

A Novel Morphological Marker for the Analysis of Molecular Activities at the Single-cell Level

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ABSTRACT. For more than a century, hematoxylin and eosin (H&E) staining has been the *de facto* standard for histological studies. Consequently, the legacy of histological knowledge is largely based on H&E staining. Due to the recent advent of multi-photon excitation microscopy, the observation of live tissue is increasingly being used in many research fields. Adoption of this technique has been further accelerated by the development of genetically encoded biosensors for ions and signaling molecules. However, H&E-based histology has not yet begun to fully utilize *in vivo* imaging due to the lack of proper morphological markers. Here, we report a genetically encoded fluorescent marker, NuCyM (Nucleus, Cytosol, and Membrane), which is designed to recapitulate H&E staining patterns *in vivo*. We generated a transgenic mouse line ubiquitously expressing NuCyM by using a ROSA26 bacterial artificial chromosome (BAC) clone. NuCyM evenly marked the plasma membrane, cytoplasm and nucleus in most tissues, yielding H&E staining-like images. In the NuCyM-expressing cells, cell division of a single cell was clearly observed as five basic phases during M phase by three-dimensional imaging. We next crossed NuCyM mice with transgenic mice expressing an ERK biosensor based on the principle of Förster resonance energy transfer (FRET). Using NuCyM, ERK activity in each cell could be extracted from the FRET images. To further accelerate the image analysis, we employed machine learning-based segmentation methods, and thereby automatically quantitated ERK activity in each cell. In conclusion, NuCyM is a versatile cell morphological marker that enables us to grasp histological information as with H&E staining.

Key words: *in vivo* imaging, histology, machine learning, molecular activity

Introduction

Two-photon excitation microscopy (TPEM) allows us to investigate the dynamic behavior of various cells in living mice. For example, labeling of specific types of immune cells has visualized how immune cells communicate with each other in time and space-specific manners (Germain *et al.*, 2012). Meanwhile, the advent of fluorescent protein (FP)-based, i.e., genetically encoded, biosensors for neural

activity has enormously widened the application of TPEM in neuroscience (Ji *et al.*, 2016; Petersen, 2017). Moreover, genetically-encoded biosensors for pH, ions, protein kinase activities, etc. are now being applied to *in vivo* imaging of tumor tissues, opening a new window in cell biology and cancer research (Miller and Weissleder, 2017).

A number of genetically encoded biosensors are based on the principle of Förster resonance energy transfer (FRET) (Aoki *et al.*, 2013; Miyawaki and Niino, 2015; Rodriguez *et al.*, 2017) and have been used in *in vivo* imaging (Hirata and Kiyokawa, 2016). Although development of transgenic mice expressing the FRET biosensor has been a difficult task, in recent years transgenic mice expressing the FRET biosensor, collectively called FRET mice, have been successfully generated either by using transposon-mediated gene transfer or a knock-in strategy. These FRET mice have permitted visualization of the activities of protein kinases and small GTPases (Andrews *et al.*, 2016;

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Abbreviations: H&E, hematoxylin and eosin; BAC, bacterial artificial chromosome; FRET, Förster resonance energy transfer; TPEM, two-photon excitation microscopy; FP, fluorescent protein; WPRE, woodchuck hepatitis virus (WHP) posttranscriptional regulatory element; DCGAN, deep convolutional generative adversarial networks.

Kamioka *et al.*, 2012; Nobis *et al.*, 2017).

In many studies using *in vivo* TPEM, the cells of interest are labeled with fluorescent markers or biosensors expressed from a cell type-specific promoter/enhancer, and the tissue architecture is visualized by second harmonic generation (SHG) signals from collagen fibers. For example, lymphocyte-specific expression of fluorescent proteins has been achieved by using promoters of *lck*, CD2, and CD19 (Maas *et al.*, 1999; Shimizu *et al.*, 2001; Singbartl *et al.*, 2001). However, by this approach, researchers can only see the cells of interest, but not the others, which may influence the biology of the cells of interest. Thus, in the other studies, most, if not all, cells are labeled with ubiquitously expressed markers and distinguished by the characteristic morphology of each cell type (Giel-Moloney *et al.*, 2007; Hasegawa *et al.*, 2013). For this purpose, FP-based markers for the cell membrane and nucleus are developed and expressed in the mice (Abe *et al.*, 2011; Rhee *et al.*, 2006; Shioi *et al.*, 2011). Notably, a number of mouse reporter lines developed by Aizawa and his colleagues utilize the ROSA26 locus for ubiquitous expression and provide researchers the unique opportunity to visualize tissue architecture *in vivo* (Abe *et al.*, 2011; Shioi *et al.*, 2011). Moreover, a T2A or P2A self-cleavable peptide can be used to connect the cell membrane marker and nucleus marker in a single polypeptide, facilitating equimolar expression of the two markers (Chen *et al.*, 2012; Trichas *et al.*, 2008).

Our aim in this study is to visualize the tissue architecture with a fluorescent morphology marker protein and monitor the activities of signaling molecules in FRET mice. Because the majority of the currently available FRET biosensors are comprised of cyan-color fluorescent protein (CFP) and yellow-color fluorescent protein (YFP), the morphology markers should use red to far-red fluorescent proteins. In this study, we generated a transgenic mouse line expressing a novel morphological marker, named NuCyM, which marks the nucleus and plasma membrane with a red-colored fluorescent protein, mCherry (Shaner *et al.*, 2004), and the cytoplasm with a far-red fluorescent protein, iRFP (Filonov *et al.*, 2011). Further, we established a pipeline for the systematic segmentation of individual cells in solid tissues with the help of machine learning. In this way, the activity of ERK MAP kinase in single hepatocytes was quantitatively measured in an unbiased manner.

Materials and Methods

Plasmids

mCherry fluorescent protein (Shaner *et al.*, 2004) and iRFP (Filonov *et al.*, 2011) were employed for the development of a cell morphological marker, NuCyM. The detailed plasmid DNA sequence and map of NuCyM are described in Fig. S1 and S2. iRFP was fused with the nuclear export signal (NES)

(LQLPPLRLTLD) of the HIV-1 rev protein (Fischer *et al.*, 1995; Klemm *et al.*, 1997). mCherry was fused with the human histone protein H1 or the CAAX domain of the KRas protein (a.a. 170–189). To construct a polycistronic vector expressing iRFP-NES, Histone-H1-mCherry, and mCherry-KRasCAAX, these cDNAs were connected with cDNAs of the self-cleaving P2A peptide (Kim *et al.*, 2011).

