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Application of POLARIC™ fluorophores in an *in vivo* tumor model

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Abstract. Fluorescent and luminescent tools are commonly used to study the dynamics of cancer progression and metastases in real-time. Fluorophores have become essential tools to study biological events. However, few can sustain fluorescence long enough during long-term studies. In the present study, we focused on a series of new amphiphilic fluorophores known as POLARIC™, which emit strong fluorescence in lipid bilayers and can be readily modified using the Suzuki-Miyaura cross-coupling reaction. Appropriate chemical modifications of substituent groups can improve target-site specificity, reduce cytotoxicity and prolong emission. Therefore, in contrast to conventional fluorescent probes, these fluorophores show promise for long-term monitoring of biological processes. In the present study, we conducted long-term observations of tumor growth and metastasis using a POLARIC derivative as a novel fluorescent probe. For this purpose, we studied the metastatic melanoma cell line A375-SM, which proliferates at a high rate. We compared the characteristics of the POLARIC probe with the commercially available fluorescent dye PKH26 and fluorescent protein mRFP1. A375-SM cells were labeled with these fluorescent probes and orthotopically implanted into nude mice. The fluorescence emitted by POLARIC was detected more than five weeks after implantation without causing detectable harmful effects on tumor growth. By contrast, fluorescence of cells labeled with PKH26 could not be detected at this same time. Furthermore, POLARIC-, but not PKH26-labeled cells, were also detected in lung metastases. These results indicate that labeling cells with POLARIC fluorophores can significantly extend the time course of *in vivo* studies on tumor cell growth.

Introduction

Fluorescent and luminescent tools are commonly used to study the dynamics of cancer progression and metastases in real-time. They are widely used to determine the spatio-temporal dynamics of intracellular molecules, organelles, and whole cells as they can provide high resolution images with great sensitivity and without causing significant cytotoxicity (1). Fluorescence-labeled cells, in particular, can be visualized using fluorescence microscopy, flow cytometry, and whole-body imaging techniques (2,3). Fluorescent probes have therefore become an essential tool to study biological events in living cells, tissues and animals (4,5).

An important example of the utility of these techniques is the generation of genetically engineered cell lines that stably express natural fluorescent proteins such as GFP (6,7) introduced using retroviral vectors.

A series of new amphiphilic fluorophores known as POLARIC™ has recently been reported (8,9). This series of fluorophores is now commercially available. These fluorophores provide advantages over other fluorescent dyes, such as strong fluorescence in lipid bilayers, and possess molecular structures that are readily modified using the Suzuki-Miyaura cross-coupling reaction. Thus, appropriate chemical modifications of substituent groups can improve target-site specificity, reduce cytotoxicity, and prolong emission. These fluorophores, therefore, show promise for monitoring long-term biological processes for prolonged periods of time compared with commonly used fluorescent probes such as PKH26 (10,11). In the present study, we described long-term observations of tumor growth and metastasis using a POLARIC derivative.

Materials and methods

Cell line and culture conditions. The super-metastatic human malignant melanoma cell line, A375-SM, was kindly provided by Prof. I.J. Fidler (M.D. Anderson Cancer Center, Houston, TX, USA). The cells were cultured in Minimum Essential Medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

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Fluorescence staining techniques and plasmid transfection. Cells were labelled using POLARIC (POLARIC-500c6F; Goryo Chemical, Inc., Sapporo, Japan) and a PKH26 Red Fluorescent Cell Linker kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The expression vectors for CS-RfA-CMV-m red fluorescent protein 1 (mRFP1) were obtained from Dr Roger Tsien (UCSD, CA, USA). The packaging vector pCAG-HIVgp and the VSV-G- and REV-expressing construct pCMV-VSV-G-RSV-REV were obtained from Dr H. Miyoshi (RIKEN, Tsukuba, Japan). For transfection, expression plasmids were incubated with FuGene HD (Roche, Basel, Switzerland) according to the manufacturer's recommendations. Lentivirus-mediated gene transfer was performed as previously described (12).

