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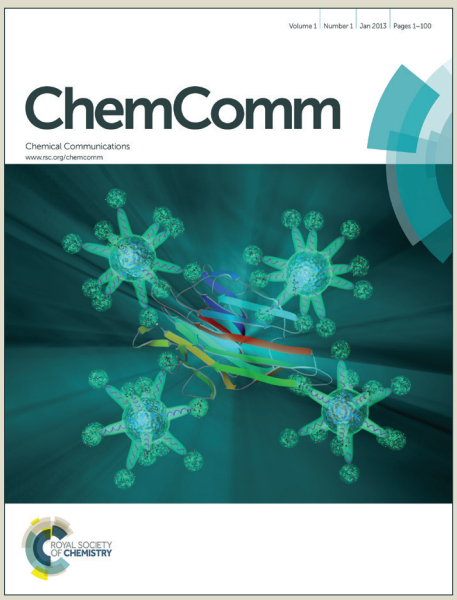

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

A Fluorescent Probe for Imaging Symmetric and Asymmetric Cell Division in Neurosphere Formation†

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

We report here a novel fluorescent chemical probe CDy5 which stains distinct neural stem/progenitor cells (NSPCs) by binding to acid ceramidase in mouse neurospheres. CDy5 is distributed evenly or unevenly to the daughter cells during multiple mitoses enabling the live imaging of symmetric and asymmetric divisions of isolated NSPCs.

Development of a multicellular organism from a single-cell zygote requires precisely orchestrated symmetric and asymmetric cell divisions. Two daughter cells with different fates are produced by an asymmetric division to generate cellular diversity, while identical daughter cells are produced by a symmetric division to proliferate. In the case of stem/progenitor cells which have the capability to self-renew and give rise to multiple types of cells, at least one daughter cell must retain the properties of the mother cell.[1] During asymmetric division, cells are polarized and certain cellular components are segregated into one half of the cell resulting in an uneven distribution of the components between two daughter cells.[2] A neurosphere generated *in vitro* from a mouse neural stem/progenitor cell (NSPC) is a particularly interesting material to study the 2 different types of cell division in mammals. A single NSPC can grow within a week to a neurosphere composed of hundreds of cells at various stages of differentiation.[3] It is known that a small number of cells in a neurosphere remain as stem/progenitor cells but a majority of the cells are differentiated cells.[4] Although fluorescent proteins genetically manipulated to be expressed only in specific type of cells have shown asymmetric cell division, there is an unmet need for easy-to-use tools for the study of asymmetric division using non-genetically modified animal cells.[5, 6]

We postulated that if a fluorescent chemical probe was bound to an unequally distributed cellular component during cell division in neurospheres, it would distinguish NSPCs and visualize their symmetric and asymmetric divisions, and investigating the function of the cellular target binding to the probe might provide insight into the mechanisms of NSPC proliferation and differentiation. By high content screening of our diversity oriented fluorescence library in various cell-based platforms, we have previously developed fluorescent chemical probes for various types of cells including pluripotent stem cells and neural stem cells.[7-9] In this study, we have synthesized sub-

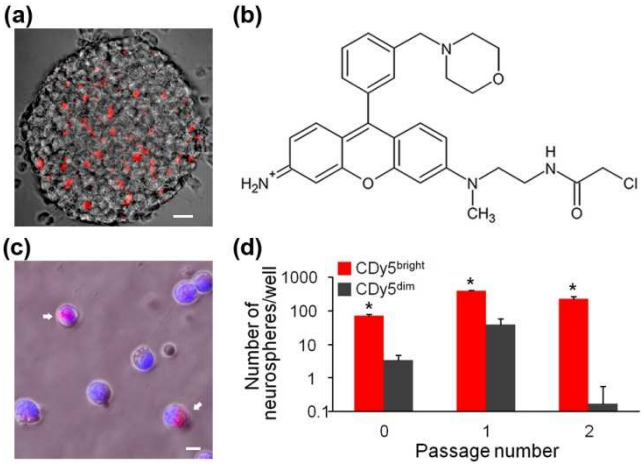

Fig 1. (a) Confocal fluorescence micrograph (taken using A1R$^+$si, Nikon) of a neurosphere fixed in paraformaldehyde. Scale bar, 10 µm. (b) Chemical structure of **CDy5**. (c) Epifluorescence micrograph of living single cells dissociated from **CDy5**-stained neurospheres. **CDy5**-stained cells are marked by white arrows. Scale bar, 10 µm. (d) Neurosphere assay of FACS sorted **CDy5**^{bright} and **CDy5**^{dim} cells. Data represent mean ± SD. * p<0.01, Student's t-test.

