Journal of Neuroscience Methods 215 (2013) 88–96

Contents lists available at SciVerse ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Basic Neuroscience

Fluorescent probes as a tool for cell population tracking in spontaneously active neural networks derived from human pluripotent stem cells

M. Mäkinen[a,1], T. Joki[a,1], L. Ylä-Outinen[a], H. Skottman[b], S. Narkilahti[a,c], R. Äänismaa[a,*]

[a] NeuroGroup, Institute of Biomedical Technology/BioMediTech, Biokatu 12, FI-33014 University of Tampere, Finland
[b] OpthalmologyGroup, Institute of Biomedical Technology/BioMediTech, Biokatu 12, FI-33014 University of Tampere, Finland
[c] The Science Center of Pirkanmaa Hospital District, Tampere, Finland

HIGHLIGHTS

- Fluorescent labels CT and DiD can be utilized with human stem cell-derived neurons.
- Labels do not affect the cell viability and minimally affect the cell proliferation.
- Labeled cells can be successfully followed for at least 4 weeks.
- Labeled cell populations form spontaneously active neural networks.

ARTICLE INFO

Article history:
Received 27 November 2012
Received in revised form 25 February 2013
Accepted 27 February 2013

Keywords:
CellTracker
DiD
Co-culture
Fluorescent probe
Labeling
Human stem cell derived neural cells
Long term

ABSTRACT

Applications such as 3D cultures and tissue modelling require cell tracking with non-invasive methods. In this work, the suitability of two fluorescent probes, CellTracker, CT, and long chain carbocyanine dye, DiD, was investigated for long-term culturing of labeled human pluripotent stem cell-derived neural cells. We found that these dyes did not affect the cell viability. However, proliferation was decreased in DiD labeled cell population. With both dyes the labeling was stable up to 4 weeks. CT and DiD labeled cells could be co-cultured and, importantly, these mixed populations had their normal ability to form spontaneous electrical network activity. In conclusion, human neural cells can be successfully labeled with these two fluorescent probes without significantly affecting the cell characteristics. These labeled cells could be utilized further in *e.g.* building controlled neuronal networks for neurotoxicity screening platforms, combining cells with biomaterials for 3D studies, and graft development.

1. Introduction

For the development of *in vitro* cell and tissue models it is often desirable to distinguish different cell populations within co-cultures in real time. This can be performed on the basis of differences in the cell morphology. However, morphological differences can vary even within the same cell type and become difficult to observe in a three dimensional culture environment. Fluorescent probes are an alternative to morphology-based identification. These probes have been tested with animal-derived cell cultures (Honig and Hume, 1989) but only one study has earlier described the use of fluorescent probes in human-derived neural cell cultures (Rizvanov et al., 2010). Furthermore, the possible cytotoxic effects should be assessed separately for human or primate cells due to species differences in neurotoxicity (Boyce et al., 1984).

The objective of this research was to find suitable fluorescent probes for the long term labeling of human embryonic stem cell (hESC)-derived neural cell co-cultures. The suitability of two widely used dyes, CellTracker (CT, also known as chloromethylfluorescein diacetate, CMFDA) and long chain carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD or DiIC18(5)) were investigated. CT has been widely used to label living cells (Redelman et al., 1988), and has been utilized in tracking both animal (Silverman et al., 2000) and human derived (Jablonska et al., 2010) cell transplants. Lipophilic long chain dicarbocyanine dyes belong to large family of cyanine dyes (for review see Mishra et al., 2000). DiD and its analogs have been used for cell visualization both in tissue slices and cell cultures (Honig and Hume, 1986, 1989). The impact of these dyes on human-derived

* Corresponding author at: NeuroGroup, Institute of Biomedical Technology/BioMediTech, University of Tampere, Biokatu 12, 6th floor, 33520 Tampere, Finland. Tel.: +358 40 1782942; fax: +358 3 35518498.
E-mail addresses: meeri.makinen@uta.fi (M. Mäkinen), tiina.joki@uta.fi (T. Joki), laura.yla-outinen@uta.fi (L. Ylä-Outinen), heli.skottman@uta.fi (H. Skottman), susanna.narkilahti@uta.fi (S. Narkilahti), riikka.aanismaa@uta.fi (R. Äänismaa).
[1] These authors contributed equally to this work.

0165-0270/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jneumeth.2013.02.019

neural cells has not been previously studied although they have been used in cell cultures from both animal and human origin (Packard et al., 1984; Honig and Hume, 1986; Jablonska et al., 2010; Markiewicz et al., 2011).

The optimum labeling parameters and the characteristics of cell population labeled with each of these dyes were defined. Furthermore, the effect of labeling on the proliferation and viability was studied. The behavior of CT and DiD during immunocytochemistry as well as in co-cultures was also studied. Finally, the ability of CT- and DiD-labeled hESC-derived co-cultures to form functional neural networks was investigated.

2. Materials and methods

2.1. Cells

2.1.1. HESC line derivation and maintenance

A number of hESC lines (Regea 08/056, 06/040, 08/023) were used in this study. The Regea lines were derived at Institute of Biomedical Technology (IBT), University of Tampere, Finland. IBT holds an approval from the Ethics Committee of Pirkanmaa Hospital District for derivation, characterization, and differentiation of hESC-lines (R05051, R05116) as well as an approval of Valvira, the Finnish National Supervisory Authority for Welfare and Health, for research on human embryos (1426/32/300/05). The derivation and characterization of Regea lines has been described previously (Rajala et al., 2010; Skottman, 2010). The maintenance of hESC lines was done as described earlier (Rajala et al., 2007). The hESC lines were quality controlled with frequent gene and protein expression analysis, karyotype, and mycoplasma assays.