Microscopy. Images were acquired using an FV-10i confocal microscope (Olympus, Tokyo, Japan). The 572-nm (PKH) and 520-nm (for POLARIC) emission filter sets were used. To assess bleaching rate, each stained cell was exposed to a 473-nm laser beam at a power setting of 100% output (11.9 mW). Fluorescent intensity was calculated using ImageJ Software (NIH, Bethesda, MD, USA).

Flow cytometry. To assess fluorescence intensity, the cells were trypsinized at 1, 2 and 3 weeks after staining. To evaluate fluorescence intensity of primary tumors or to detect lung metastases, excised tissues were minced and digested with collagenase II (Gibco). Red blood cells were removed by lysing them with Lysis Buffer (BD Biosciences, Franklin Lakes, NJ, USA). Fluorescence intensity was measured using a FACS Aria II (BD Biosciences) with emission at 488 nm as well as 572-nm or 650-nm filter sets. Dead cells were distinguished from viable cells using DAPI (100 ng/ml).

Animal model. The local animal research authorities approved all procedures for animal experimentation. The protocol for animal care was in accordance with institutional guidelines. Six-week-old female nude mice (BALB/c Slc-nu/nu) were obtained from Sankyo Labo Service Corporation (Tokyo, Japan). The A375-SM human melanoma xenograft model was generated by subcutaneous injection of 1×10^6 fluorophore-labeled A375-SM cells.

In vivo fluorescence imaging. *In vivo* fluorescence imaging was performed using an IVIS Spectrum (Caliper Life Science, Hopkinton, MA, USA) on the indicated days. Mice were anesthetized with 2% isoflurane and placed onto the warmed stage inside of the IVIS light-tight chamber and anesthesia was maintained with 1.5% isoflurane. Mice were imaged in ventral positions. Fluorescence signals were acquired using the respective band pass excitation and emission filter sets as follows: POLARIC, 500 nm (30-nm bandwidth) and 620 nm (20-nm bandwidth); PKH, 535 and 580 nm; and mRFP1, 570 and 620 nm. Photon acquisition time was 1 sec, small binning, and F/Stop 4. After the mice were sacrificed, primary tumors and lungs were surgically resected. To examine spontaneous lung metastasis, lungs were subjected to *ex vivo* fluorescent imaging. Fluorescence signals were quantified using Living Image 3.0 (Caliper Life Science). Signal intensity data are expressed as the total photons/s within a uniform region of interest positioned over specific tumor sites.

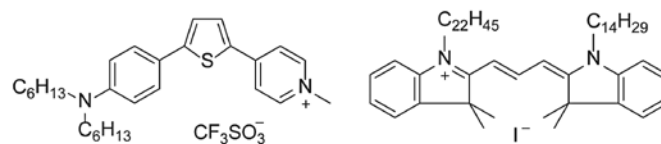


Figure 1. Molecular structure of POLARIC™ (left) and PKH26 (right) probes.

Statistical analysis. Differences among experimental groups were evaluated using the Mann-Whitney U test and $P < 0.01$ was considered to indicate a statistically significant difference.

Results

Selection of a POLARIC derivative suitable for studying tumor cells. The central aromatic moiety of POLARIC derivatives absorbs and emits light. The terminal alkyl chains have essentially no effect on optical properties such as absorption and emission wavelengths. However, the alkyl chains strongly influence the fluorescence intensities in the lipid membrane due to the dispersion of dye (13). We first stained tumor cells using POLARIC derivatives with different alkyl chains and used the brightest derivative, which had two hexyl groups (Fig. 1). Since the dispersed fluorophore suppresses the fluorescence quenching and decomposition reactions, the dihexyl derivative is expected to be suitable for long-term monitoring. We compared the characteristics of the POLARIC probe with those of PKH26 (10,11), which is commercially available from Sigma-Aldrich, and mRFP1, which was introduced using a lentivirus vector as described above.