Cells and reagents

MDCK cells were purchased from the RIKEN BioResource Center (no. RCB0995), and maintained in MEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS), 1% non-essential amino acids (Life Technologies), 1% GlutaMAX Supplement (Life Technologies) and 1 mM sodium pyruvate (Life Technologies) at 37 degrees c, 5% CO₂. PD0325901 was purchased from Calbiochem (San Diego, CA). For immunoblotting analysis, anti-mCherry (#ab167453; Cambridge, UK) and anti-pan ERK (#610123; BD Biosciences, Bedford, MA) were applied as primary antibodies. Signals were detected with anti-rabbit or anti-mouse secondary IgG antibodies (#926-68021 and #926-32212; Li-COR Biosciences, Lincoln, NE).

Establishment of cell lines

To obtain MDCK cells expressing NuCyM, cDNAs of NuCyM were inserted into a pT2Apuro vector, which consisted of a Tol2 transposon element (Kawakami and Noda, 2004), a CAG promoter (Niwa *et al.*, 1991), and IRES-*pac* (a puromycin-resistance gene). MDCK cells were co-transfected with a pT2Apuro-NuCyM vector and pCAGGS-T2TP by electroporation and selected in the presence of 2 µg/ml puromycin.

Imaging of MDCK cells

MDCK cells expressing NuCyM were plated on 35-mm glass-based dishes (Asahi Techno Glass, Tokyo) with Medium 199 (Sigma-Aldrich, St. Louis, MO) supplemented with 20 mM HEPES and 0.1% bovine serum albumin. Cells were imaged with an IX83 inverted microscope (Olympus, Tokyo, Japan) equipped with a PlanApo 60x/1.40 oil objective lens, a Cool SNAP K4 CCD camera (Roper Scientific, Tucson, AZ), a CoolLED precis-Excite LED illumination system (Molecular Devices, Sunnyvale, CA), an IX2-ZDC laser-based autofocus system (Olympus), and an MD-XY30100T-Meta automatically programmable XY stage (SIGMA KOKI, Tokyo). The filters and dichroic mirrors used for imaging were as follows: for mCherry, a 575/25 excitation filter (Semrock, Rochester, NY), a U-MREF glass reflector (Olympus), and an FF01-624/40-25 emission filter (Semrock); for iRFP, a 632/22 excitation filter (Semrock), a U-MREF glass reflector (Olympus), and an FF01-692/40-25 emission filter (Semrock). The microscope was controlled by MetaMorph software (Molecular Devices).

Three-dimensional culture and time-lapse FRET imaging

MDCK cysts were generated as previously described (Martin-Belmonte *et al.*, 2007; Pollack *et al.*, 1998; Yagi *et al.*, 2012). Briefly, 7.5×10^3 MDCK cells were placed on a glass coverslip (13 mm in diameter) coated with 80 μ l of polymerized Matrigel (BD Biosciences), and then supplied with culture medium containing 2% Matrigel. For time-lapse imaging, 4 to 6-day-old cysts were incubated with 1.25 mM EDTA/PBS at 37 degrees c for 5 to 10 min to depolymerize the Matrigel, followed by washing three times with PBS. The cysts were centrifuged and suspended in 100 μ l of collagen solution containing 66% collagen gel (Cellmatrix Type I-A; Nitta Gelatin, Osaka, Japan), 24% reconstitution buffer (Nitta Gelatin), and 10% 10x culture medium. Cysts suspended in the collagen solution were placed into a 15-mm diameter glass-bottom well in a 35-mm diameter plastic dish, and the collagen was allowed to polymerize at 37 degrees c for 20 min. Cysts were imaged with an LCV110-MPE incubator microscope (Olympus) equipped with a 25x/1.05 water-immersion objective lens (XLPLN 25XWMP2; Olympus), and an InSight DeepSee Laser (Spectra Physics, Mountain View, CA). The excitation wavelengths for mCherry and iRFP were 1040 nm and 1300 nm, respectively. The filters and dichroic mirrors used for imaging were as follows: an IR-cut filter (BA685RIF-3) and a DM505 dichroic mirror (Olympus).

Transgenic mice

For generating the transgenic mice expressing NuCyM, the BAC containing the murine ROSA26 locus was employed (Giel-Moloney *et al.*, 2007). Briefly, cDNA of NuCyM was subcloned into a shuttle vector including an insulator (Ottaviani *et al.*, 2009), CAG promoter, and WPRE (Klein *et al.*, 2006). The shuttle vector was introduced into the SW102 strain (Lee *et al.*, 2001; Warming *et al.*, 2005) with a BAC clone retaining RP23-401D9. Purified BAC plasmid was injected into fertilized eggs obtained from C57BL/6J mice. Founder animals were bred with Jcl:ICR mice to produce stable lines. Mice of the resulting mouse line, NuCyM, were then crossed with B6N-Tyrc-Brd/BrdCrCrl mice, also known as B6 Albino mice (Japan SLC, Hamamatsu, Japan), for more than four generations. Newborn mice were illuminated with a green flashlight LED530-3W (Optocode, Tokyo) and inspected for red fluorescence through red-colored glasses. Transgenic mice expressing the FRET biosensor for ERK activity have been reported previously (Komatsu *et al.*, 2018). The transgenic mice were backcrossed for more than ten generations with C57BL/6 mice. Newborn mice were illuminated with a blue flashlight LEDGFP-3W (Optocode) and inspected for green fluorescence through yellow-colored glasses. Mice were housed in a specific-pathogen-free facility in temperature-controlled rooms with a 14 h light/10 h dark cycle, and received a routine chow diet and water *ad libitum*. To date, no disease or anomaly has been associated with the expression of NuCyM or the FRET biosensor used in this study. For imaging, 8 to 30-week-old male or female mice were

used. The animal protocols were reviewed and approved by the Animal Care and Use Committee of the Kyoto University Graduate School of Medicine (No. 10584).

Immunoblotting

Liver, pancreas, and lung tissues were lysed in lysis buffer [50 mM Tris-HCL (pH 7.5), 150 mM NaCl (Nacalai tesque, Tokyo, Japan), 1% Triton X-100 (Nacalai tesque), 1 mM EDTA (Dojindo, Kumamoto, Japan), 2 mM DTT (Wako Pure Chemical Industries, Osaka, Japan), 0.5 mM PMSF (Sigma-Aldrich), 4 μ g/ml leupeptin (Sigma-Aldrich), 10 μ g/ml aprotinin (Sigma-Aldrich), 50 mM NaF (Kanto Chemical, Tokyo, Japan), and 2 mM Na_3VO_4 (Sigma-Aldrich)] by homogenizing with a Bioruptor USD-200TM homogenizer (Cosmo Bio, Tokyo) 8 times for 30 sec each. Insoluble material was removed by centrifugation at 15000 rpm for 10 min at 4 degrees c. The supernatant was resolved by SDS-PAGE on SuperSep Ace 5–20% precast gels (Wako), and transferred to PVDF membranes (Merck Millipore, Darmstadt, Germany). The membranes were incubated for 2 h at room temperature with primary antibodies. The immunoreactivities were visualized with secondary IgG antibodies. All antibodies were diluted in milk. Proteins were detected by an Odyssey Infrared Imaging System (LI-COR Biosciences) and analyzed using the Odyssey imaging software.

