time consuming and often disruptive to the normal cell functions. Besides, the expression efficiency and consistency of these reporters can be other practical problems.[6] Among chemical probes, DNA staining fluorescent dyes such as Hoechst dyes has been popularly used for nucleus staining. While robust, all the known DNA binding dyes are toxic to the cells especially during the cell replication stage.[7] Therefore, there is an urgent need for the development of a non-toxic fluorescent probes for real-time monitoring of cell mitosis by visualizing the DNA dynamics.[8]

In recent years, several fluorescent probes based on BODIPY,[9a] rosamine,[9b] fluorescine,[9c] coumarin[9d,e] scaffold have been widely used for real-time analysis of various biological events in fluorescence microscopy and flow cytometry. However, the potential bioimaging applications of xanthones were not fully explored in spite of their high extension coefficient, bright fluorescence and excellent photo stability.[10] In our previous study, we have reported the first synthesis of solid phase fluorescent xanthone library (CX) using click chemistry[11] and its potential biological application as an embryonic stem cell probe.[12] These xanthone compounds showed in general low cytotoxicity and good cell permeability. Encouraged by the result, we designed and constructed a new fluorescent xanhtone library (AX) utilizing acid chlorides as the key diversity component and herein we report a novel low toxicity nucleus staining bioimaging probe.



Scheme 1 Synthesis of AX library. Reagents and Conditions: (a) DIEA, DMF/DCM, r.t, 12 h; (b) CuI, Ascorbic acid, DMF/Piperidine (4:1), r.t, 24 h; (c) DIEA, RCOCl DCM, r.t, 4 h; (d) 2% TFA in DCM, r.t, 15 min.

As described in scheme 1, the general synthetic strategy of AX library involves three steps. First, the propargyl amine is loaded to the freshly prepared chlorotrityl chloride resin. Then the reaction between xanthone azide and the propargyl amine on solid support in the presence of copper (I) iodide and ascorbic acid provides the click xanthone compound (intermediate **2**). The Addition of acid chlorides to the intermediate **2**, followed by the cleavage of the compounds from the resin provides crude acyl xanthone (AX) products. The compounds were then purified using a short silica gel filtration. The final compounds were characterized using HPLC-MS and eighty relatively pure compounds were isolated for further study with an average purity over 95% at 254 nm wavelength. These AX

compounds have an excitation max at around 360 nm and the emission max at around 490 nm (Table S1).

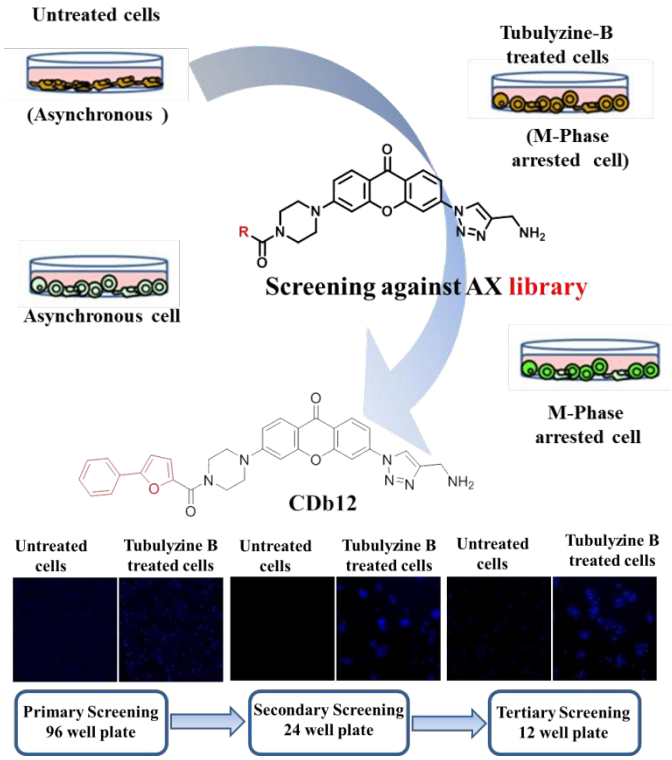


Fig. 1 The workflow of imaging-based screening for the discovery of CDb12. Blue, **CDb12**; excitation/emission = 405 nm/430 – 530 nm.