In vitro characterization of POLARIC and PKH probes. We found that the fluorescence intensity of cells labeled for 12 h with POLARIC was higher than that of cells labeled with PKH after staining for 12 h (Fig. 2A). Next, the stained cells were exposed to a 473-nm laser beam and it was found that the fluorescence decay depended on the frequency of the number of pulses and that the fluorescent decay rate of POLARIC was almost equal to that of PKH (Fig. 2B). We next quantified the fluorescence intensity for each dye every two days in cultures grown to sub-confluence for three weeks (Fig. 2C).

Whole-body imaging of tumors. A375-SM tumor cells labeled with POLARIC or PKH were engrafted subcutaneously into nude mice. As a positive control, cells transfected with RFP inserted into lentivirus were used. Primary tumor lesions labeled with each dye were externally imaged using an IVIS Spectrum on the indicated days after implantation (Fig. 3A). PKH-labeled tumors showed low intensity from the beginning and diminished over time, while fluorescence signals emitted by RFP-labeled tumors increased, even when the tumors grew progressively larger.

The fluorescence intensity of tumors labeled with POLARIC also gradually decreased due to cell division; however, the signal intensity was stronger than that of PKH-labeled tumors as the initial signal was much brighter than that of the PKH-labeled tumors. Therefore, POLARIC-labeled cells could be detected >5 weeks after injection (Fig. 3A and B). The rates of decay in fluorescence were comparable between

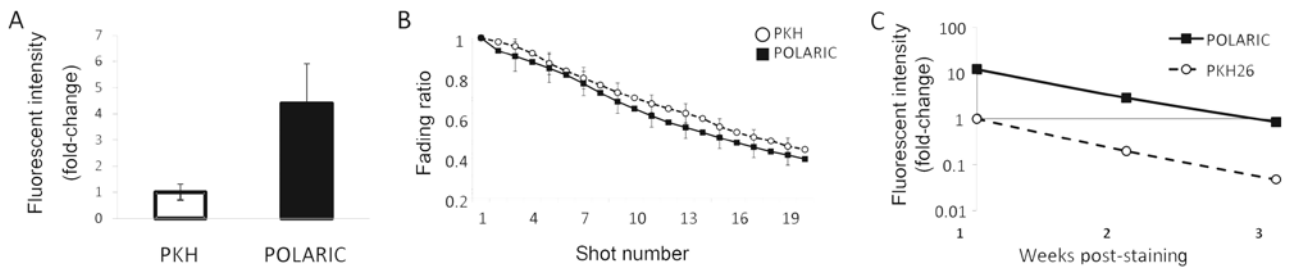


Figure 2. Analysis of cell lines labeled with POLARIC or PKH. (A) The fluorescence intensity of cells labeled with POLARIC or PKH was measured using fluorescence microscopy. (B) Effects of excitation light on fluorescence decay. The decay rates of POLARIC and PKH were nearly equivalent and depended on the number of pulses. (C) Fluorescence intensities of cells labeled with each dye were measured using flow cytometry ~1, 2 and 3 weeks after staining. The intensities gradually decreased, but no significant differences were observed between cells labeled with POLARIC or PKH.