Tissue-clearing and imaging

ScaleCUBIC-1 (reagent-1A) was prepared as a mixture of 10 wt% urea (Nacalai Tesque), 5 wt% N,N,N',N'-tetrakis (2-hydroxypropyl) ethylenediamine (Tokyo Chemical Industry, Tokyo), 10 wt% Triton X-100 (Nacalai Tesque), and 25 mM NaCl (Nacalai Tesque) (Susaki and Ueda, 2016). ScaleCUBIC-2 (reagent 2) was prepared as a mixture of 50 wt% sucrose (Nacalai Tesque), 25 wt % urea, 10 wt% 2,2',2''-nitrilotriethanol (Wako), and 0.1% (v/v) Triton X-100 (Susaki *et al.*, 2014). A 10-week-old mouse was perfused with 20 ml 4% PFA in PBS via the left ventricle. Tissues of perfused NuCyM mice were excised and fixed with 4% PFA in PBS for 16 h at 4 degrees c. The fixed organs were immersed in CUBIC-1 reagent for 7 days and then further immersed in CUBIC-2 reagent. Images of cleared organs were acquired with a Zeiss Lightsheet Z.1 microscope equipped with a single side light sheet and two pairs of lenses: an LD Plan-Apochromat 20x/1.0 detection objective lens and LSFM clearing 10x/0.2 illumination objective lens, and an EC Plan-Neofluar 5x/0.16 detection objective lens and LSFM clearing 5x/0.1 illumination objective lens. The excitation wavelength for mCherry was 561 nm. The light sheet thickness was 2 to 6 μ m. A laser blocking filter, LBF 405/488/561/640, a secondary beam splitter, SBS LP640, and an emission filter, BP 575-615, were used. Images were saved in the multilayer 16-bit tagged image file format. ZEN software (Zeiss, Oberkochen, Germany) was used to control the microscope and to acquire images. A three-dimensional image of a cleared brain was acquired with MVX10-LSFM (Olympus, Tokyo) equipped with a single side light sheet and a 1x objective lens (MV PLAPO 1X;

Olympus). The excitation wavelength for mCherry was 561 nm. A dichroic mirror, DM595 (Olympus), and an emission filter, BA 600-690 (Olympus), were used. MVX10-LS-SW software (Olympus) was used to control the microscope and to acquire images. Samples were immersed in a 7:3 mixture of silicon oil TSF4300 (Momentive Performance Materials Japan, Tokyo) and mineral oil (Sigma-Aldrich) during image acquisition.

Image processing of LightSheet images

To generate clear images, raw images acquired by LightSheet microscopy were filtered with CLAHE (enhance local contrast) by ImageJ.

TPEM and image processing

We used an FV1000MPE-BX61WI upright microscope (Olympus) equipped with a 25x/1.05 water-immersion objective lens (XLPLN 25XWMP; Olympus), and an InSight DeepSee Ultrafast laser (0.95 W at 900 nm; Spectra Physics, Mountain View, CA). The excitation wavelength for cyan fluorescent protein (CFP) was 840 nm and that for mCherry was 1040 nm. An IR-cut filter, BA685RIF-3, three dichroic mirrors, DM450, DM505 and DM570, and four emission filters, FF01-425/30 (Semrock) for the second harmonic generation (SHG), BA460-500 (Olympus) for CFP, BA520-560 (Olympus) for YFP by FRET, and 645/60 (Chroma Technology, Bellows Falls, VT) for mCherry, were used.

The microscope was equipped with a two-channel GaAsP detector unit and two multi alkali detectors. FluoView software (Olympus) was used to control the microscope and to acquire images, which were saved in the multilayer 16-bit tagged image file format. Metamorph software was used for background noise subtraction and image analysis. After background subtraction, the FRET/CFP ratio images were represented in the intensity-modulated display (IMD) mode. In the IMD mode, eight colors from red to blue are used to represent the FRET/CFP ratio, with the intensity of each color indicating the mean intensity of FRET and CFP channels.

Preparation of mice for intravital imaging

Mice were anesthetized with 1.0 to 1.5% isoflurane (Abbott Laboratories, North Chicago, IL) inhalation and placed in the prone or supine position for imaging of tissues on an electric heat pad maintained at 37 degrees c. Imaging of ear skin was performed as previously described (Hiratsuka *et al.*, 2015). Briefly, the mouse ear was sandwiched between a cover glass and a thermal conductive silicon gum sheet. Hairs were removed by depilation cream 24 h before the experiments. For liver and pancreas imaging, each tissue was pulled out of the abdominal cavity through an abdominal incision and immobilized on a custom-made vacuum-stabilized imaging window (Olympus Engineering, Tokyo), which was connected to a vacuum aspirator and designed to continuously pull on the tissue. The suction force was set to approximately 20 kPa, and the blood flow in the liver was not detectably

affected under this condition. The bowl of the imaging window was warmed to 37.5 degrees c with a lens heater (TP-LH; Tokai Hit, Shizuoka, Japan). In some experiments, 5 mg/kg MEK inhibitor PD0325901 in PBS solution was injected intravenously.

Segmentation of the plasma membrane by deep-neural networks

Segmentation of NuCyM images by deep-neural networks has been reported by several groups (Isola *et al.*, 2016; Murata *et al.*, 2018; Sato *et al.*, 2018). In the present study, we tested three different deep-neural networks, U-Net (Ronneberger *et al.*, 2015), branched U-Net, and pix2pix. The advantage of U-Net is that it integrates the features at shallow layers and at deep layers, and features which are lost by convolution are used at deeper layers effectively. In the case of the branched U-Net, the decoder part is divided into three branches. Each branch has a unique role that generates a probability map for the cell nucleus or cell membrane, or background from the feature maps obtained by the encoder. Pix2pix, an improved method of deep convolutional generative adversarial networks (DCGAN), uses two networks: a generator and discriminator. The generator makes a segmentation result from an input image and the discriminator classifies whether the generated segmentation result is real or fake.