DNA is condensed and segregated during the mitosis stage demonstrating a dynamic structural changes. As a screening platform to discover novel mitosis probe, human retinal pigment epithelial (RPE1) cell line was chosen. Since RPE1 cells were immobilized as a diploid, it is suitable to induce mitotic arrest in RPE1 cells rather than using other cell types[13]. We used M-phase arrested RPE1 cells by tubulyzine-B as positive control,[14] along with asynchronous cells as negative control. Starting from 96 well plate test, brighter compounds for M-phase arrested cells over negative control cells were collected as primary hits, and the hits were confirmed in larger cell populations using 24 well and 12 well plates sequentially (Fig. 1). Among eighty AX library compounds, eight compounds were selected from the primary screening by showing increased fluorescent responce in m-phase arrested cells. The hit compounds have either a hetero-aromatic ring or a hetero atom attached to the phenyl ring (see supporting chart 1. building block no. 66, 80, 83, 117, 119, 120, 128 and 130). Based on the selectivity to the m-phase astrestd cells, **CDb12** (**C**ompound of **D**esignation **b**lue **12**) was selected as a nucleus staining probe.(CDb12: λ_{ex} = 365 nm and λ_{em} = 500 nm in DMSO, Fig. S1).

Confocal images of **CDb12**-stained RPE1 cells clearly showed **CDb12** signal clearly in the nucleus (Fig. 2). During the interphase, the signal was dispersed in the whole nucleus, but during the mitosis, the staining was condensed aligned with DNA structures, showing much higher fluorescence intensity than that of interphase cells (Fig. S2). Although the fluorescence signal was faint, CDb12 stained nucleus clearly even in the interphase cells (Fig.2b). Futher *in vitro* test confirmed that **CDb12** binds DNA by increasing its fluorescence in a dose dependent manner, but no response to RNA (Fig. S3). The dissociation constant (K_d)of **CDb12** with DNA is 0.13 mg/mL (Fig. S4), and this result suggests that though **CDb12** shows strong fluorescent intensity at nucleus during mitosis, surprisingly it has a weak *in vitro* binding affinity for DNA.

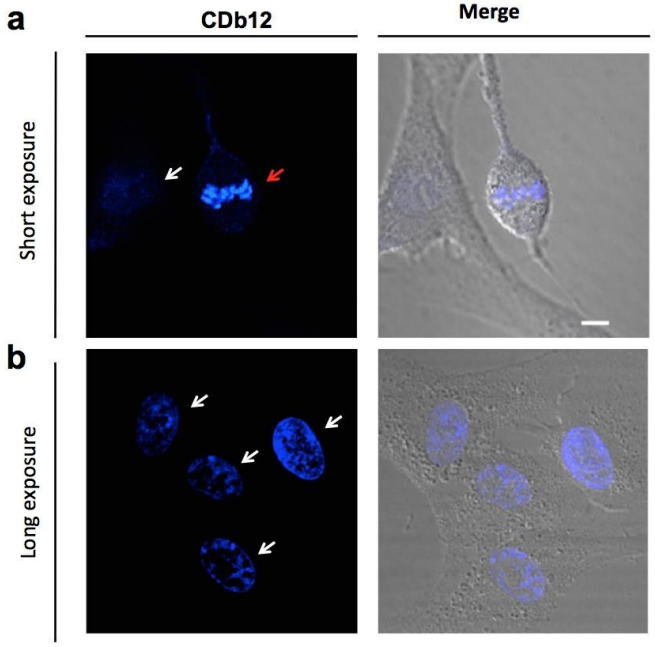


Fig. 2 The nuclear localization of CDb12 in RPE1 cell. (**a**) A red arrow indicates a cell in mitosis and a white arrow indicates a cell in interphase. (**b**) A long exposure image shows the nuclear localization of CDb12 even in the interphase cells. (**CDb12**, blue; Size bar, 10 μm).

To confirm the **CDb12**-DNA binding behaviour in live cells, we performed a flow cytometry experiment compared with known standard DNA dye Vybrant®DyeCycle Ruby. As expected, **CDb12**/Vybrant®DyeCycle Ruby dual parameter dot-plot demonstrated three populations of G1, S, and G2/M of the cell cycle in live cells (Fig. 3).