libraries of the stem cell probes and screened them in mouse neurospheres on a fluorescence microscope. We observed certain cells in neurospheres distinctly stained by a rosamine compound which contains a chloroacetamide moiety (Fig. 1a and S1). We named the compound **CDy5** (compound of designation yellow 5, $\lambda_{ex}/\lambda_{em}$=530/575 nm) and examined the relationship between neurosphere formation and **CDy5** staining by FACS-sorting **CDy5**^{bright} and **CDy5**^{dim} cells and culturing them (Fig. 1b,c, Scheme S1 and Data S1).

CDy5^{bright} cells generated more than 10 times more neurospheres than **CDy5**^{dim} cells in 3 independent experiments conducted with cells of different passage numbers (Fig. 1d). In addition, when the neurospheres were randomly differentiated and immunostained against astrocyte, neuron and oligodendrocyte markers, more numbers of neurospheres generated from **CDy5**^{bright} cells differentiated into 3 or 2 types of cells than those from **CDy5**^{dim} cells (Fig. S2a,b). These data suggest that **CDy5** selectively stains proliferative NSPCs in heterogeneous cell populations of different stages of differentiation.

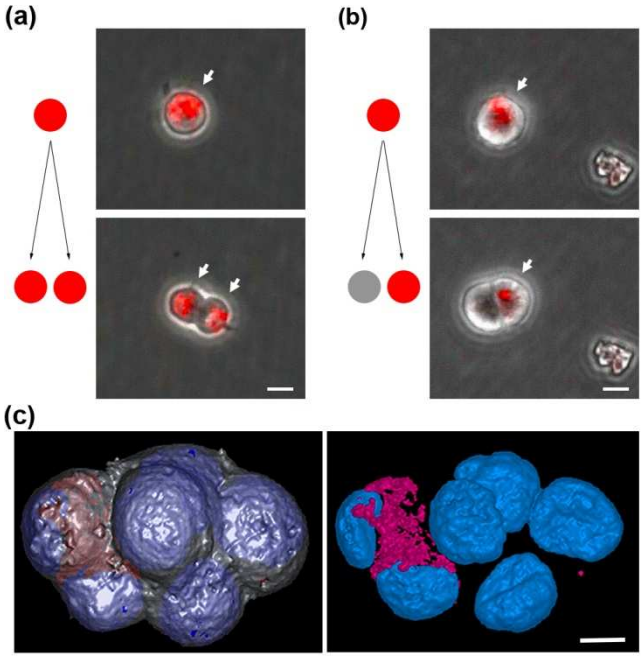

Fig 2. (a) Time-lapse live imaging of an even distribution of **CDy5** during cell division. Scale bar, 10 μm. (b) Time-lapse live imaging of an uneven distribution of **CDy5** during cell division. **CDy5** distributed into only one daughter cell was marked by a white arrow. Scale bar, 10 μm. (c) Three dimensional image of a neurosphere generated from a **CDy5**-stained cell. Two out of six cells remained stained by **CDy5**. Right, whole morphology of a neurosphere; left, only nuclei and **CDy5**-stained cytoplasm are shown. Blue, Hoechst 33342; red, **CDy5**; gray, CellTracker™ Green. Scale bar, 5 μm.