Image-processing filters

Two morphological filters were used to stitch the missing membrane information in the automated images. One was a dilate filter, which grows membrane lines in a binary image, and has a circular shape with a diameter of 6 pixels. The other was a watershed lines filter, which segments objects that are touching one another. The filter size was defined as between 5% to 95% of the size of the region in ground truth images. Automated regions were sorted within the range from 290 to 1290 pixels. The nucleus filter was defined by the size of the nucleus generated by a machine learning process. We regarded a nucleus of size 33 to 433 pixels as correct based on the ground truth images. Metamorph software was used for image processing with two morphological filters and calculation of the area size.

Results

NuCyM visualizes cell morphology in three-dimensional imaging

H&E staining-based histology provides invaluable information across a wide array of biological fields. In order to recapitulate the H&E staining-like images in living animals, we generated a fluorescent marker for labeling of the nucleus, cytoplasm, and plasma membrane (Fig. 1A). The marker is comprised of an infrared fluorescent protein, iRFP, fused with a nuclear export signal (NES), a red fluorescent protein, mCherry, fused with Histone H1, and

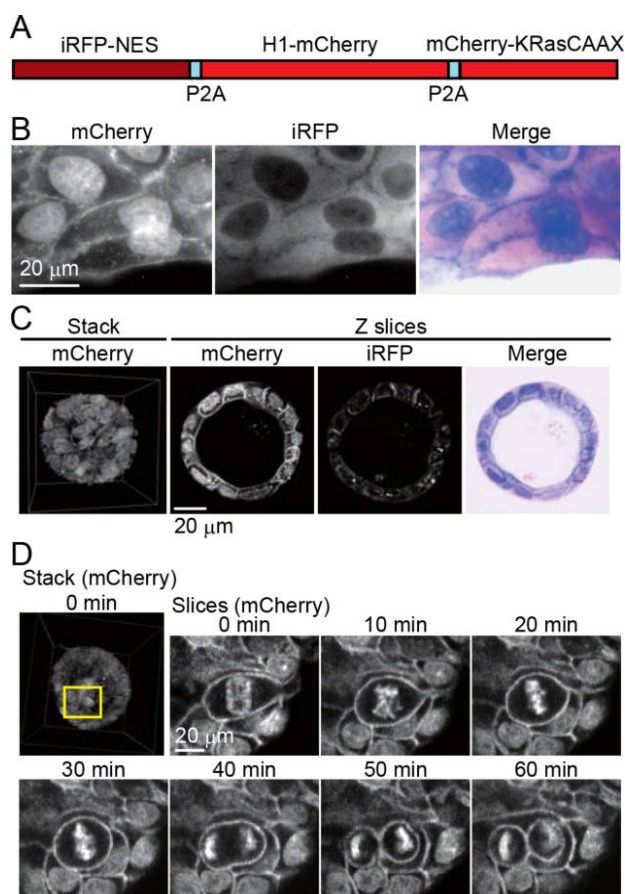


Fig. 1. A cell morphological marker, NuCyM, provides information of cell morphology in three-dimensional imaging. (A) The structure of the cell morphological marker, NuCyM, is shown. (B) MDCK cells were transfected with the plasmid of NuCyM and acquired images. Scale bar, 20 μ m. (C and D) MDCK cells stably expressing NuCyM were cultured in Matrigel for 4 days and acquired z stack images. An XYZ image of the mCherry signal (left) and z planes (right) are shown (C). Representative images at the indicated time points are also shown (D).

mCherry fused with the CAAX domain of KRas. These three fusion proteins were linked with P2A self-cleaving peptides. We named this marker NuCyM (Nucleus, Cytosol, and Membrane). To validate its expression and localization, NuCyM was expressed in MDCK cells (Fig. 1B). As expected, mCherry was detected at the nucleus and plasma membrane, and iRFP was detected at the cytoplasm. An overlay image with pseudocolors generated images similar to those by H&E staining. Our aim in developing NuCyM is to examine the cell morphology in three dimensions. To demonstrate this possibility, we examined the morphological changes of MDCK cells that formed cysts in collagen gel (Fig. 1C). MDCK cells expressing NuCyM revealed the structure of each cell even in the three-dimensional objects, supporting the notion that NuCyM is a helpful tool for extracting histological information in living

animals. To further investigate the potential of NuCyM, we attempted to visualize the cell morphology during mitosis (Fig. 1D). With the NuCyM image, a cell was clearly identified in five basic phases during mitosis: prophase, prometaphase, metaphase, anaphase, and telophase. These results indicate that NuCyM is a powerful tool for visualizing cell morphology via three-dimensional imaging.

The cellular architecture can be visualized in NuCyM mice

After the preliminary analysis of MDCK cells by epifluorescence microscopy, we next validated whether NuCyM could be applicable for *in vivo* imaging by TPEM. For our purpose to use NuCyM as an alternative to H&E staining, the expression of NuCyM should be ubiquitous in all of the cell types. Thus, the expression unit of NuCyM was inserted into the ROSA26 locus of a BAC (Fig. 2A). The expression unit was comprised of the NuCyM-coding sequence, CAG promoter, polyA sequence of SV40, D4Z4 insulator, and WPRE sequence. The recombinant BAC DNA was injected into mouse oocytes to obtain transgenic mouse lines. We established three independent transgenic mouse lines expressing NuCyM. Mice expressing NuCyM, named NuCyM mice, were born at the expected Mendelian frequency and displayed no overt phenotype, with normal development and fertility. In a preliminary screening, the pattern of NuCyM expression did not change significantly in any of the three lines; therefore, we chose a mouse line with the highest NuCyM expression in the skin for further analysis.

Under a two-photon excitation microscope, NuCyM was expressed ubiquitously in mouse tissues and could be readily used to draw the histological architecture by live imaging (Fig. 2B and D). As expected, mCherry was detected at the nucleus and plasma membrane. On the other hand, in the iRFP channel, signals were detected not only in the cytoplasm but also in the nucleus, suggesting insufficient cleavage of the P2A peptide (Fig. 2B). To confirm this, immunoblotting was performed using the tissues of NuCyM mice and HeLa cells as control (Fig. 2C). Some amount of uncleaved products, i.e., iRFP-H1-mCherry-mCherry-CAAX (Cy-Nu-M), H1-mCherry-mCherry-CAAX (Nu-M), and/or iRFP-H1-mCherry (Cy-Nu), was observed in all samples. Cy-Nu was detected more in the pancreas than the other tissues (blue arrowhead), strongly suggesting that Cy-Nu accumulated in the nucleus. This result also suggested that the cleavage efficiency of the P2A peptide depends on the tissue. Moreover, we noticed that the cytoplasmic localization of iRFP did not add much information because the perimeter of the cell was visible by the plasma membrane-targeted mCherry. Therefore, hereafter, we show only the images of mCherry, which marks the nucleus and the plasma membrane. In the liver, the portal veins, hepatic arteries, and hepatic cords were clearly