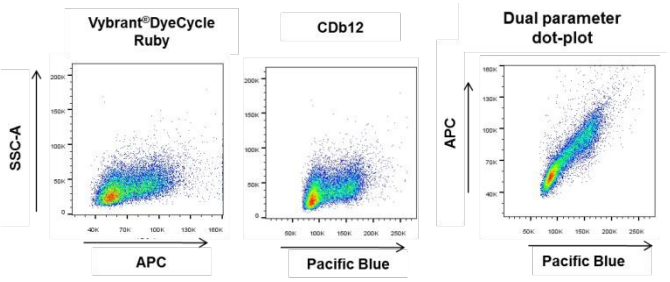


Fig. 3 Dual parameter dot-plot of CDb12 and Vybrant®DyeCycle Ruby. Jurkat cells were stained with **CDb12** (5 μM) or Vybrant®DyeCycle Ruby (5 μM). The specificity and efficiency of dyes on cell cycle distributions of live cells were

analyzed by BD FACS Aria liu SORP cell sorter with Pacific blue (Ex:405 nm; Em: 450/50 nm), APC (Ex: 640 nm; Em: 670/14 nm).

Next, we examined whether **CDb12** could visualize the dynamics of cell division in comparison with conventional Hoechst dye. Although the fluorescence intensity of CDb12 was weaker than that of Hoechst, we could observed **CDb12**-labeled cells clearly during mitosis from metaphase through anaphase to telophase (Fig. 4 upper). Even the divided daughter cells could enter another cell division, when observed longer term. On the other hand, Hoechst 33342 labelling induces the cell death at the onset of mitotic progression (Fig. 4 lower). These finding demonstrates that **CDb12** enables the real time visualization of nuclear dynamics with fluorescence in live mitosis without prominent cytotoxicity (Fig. S5).

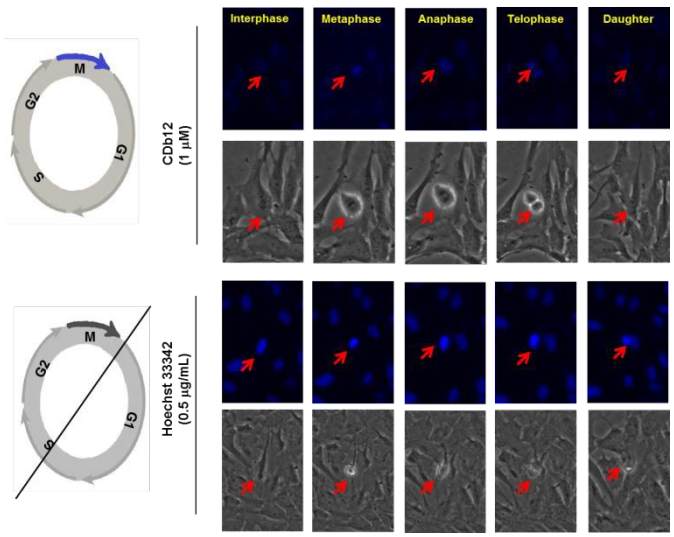


Fig. 4 Time-lapse imaging of CDb12, compared to Hoechst 33342. RPE1 cells were stained with **CDb12** (1 μM) or Hoechst 33342 (0.5 μg/mL). The mitosis was monitored for 24 h with frames taken every 3 min on a time-lapse imaging system Nikon Biostation IM (Nikon) with DAPI filter (405 nm).

In conclusion, we have designed and synthesized a new fluorescent xanthone library (AX) using acid chloride as the diversity component. Through imaging based screening, we discovered a novel DNA binding dye **CDb12**, and demonstrated a dynamic real time visualization of cell division, thanks to the low toxicity of **CDb12**. By **CDb12** staining, it will be easy to track the silhouette of individual cells in order to provide important information of nuclear dynamics. The unique low toxicity of **CDb12** seems to be due to the low binding affinity to DNA.

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Notes and references

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†Electronic Supplementary Information (ESI) available: General synthetic procedures, chemical structures and characterization of the AX library compounds and **CDb12**. Procedure for DNA. *In vitro* screening, m-phase arrested cells, and live cell screening against **CDb12**.
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Rapid and efficient synthesis of a novel fluorescent xanthone library (AX) and its applications for the development of a new nucleus staining fluorescent probe (**CDb12)** for monitoring real-time mitosis progression in live cells is presented.