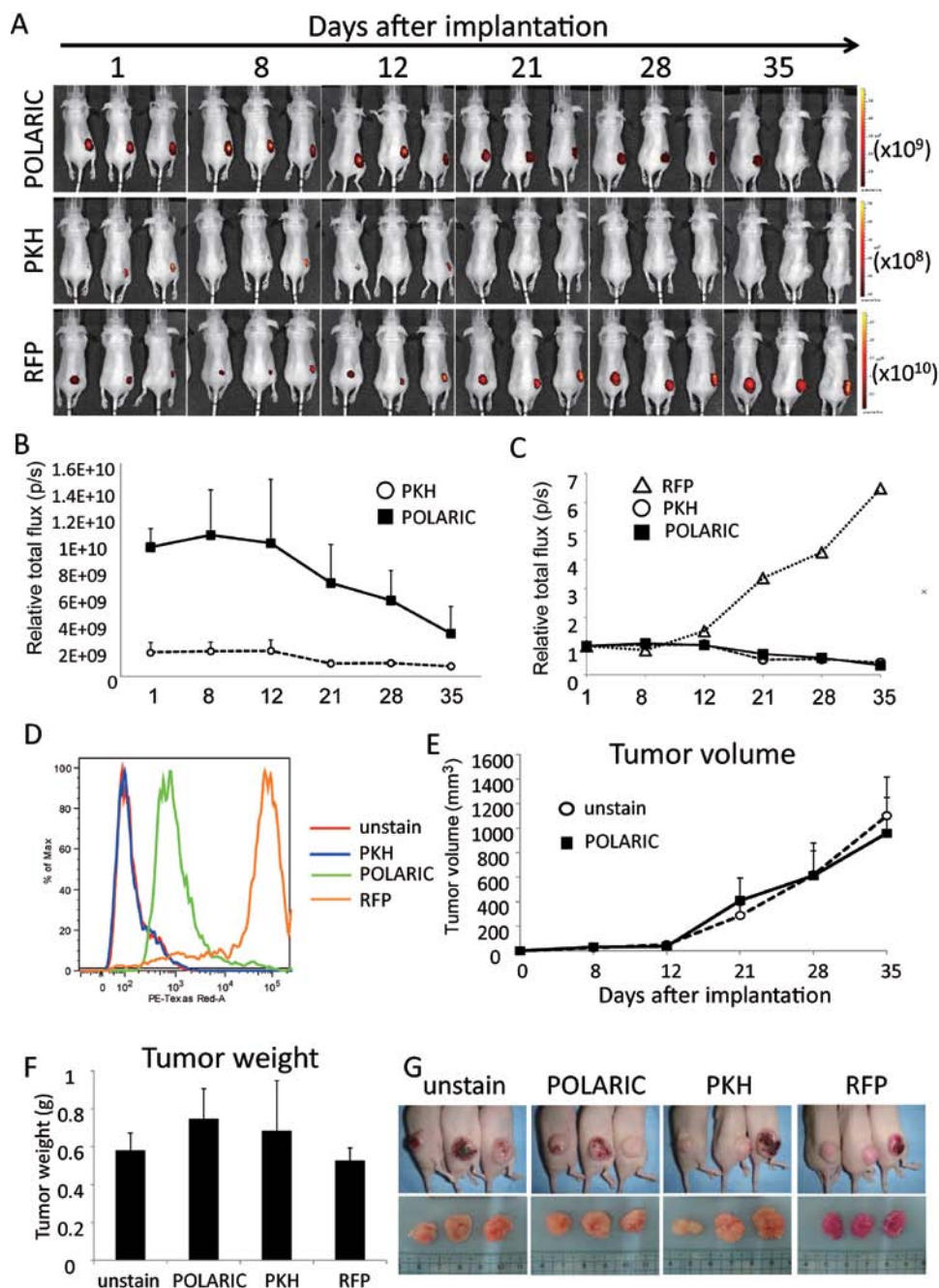


Figure 3. Analysis of nude mice engrafted with A375-SM cells labeled with POLARIC or PKH. (A) Optical imaging of mice bearing tumors induced by subcutaneous injection of A375-SM cells into the right flank. The time course is indicated. (B) Quantitation of tumor fluorescence on the basis of region of interest (ROI) analysis. (C) Ratio of relative total flux (compared with day 1) based on ROI analysis. (D) Representative fluorescence intensities of resected tumors were analyzed using flow cytometry. (E) Analysis of volumes of tumors induced with POLARIC-labeled A375-SM cells. (F) Average resected tumor weights showed no significant differences between tumors induced by cells labeled with either POLARIC or PKH. (G) Representative mice and their tumors.