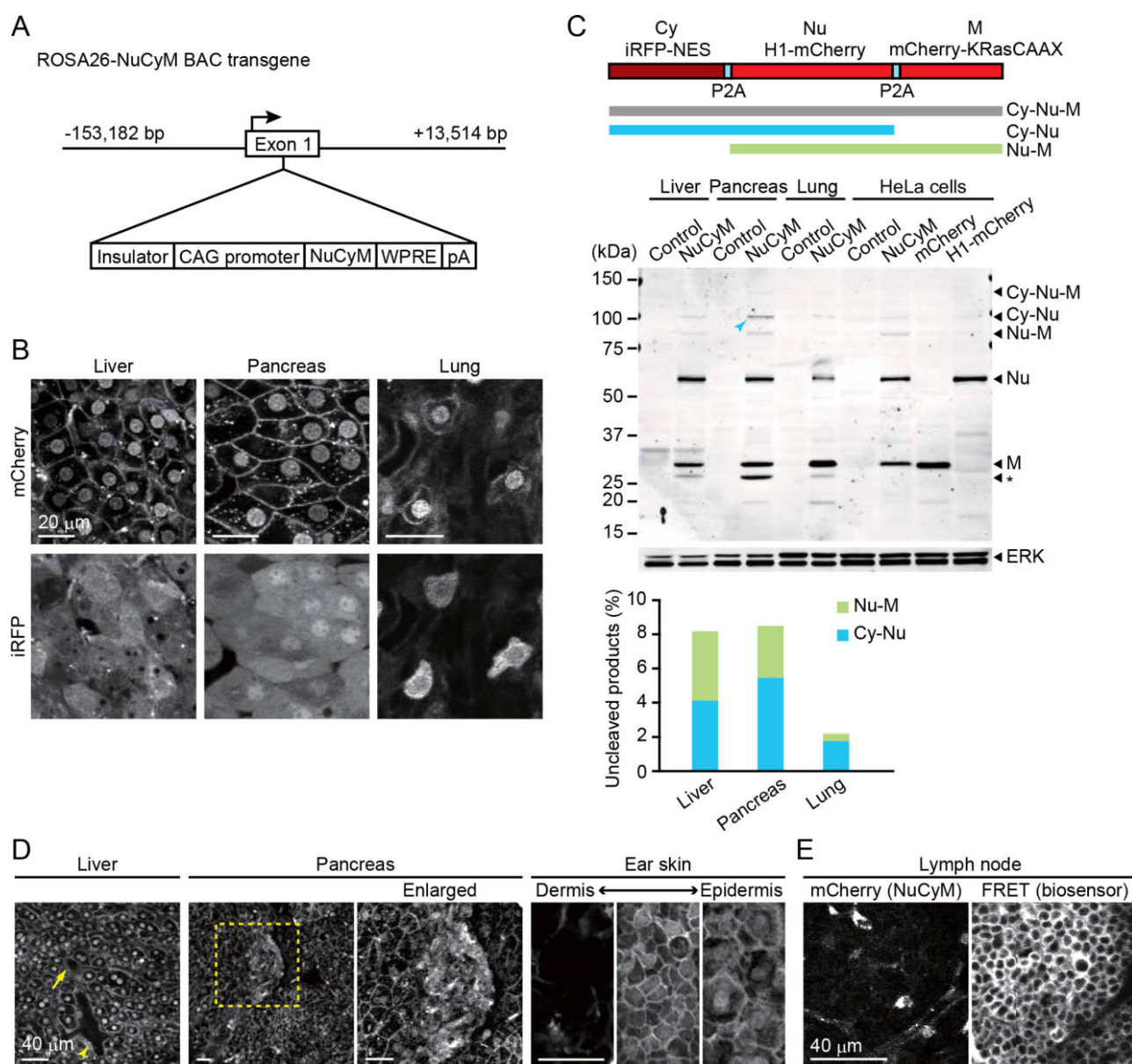
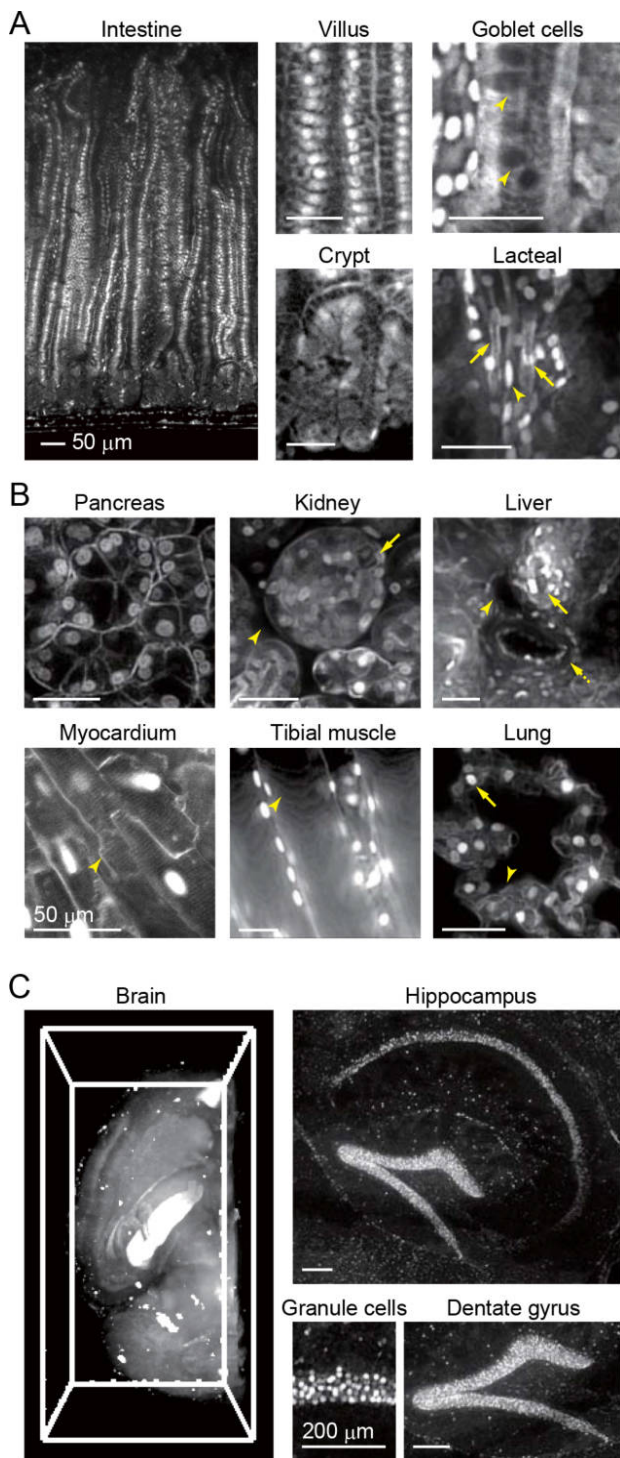