2.1.2. Derivation of neural cultures

The neural differentiation of hESCs was performed as described by Lappalainen et al. (2010). Basic fibroblast growth factor (bFGF) was used in 20 ng/ml concentration during the neurosphere culturing step. For the final neural maturation, the neurospheres were mechanically dissected and plated onto 24- or 48-well plates coated (overnight in +4 °C or two hours in +37 °C) with laminin (10 μg/ml, mouse or human, Sigma–Aldrich, St. Louis, MO). The bFGF was withdrawn at the beginning of adherent culture. Half of the medium was replaced 2-3 times a week. For co-culturing, the adherent fluorescent labeled cultures were dissected enzymatically (TrypLe Select™, Gibco).

2.2. Fluorescent dyes

Fluorescent probe 5-chloromethylfluorescein diacetate (CellTracker Green CMFDA, CT, C2925, Molecular Probes®, Life Technologies, UK) was prepared by dissolving it to DMSO (10 mM stock). Lipophilic carbocyanine 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD, D-307, Molecular Probes®, Life Technologies) was prepared by dissolving it to 99.8% ethanol (20 mM stock). On the day of use, a labeling medium was prepared by diluting stock solution to fresh culture medium. The final concentrations recommended by the manufacturer, Molecular Probes®, were 0.5–25 μM for CT and 25 μM for DiD. However, during the experiments we came to test a wider range for CT (0.5, 2, 4, 5, 8, 10, 16, 20, 30, 40 or 60 μM). Based on the literature (Honig and Hume, 1986, 1989; Potter et al., 1996) we decided to test a larger range for DiD as well (0.2, 0.5, 1, 5, 10, 20 or 50 μM). The culture medium on the cells was replaced with the labeling medium and incubated (CT: 15, 30 or 60 min or 2, 4, 24, 48, or 72 h; DiD: 2, 10, 30 or 60 min or 2, 4, 8, 16 or 24 h) at +37 °C, 5% CO_2, humidified atmosphere. After incubation, the labeling medium was replaced with fresh culture medium and the cells were imaged at varying periods after labeling. Every parameter set was tested at least once with 2 parallel samples for both probes and the optimal parameters were tested at least 8 times with 2–20 parallel samples.

2.3. Cell viability

The LIVE/DEAD® Viability/Cytotoxicity Kit (L-3224, Molecular Probes®, Life Technologies) or the components of the kit purchased individually (Calcein AM; C1430, EthD-1; E1169, Molecular Probes®, Life Technologies) were used to assess the cell viability (Althouse and Hopkins, 1995). Briefly, Calcein AM and EthD-1 were diluted to culture medium to the final concentrations of 0.1 μM and 0.5 μM, respectively. After 30 min of dark incubation, the cells were imaged with a fluorescence microscope. Analysis was performed by manually counting the number of Calcein AM and EthD-1 labeled cells from the images.

2.4. Immunocytochemistry

Cells were fixed with cold 4% paraformaldehyde for 15 min in room temperature. To prevent unspecific binding of antibodies, cells were permeabilized with 0.1% Triton X-100 (Sigma) or 0.1% saponin (Sigma–Aldrich) and blocked with 10% normal donkey serum (NDS, Millipore) in 1% bovine serum albumin (BSA, Sigma) in DPBS. Primary antibodies were diluted to 1% NDS, 1% BSA in DPBS with 0.1% Triton X-100 or 0.1% saponin. The primary antibody solution was kept on cells overnight at +4 °C. The excess primary antibodies were removed by washing with 1% BSA in DPBS without or with 0.1% saponin. Next, the secondary antibodies were added in 1% BSA in DPBS without or with 0.1% saponin. Secondary antibodies were incubated at room temperature for one hour. Immunocytochemical staining protocol was also performed with no permeabilization. The excess secondary antibodies were removed by washing with PBS and phosphate buffer without saline. For nuclear staining and mounting, Vectashield with 4,6-diamidino-2-phenylindole (DAPI, H1200, Vector laboratories, Peterborough, UK) was utilized. Primary antibodies anti-MAP-2 (rabbit, 1:600, AB5622, Millipore, Billerica, MA, USA) for detecting neuronal cells as well as their processes, anti-GFAP (sheep, 1:600, AF2594, R&D Systems, Minneapolis, MN, USA) for detecting astrocytes and anti-Ki-67 (rabbit, 1:800, AB9260, Millipore) for detecting proliferating cells were used. AlexaFluor-488, -568 or -680 conjugates of anti-rabbit or anti-sheep secondary antibodies (1:600, Molecular Probes Invitrogen) were used. In order to quantify proliferation, DAPI and Ki-67 positive cell nuclei were counted from the microscope images.

2.5. Imaging system

Cells were visualized and imaged with a fluorescent microscope set (Olympus IX51 inverted microscope, PlanFLN 4×, 10×, 20×, and 40× objectives, Olympus DP30BW CCD camera). The light was filtered with U-MNU2 (excitation filter 360–370; emission filter 420; dichromatic filter 400), U-MNB2 (excitation filter 470–490; emission filter 520; dichromatic filter 500), U-MWG2 (excitation filter 550-510; emission filter 590; dichromatic filter 570), or U-N41023 (excitation filter 625–675; emission filter 710; dichromatic filter 680) filter cube. The images were processed with Adobe Photoshop. Briefly, the grayscale images were converted to an RGB format and the output levels were adjusted to zero, except for the channel corresponding to the color of the fluorescent light.

2.6. Microelectrode array system

The electrical activity of the neural networks were measured by culturing cells on microelectrode array (MEA)-dishes (Multi