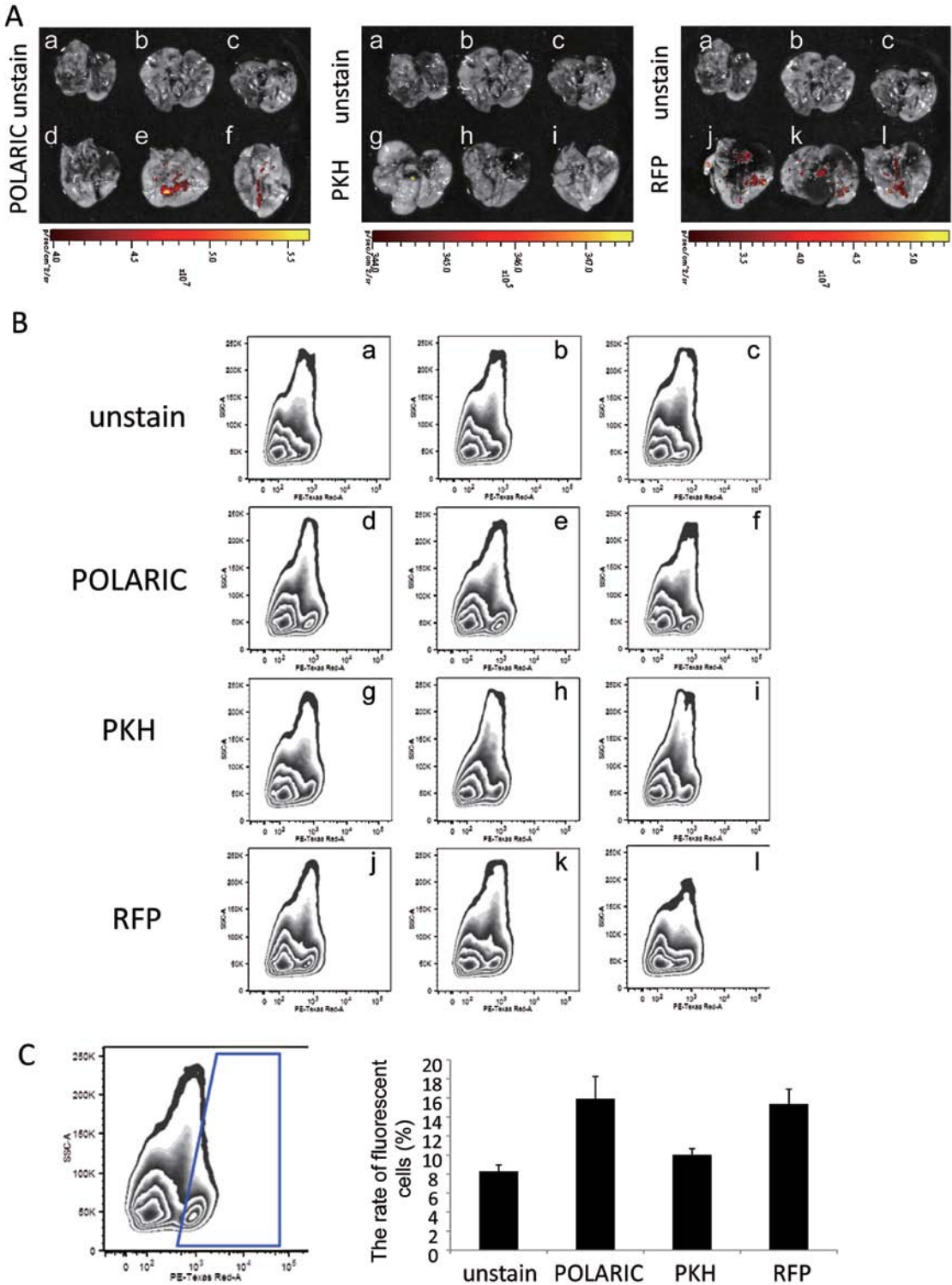


Figure 4. Detection of POLARIC-stained metastatic tumor cells in the lung. (A) *Ex vivo* imaging of lung metastases derived from tumors induced by A375-SM cells labeled with POLARIC, PKH and RFP. (B) Flow cytometric analysis of lung metastatic cells. (C) Determination of the percentage of lung cells labeled with POLARIC, PKH or mRFP1. The percentage of cells labeled with POLARIC and RFP was significantly greater than that of control cells and cells labeled with PKH.

the POLARIC- and PKH-labeled tumors (Fig. 3C), and this finding was consistent with the *in vitro* data (Fig. 2C).