Fig. 2. NuCyM mice enable us to identify individual cells through cell morphology. (A) The structure of the plasmid used to generate NuCyM mice is shown. (B) The liver, pancreas, and lung in a NuCyM mouse were imaged by TPME. Scale bars, 20 μ m. (C) The liver, pancreas, lung, and HeLa cells were analyzed by immunoblotting with antibodies against mCherry and pan ERK. Quantification of the localization patterns of uncleaved products: H1-mCherry-mCherry-CAAX (Nu-M) and iRFP-H1-mCherry (Cy-Nu). An image was subjected to gamma correction 0.6. *: byproduct. (D) The portal vein and hepatic artery are indicated with an arrow and an arrowhead, respectively (left). Islets of Langerhans in the pancreas (center) and from the depth to surface in the auricular epidermis (right) were imaged. The boxed areas in the center panel are enlarged in the right panel. Scale bars, 40 μ m. (E) A lymph node in a TG mouse expressing both NuCyM and the ERK biosensor was imaged with TPME. Scale bar, 40 μ m.

distinguished in living mice. In the pancreas, islets of Langerhans could be identified by the high cell density. In the basal layer of the ear skin, each cell could be clearly distinguished owing to plasma membrane staining, which is usually difficult in H&E staining (Fig. 2D). Against our expectation, NuCyM was not expressed in lymphocytes,

although a FRET biosensor for ERK was expressed by the CAG promoter (Fig. 2E). In conclusion, NuCyM mice provide H&E staining-like images in live imaging of many, but not all, tissues with rooms for future improvement.



NuCyM mice are compatible with the tissue-clearing technique CUBIC

We sought to expand the application of NuCyM mice by the tissue-clearing technique. To this end, we cleared the tissues with the CUBIC reagent and observed several

Fig. 3. Cleared NuCyM mice enable us to identify cell and tissue morphology from the surface to the depth. (A) A cleared intestine of a NuCyM mouse was imaged with LightSheet microscopy and adapted histogram equalization (CLAHE) with ImageJ. The XY image of the jejunum (left) contains almost all the structures of the small intestine: the muscle layer, crypt, and villus. Expanded images of the villus (upper center), crypt (lower center), goblet cells (upper right, arrowheads), and lacteal (lower right, arrows), as well as an individual smooth muscle cell (lower right, an arrowhead) are shown. Scale bars, 50 μ m. (B) Cleared tissues were imaged with LightSheet microscopy. In the pancreas, acinar cells with polarized nuclei are shown. In the kidney, Bowman's capsule (arrowhead) and efferent and afferent arterioles (arrow) are shown. In the liver, the portal region with the vein (arrowhead), bile duct (arrow), and hepatic artery (dotted arrow) is shown. In the myocardium and tibial muscle, cross striations are shown (arrowheads). In the lung, type I (arrowhead) and type II (arrow) pneumocytes are shown. Scale bars, 50 μ m. (C) A cleared brain was imaged with LightSheet microscopy. The XYZ image (left) was constructed with Imaris software. Expanded images of the hippocampus (upper right), granule cells in the CA1 region (lower center), and dentate gyrus (lower right) are shown. Scale bars, 200 μ m. For contrast enhancement, some images were subjected to gamma correction from 0.8 to 2.0.

organs under light-sheet microscopes. In the small intestine, cells within villi and crypts were clearly identified with a single cell resolution (Fig. 3A). Moreover, goblet cells could be observed based on their characteristic morphology. In H&E staining, the central lacteal is usually difficult to identify because plasma membrane could not be detected by this method. In contrast, the membrane localization of NuCyM allowed us to identify the luminal space of the central lacteal.

To demonstrate a further advantage of NuCyM, we tried to identify a distinctive structure related to histological analysis (Fig. 3B). In the pancreas, acinar cells lining an acinus were identified and showed polarized nuclei. In the kidney, a glomerulus was identified as a ball-like structure with Bowman's capsule. The efferent arterioles and the afferent arterioles were also identified with endothelial cells. In the liver, the portal area—including the portal vein, hepatic artery, and bile duct—was recognized. In muscle, the tibialis anterior muscle and the myocardium were analyzed. Both muscles showed cross striation, probably due to localization of mCherry-CAAX to the endomembranes. The tibialis anterior muscle showed multiple flattened nuclei at the periphery of myofibers, whereas the myocardium showed one central nucleus in each cardiac cell. In the lung, type I and type II pneumocytes in alveoli were recognized by referring to the nuclear size and shape. Based on H&E staining, the type I pneumocytes showed a flatter nucleus, whereas the type II pneumocytes showed a round nucleus. Also, in a cleared brain, the structures of the whole brain were identified (Fig. 3C). Under this condition, granule cells and the dentate gyrus in the hippocampus were observed at the single-cell level, although membrane signals of NuCyM were not observed in granule cells. Collectively, almost all of these findings represent advantages of NuCyM mice for histology.