To determine if **CDy5** forms a covalent bond with a protein, we fixed the neurospheres stained with **CDy5** and Hoechst 33342 using 4% paraformaldehyde followed by absolute methanol which extracts organic dyes bound to their targets by non-covalent bonding. Both **CDy5** and Hoechst signals were detected when observed after fixation with paraformaldehyde. However, following methanol treatment, Hoechst 33342 was completely washed out while **CDy5** remained without losing its signal intensity. This result together with the neurosphere assay described above indicates that **CDy5** binds to a protein which is more highly expressed in NSPCs than in differentiated cells forming a covalent bond (Fig. S3). The NSPC specificity of **CDy5** and its strong binding to a protein led us to assume that **CDy5** might enable imaging of the symmetric or asymmetric distribution of the target protein during cell division. For time-lapse imaging of single cells, we dissociated **CDy5**-stained neurospheres into single cells, identified brightly stained cells and acquired images periodically using a microscope equipped with a cell incubator system without additional **CDy5**. The phase contrast images showed single cell divisions which gave rise to 2 morphologically identical daughter cells. But fluorescence images acquired in parallel revealed an even distribution of **CDy5** in certain cell divisions and uneven distribution in some other cell divisions reflecting symmetric and asymmetric divisions (Fig. 2a,b). Long-term image acquisitions for 2 consecutive days showed the growth of **CDy5** stained single cells into multi-cell neurospheres by both symmetric and asymmetric cell divisions (Fig. S4 and Movie S1). The 3D image reconstructed from z-stack confocal fluorescence images of a multi-cell neurosphere

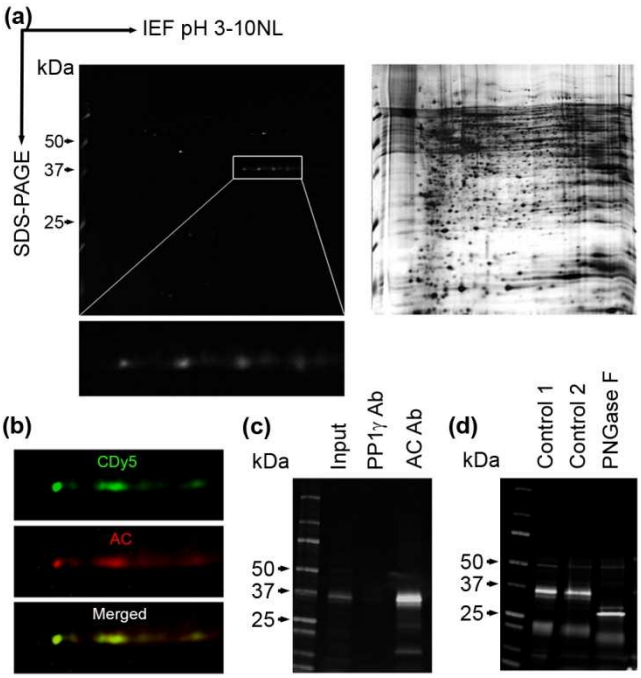

Fig. 3. (a) Fluorescence image of **CDy5**-stained neurosphere protein extract separated by isoelectric focusing (pH 3-10) and SDS-PAGE. The major fluorescent spots of ~35 kDa were marked with a rectangle (left upper panel) and magnified (left lower panel). The proteins in the gel was detected by silver staining (right panel). (b) Western blot of **CDy5**-labeled protein with acid ceramidase (AC) antibody. **CDy5** signal (upper panel, 532/580 nm) and signal from secondary antibody that recognizes the AC antibody (middle panel, 633/670 nm) overlapped (lower panel). (c) Pull-down assay of **CDy5**-labeled protein using acid ceramidase antibody (AC Ab). (d) Fluorescence image of **CDy5**-stained neurosphere protein extracts separated by SDS-PAGE. Control 1, protein extract in lysis buffer; control 2, protein extract in enzyme reaction buffer.

more clearly showed cells remained stained by **CDy5** with a good morphological structure of a whole neurosphere (Fig. 2c).

We then analyzed the neurosphere proteins to identify the cellular binding target of **CDy5** by a proteomics approach. When the proteins extracted from **CDy5**-stained neurospheres were separated by 2-dimensional SDS-PAGE and scanned on a fluorescence scanner, we were able to detect 4 major fluorescent spots of ~35 kDa among many different proteins which were detected by silver staining (Fig. 3a).We excised out the major spots from the gel for in-gel tryptic digestion and peptide extraction. LC MALDI TOF/TOF mass spectrometry analysis of the peptide sample provided a list of candidate proteins including protein phosphatase 1 gamma catalytic subunit (PP1γ) and N-acylsphingosine amidohydrolase (acid ceramidase; AC) β subunit whose molecular weights are ~35 kDa. By a 2-color fluorescence 2D Western blot analysis, AC was determined to be the protein that binds to **CDy5** (Fig. 3b). This was confirmed by pull-down assay that showed strengthened **CDy5** signal intensity in a sample pulled down by AC antibody but not by PP1γ antibody (Fig. 3c). AC is synthesized as a precursor polypeptide of 395 amino acids in human and 394 amino acids in mouse, which is processed into non-glycosylated α subunit and glycosylated β subunit.[10-12] As the mouse β subunit of 253 amino acids has 5 potential N-glycosylation sites, we treated the **CDy5**-stained neurosphere cell lysate with peptide-N-glycosidase (PNGase) F which removes N-