Next, we determined the fluorescence intensities of tumors using flow cytometry. The fluorescence of tumors labeled with POLARIC was readily detected in contrast to the fluorescence of tumors labeled with PKH (Fig. 3D). The fluorescent indicators did not affect tumor size (Fig. 3E) or

tumor weight (Fig. 3F), suggesting that POLARIC did not inhibit tumor growth or cell proliferation, similar to other fluorescent proteins. POLARIC did not induce significant toxicity in this mouse model. Therefore, each of the fluorescent dyes studied here can be used for *in vivo* experiments without any harmful effects on mice or their tumors (Fig. 3G).

POLARIC-stained tumor cells are useful for in vivo detection of lung metastases. We found that A375-SM cells metastasized to the lungs at a high frequency when they were engrafted in immunocompromised mice (14-16). *Ex vivo* evaluation of lungs excised from engrafted mice revealed metastasis in 2/3, 0/3 and 3/3 lungs excised from mice engrafted with POLARIC-, PKH-labeled, or mRFP1-expressing tumors, respectively (Fig. 4A). To confirm these imaging data, lung tissues were homogenized and analyzed by flow cytometry to quantify the rate of fluorescent metastatic tumor cells (Fig. 4B and C). The percentages of fluorescent cells in dissociated lung cells were high in POLARIC-labeled and mRFP1-expressing tumor-bearing mice, whereas those in the lungs of PKH-labeled tumor-bearing mice were hardly detectable (Fig. 4B and C).

Discussion

In the present study, we investigated the utility of the novel fluorescent POLARIC probes for *in vivo* imaging studies and compared the results with those of other fluorescent probes used most frequently for cancer research (17). The fluorescence intensity of A375-SM cells labeled with POLARIC was higher than that of A375-SM cells labeled with PKH, and this intensity could be detected even after three weeks of culture. Dilution of the probes by cell division *in vitro* caused the loss of fluorescence intensity (10,18,19); however, there was no detectable difference between cells labeled with either POLARIC or PKH. Furthermore, the rate of division of cells labeled with POLARIC was almost the same as that of cells labeled with PKH or unlabeled control cells, suggesting that POLARIC did not affect cell proliferation. Several fluorescent indicators can only be used for relatively limited periods, and the duration of the signal emitted by POLARIC-labeled cells represents a significant advancement in this technique. Although the fluorescence emitted by PKH persisted for as long as that emitted by POLARIC, the initially greater intensity of the POLARIC signal made its detection much easier than that of the PKH signal.

POLARIC labeling was evaluated in the orthotopic melanoma-xenograft mouse model. Image analysis of the mice showed that POLARIC-labeled primary tumors were clearly visible, even as long as five weeks after injection. *Ex vivo* imaging demonstrated that labeling with POLARIC also facilitates the visualization of metastatic nodules in the lungs. In addition, POLARIC showed no harmful effects on tumor progression and metastasis. However, the emission maximum of POLARIC at 592 nm is relatively low for the IVIS Spectrum (3). Therefore, fluorescent probes that absorb red light more efficiently must be developed to detect signals emitted by deeper organs. Since the absorption and emission wavelengths of POLARIC fluorophores are altered by replacing the central aromatic moiety (8,9), the proper probes may be designed and synthesized in a manner similar to the derivatives of POLARIC using a more extended aromatic moiety.

Cell lines that stably express fluorescent proteins are commonly used for *in vivo* experiments (17). However, this requires sophisticated molecular genetic manipulation and laboratory facilities. Furthermore, these techniques are not always adequate for primary cells, which are notoriously

difficult to transfect (20). Therefore, DNA transfection is likely to be unsuitable for labeling primary cells soon after harvesting from animals. Using cultured cells can introduce artifacts as their phenotypes differ from their cells of origin (21). Our present study suggests that labeling cells with POLARIC fluorophores can be easily accomplished in a short time without using advanced techniques and facilities. Therefore, it should be possible to adapt this technique to a variety of applications.

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