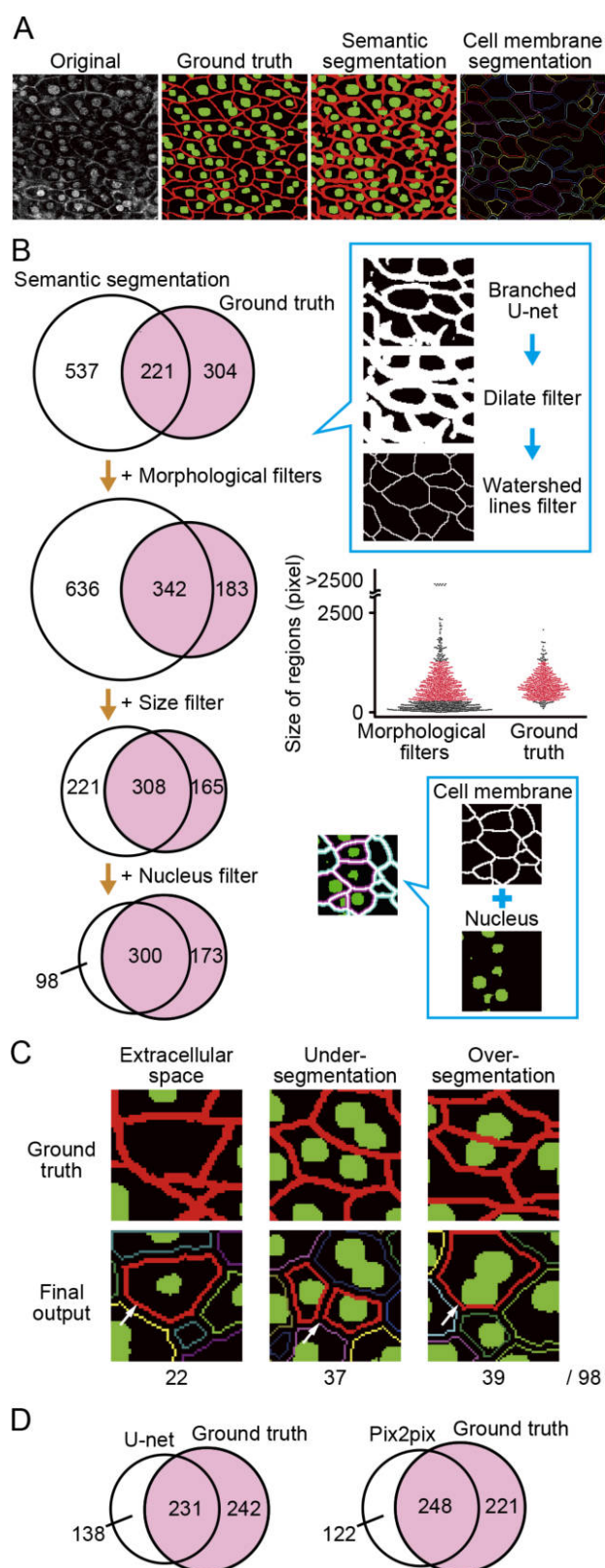


Fig. 4. Deep learning-based algorithms delineate the cell membrane in NuCyM images. (A) On the fluorescent images of the liver of NuCyM mice (left), the cell membrane and nucleus were manually traced with red lines and green area, respectively, for the preparation of ground truth images (left middle). After training, NuCyM images were segmented by the branched U-net method to generate the output images of semantic segmentation (right middle). On the output images, regions corresponding to single cells were generated by using morphological and watershed filters (right). (B) Overview of the procedure. Left, the results of visual inspection of the overlapped regions are shown in Venn graphs. Right, the output images of semantic segmentation were first processed with two morphological filters, a dilate filter and watershed lines filter (top). Next, outliers in the size were eliminated by a size filter. Finally, regions without a nucleus were eliminated to generate the final regions corresponding to single cells. (C) Three types of false-positive regions were eliminated by the last step. Left, a region without a nucleus. Center, a binuclear cell divided into two regions. Right, failure to divide two cells due to overlapped nucleus images. (D) Images were processed similarly except that U-net or pix2pix was used for the semantic segmentation.

Machine learning-based algorithms delineate the cellular perimeter in NuCyM images

An object of NuCyM development is to provide a tool to study cell-to-cell heterogeneity in the signaling of molecular activities. For this purpose, automatic segmentation of a cell boundary is essential to accumulate data from a number of cells. We attempted this task by adding liver images as an example. Even though experienced biologists could easily distinguish hepatocytes in the images, simple binarization of the images failed to delineate the cell boundary, partly because NuCyM at the plasma membrane did not necessarily distribute evenly on the plasma membrane, and partly because signals of the nuclear NuCyM could not be separated from that at the plasma membrane (Fig. S3A).

To overcome this problem, we recently developed a method called branched U-Net to segregate the plasma membrane and nucleus (Murata *et al.*, 2018). Briefly, in 50 fluorescent images of the liver of NuCyM mice (Fig. 4A, left), the plasma membrane and nucleus were traced manually to generate “ground truth” images (Fig. 4A, left middle panel). The ground truth images were then fed into the encoder part of the branched U-Net to yield output images (Fig. 4A, right middle panel). Because only 221 of the 758 regions identified in the output images overlapped the cells in the ground truth images, we processed the output images sequentially with dilate and watershed lines filters (Fig. 4A, right panel; Fig. 4B, right upper panel). These morphological filters increased the sensitivity from 221/525 to 342/525. Because the output images contained many over-segmented cells and sinusoids, the regions were filtered by the pixel size; i.e., sizes above and below 5% were eliminated (Fig. 4B, middle panel). To further eliminate blood vessels and sinusoids, regions without a nucleus described by machine learning-based algorithms were eliminated (Fig. 4B, lower panel). In this way, the branched U-net method

with morphological filters achieved 63% sensitivity (300/473) and 75% specificity (300/398). Notably, the 98 false-positive regions were classified into regions without a nucleus (22/98), fragmented regions of multi-nuclear cells (37/98), and regions containing multiple cells (39/98) (Fig. 4C). If we assume that regions without a nucleus are the true negative regions, then the specificity is increased to 94% (376/398). We also tested two different machine learning algorithms based on U-Net, the original U-Net and the pix2pix as described in the Methods section (Fig. 4D). The original U-Net showed 49% sensitivity (231/473) and 63% specificity (231/369). The pix2pix showed 53% sensitivity (248/469) and 67% specificity (248/370). Thus, we concluded that the branched U-Net was appropriate for our experimental conditions.

Automatic segmentation enables us to quantify heterogeneity in the ERK activity of hepatocytes

Finally, as a proof of concept, we quantified ERK activity in hepatocytes by using transgenic mice expressing NuCyM and the ERK FRET biosensor. The FRET biosensor is comprised of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) and the FRET signal is correlated with ERK activity within the cells (Komatsu *et al.*, 2011). For the measurement of ERK activity with the FRET biosensor, fluorescence images of CFP and YFP were acquired to quantify FRET. We used the YFP fluorescence image as the FRET image because YFP fluorescence is primarily evoked by FRET.

The transgenic mice were anesthetized and observed under a two-photon excitation microscope to acquire images of CFP, FRET, and mCherry (Fig. 5A). During imaging, the mice were inoculated with a MEK inhibitor. As expected, the FRET ratio in hepatocytes was decreased by the MEK inhibitor. We then segmented the CFP and YFP images using the NuCyM image and branched U-Net. The segmented regions were used to calculate the FRET/CFP ratio in each cell (Fig. 5B). The FRET ratio in hepatocytes expressing the ERK biosensor was decreased by the MEK inhibitor in single cell analysis. In summary, the application of machine learning to mice expressing NuCyM and the FRET biosensor enable us to analyze molecular activities systematically.