glycan from the protein. This resulted in a downward shift of the fluorescent band from ~35 kDa to ~25 kDa due to a faster migration of the deglycosylated protein in SDS-PAGE further confirming that the fluorescence signal is from the **CDy5** bound to the β subunit of AC (Fig. 3d). Furthermore, MS/MS fragment analysis revealed that **CDy5** binds to the first N-terminal amino acid residue cysteine of AC β subunit (Fig. S5).

Having found that **CDy5** preferably stains proliferative NSPCs in neurosphere by binding to AC, we examined the expression levels of AC gene *Asah1* and 38 other genes associated with NPSC and its differentiation in **CDy5**^{bight} and **CDy5**^{dim} neurosphere cells by single cell quantitative RT-PCR.[4, 13] The expressions of most genes analyzed including *Asah1* were higher in **CDy5**^{bright} cells compared to the levels in **CDy5**^{dim} cells. Noticeably, expressions of the genes directly involved in Notch signaling such as *Jag1, Dll1 and Hes1* were particularly higher in **CDy5**^{bright} cells (Fig. S6). To investigate the role of AC in neurosphere formation, we treated dissociated neurosphere cells with AC inhibitors Carmofur and Ceranib-2 ranging from 0.01 to 10 μM.[14, 15] When we counted the numbers of neurospheres generated in the presence of these inhibitors, significant inhibition of neurosphere formation with IC_{50}s of 0.92 μM for Carmofur and 0.78 μM for Ceranib-2 was observed (Fig. S7).

As the AC inhibitors reduced neurosphere formation, we examined if **CDy5** exerts adverse effects on the proliferation of neurospheres by culturing neurospheres in the presence of **CDy5**. The numbers of neurospheres grown in the medium containing 2 and 4 μM of **CDy5** were 229 ± 72 and 228 ± 83 which were not significantly different from 189 ± 40 grown in the vehicle-added control group suggesting that **CDy5** does not affect normal proliferation and growth of NSPCs.

AC hydrolyzes ceramide into fatty acid and sphingosine at a pH of ~4.5 and is highly active particularly in the brain and kidney among the organs of a mouse.[12, 16, 17] Deficiency of this enzyme activity causes systemic accumulation of ceramide leading to a lysosomal lipid storage disorder known as Farber disease which involves impairment in cognitive and motor functions.[18, 19] In embryonic development, *Asah1* starts to be expressed from the 2-cell stage, and if the gene is completely knocked out, the 2-cell embryo does not divide but undergoes apoptotic death.[20] On the other hand, more proliferative and drug resistant cancer cells express increased level of AC which has been known to be functionally important for cancer cell proliferation and has hence been proposed as an attractive target for cancer therapy.[21, 22] Our results from single cell gene expression analysis and neurosphere assay with AC inhibitors imply that AC is one of the important molecules highly expressed to render the NSPCs more proliferative in the early stage of neurosphere formation.

In conclusion, we developed a novel fluorescent chemical probe **CDy5** that stains NSPCs in heterogeneous population of cells in a neurosphere by binding to AC which was revealed in this study as an important molecule for NSPC to proliferate and form a neurosphere. Our work described here will provide an invaluable tool to understand mammalian neural development.

We thank Dr. Clement Khaw (SBIC-Nikon Imaging Centre) for help with confocal microscopy. This study was supported by an intramural funding from A*STAR (Agency for Science, Technology and Research, Singapore) Biomedical Research Council and a Singapore Ministry of Education Academic Research Fund Tier 2 (MOE2010-T2-1-025).

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† Electronic Supplementary Information (ESI) available: supplementary figures S1-S7, CDy5 synthetic scheme S1, CDy5 characterization data S1, time-lapse movie S1 and experimental procedures. See DOI: 10.1039/b000000x/

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