Discussion

The aim of this study was to develop fluorescent morphological markers that will facilitate quantitative analysis of cytoplasmic biosensors by TPTEM. A number of FP-tagged markers for organelles have already been developed (Abe *et al.*, 2011; Shioi *et al.*, 2011). Among them, markers for the plasma membrane and nucleus are particularly useful for identifying individual cells and cell boundaries (Abe *et*

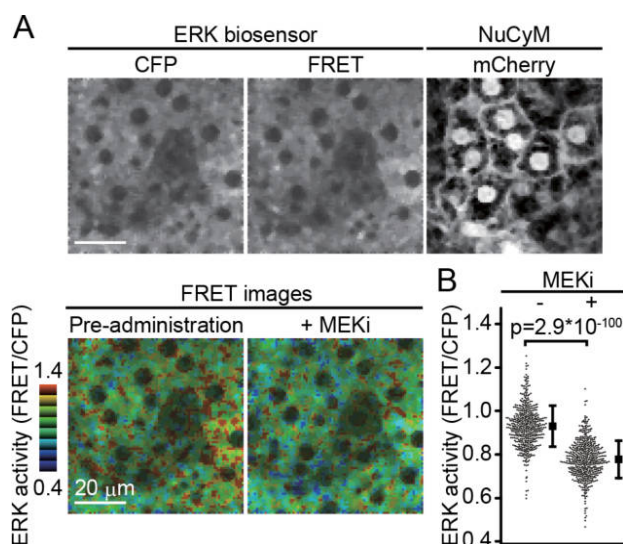


Fig. 5. A deep learning-based algorithm enables us to analyze the heterogeneity of molecular activities in hepatocytes. The livers of the transgenic mice expressing both NuCyM and ERK biosensor were observed by TPTEM. CFP (upper left), FRET (upper center), and NuCyM (upper right) images were acquired simultaneously. (A) Representative FRET ratio images of before (lower left) and 30 min after (lower right) the administration of MEK inhibitor PD0325901 (5 mg/kg) are shown in the intensity-modulated display mode with the ratio range of 0.4 to 1.4. A NuCyM image of the same viewfield is also shown. Scale bar, 20 μm. (B) On the NuCyM images, regions for single cells were generated by the branched U-net method. The regions were transferred to CFP and FRET images to calculate the ratios (FRET/CFP), which are shown in beeswarm plots. Statistical analysis was performed by Mann-Whitney U test. Solid squares and error bars represent means and SDs.

et al., 2011; Choy *et al.*, 1999; Rhee *et al.*, 2006; Shioi *et al.*, 2011). For simultaneous labeling of the nucleus and plasma membrane, Trichas *et al.* developed a self-cleavable fusion protein comprised of a nuclear marker, H2B-GFP, and a plasma membrane marker, myristoylated tdTomato (Trichas *et al.*, 2008). Similarly, Chen *et al.* developed a self-cleavable fusion protein comprised of H2B-EGFP and GPI-anchored mCherry (Chen *et al.*, 2012). However, these fusion proteins are not compatible with many FRET biosensors that utilize CFP and YFP as the FRET pair, because precedent markers use the green-to-yellow fluorescence range. In this study, we developed a new morphological marker, NuCyM, comprised of mCherry and iRFP, which is compatible with FRET biosensors carrying CFP and YFP as the FRET pair (Fig. 5). On the other hand, in some tissues of transgenic mice expressing NuCyM, an iRFP signal was detected not only in the cytoplasm but also in the nucleus (Fig. 2B), due to insufficient cleavage of the P2A peptide (Fig. 2C). Although iRFP fluorescence showed unexpected localization in the nucleus in some tissues of transgenic mice, the nucleus and plasma membrane with mCherry fluorescence were clearly visualized in three-

dimensional culture or transgenic mice under two-photon microscopes (Fig. 1; Fig. 2).

For the ubiquitous expression, the cDNA of NuCyM was inserted into a ROSA26 BAC clone (Giel-Moloney *et al.*, 2007) and expressed from the CAG promoter (Fig. 2A). Giel-Moloney showed that this strategy increased the expression of EGFP in lymphocytes more than 10-fold in comparison to the use of transgenic mice generated by injection of the expression cassette CAGGS-EGFP (Giel-Moloney *et al.*, 2007). However, against our expectation, the expression level of NuCyM in lymphocytes was not sufficient for TPME (Fig. 2E). Another drawback of this probe is that the relative intensity of the nucleus and membrane can vary among cell types. For example, the cell membrane of nerve cells is hardly visible, probably due to the high membrane-to-nucleus volume ratio. Therefore, equimolar expression of the three markers by NuCyM is not necessarily beneficial depending on the cell types.

In our preliminary analyses with morphological filters, which were developed primarily for the 2D tissue culture images, the segmentation of individual cells of NuCyM mice was not satisfactory (Fig. S3A). On the other hand, semantic segmentation of cell membranes by deep learning has been employed for the diagnosis of HER2-positive mammary cancers (Saha and Chakraborty, 2018). Therefore, we applied three deep learning-based methods, U-net, branched U-net, and pix2pix, for the semantic segmentation of NuCyM images, and found that the branched U-net method showed the best performance (Fig. 4B; Fig. 4D) (Murata *et al.*, 2018). The branched U-net method uses three branches corresponding to the cell membrane, the nucleus, and the background for the generation of output images; therefore, this method fully takes advantage of the NuCyM, which stains the nucleus, cell membrane and cytoplasm.

It should be noted that the method for the semantic segmentation still has room for improvement (Fig. 4). It appeared that uneven or discontinuous distribution of KRas-CAAX-tagged mCherry to the plasma membrane caused under-segmentation (Fig. S3B). The size of the membrane gap is more than 500 nm, negating the possibility that the gap is the raft domain (Pham *et al.*, 2015; Yuan *et al.*, 2002). The distribution of the native H-, N-, and K-Ras is also known to be uneven on the cell membrane (Choy *et al.*, 1999). Abe *et al.* previously compared the distribution of the membrane markers lyn-EGFP, EGFP-KRas, GAP43-EGFP, Display-Venus, and Display-mCherry, and concluded that lyn-EGFP performed best (Abe *et al.*, 2011). We previously compared the ROCK biosensor tagged with the myristoylation signal of lyn, the KRas CAAX domain, and the HRas CAAX domain and found that the KRas CAAX domain worked best (Li *et al.*, 2017). We need to further explore the best membrane marker for the *in vivo* imaging.

Another possibility to improve the accuracy of the

semantic segmentation is to reduce the motion artifacts during *in vivo* imaging. The motion artifacts caused by respiration could be overcome by developing the vacuum-stabilized imaging window or similar physical immobilization apparatus. Moreover, motion compensation strategies can be also applied to intravital imaging. The combination of these methods will help us to obtain and analyze images in living mice.

In summary, we generated NuCyM, a novel morphological marker compatible with CFP/YFP-type FRET biosensors, and transgenic mouse lines. Further, the quantification of signaling molecule activities can be accelerated by applying deep learning-based semantic segmentation methods to the NuCyM images.

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