Technical note

A simple and robust fluorescent labeling method to quantify trophoblast fusion

Yang Zhang[a,b], Huanghe Yang[a,b,*]

[a] Department of Biochemistry, Duke University Medical Center, Durham, NC, USA
[b] Department of Neurobiology, Duke University Medical Center, Durham, NC, USA

A B S T R A C T

Trophoblast fusion into syncytiotrophoblasts is a specialized yet enigmatic cellular process, which is essential for placental development and function. To facilitate mechanistic understanding of this critical process, here we re-purposed a widely used fluorescent membrane potential dye, Di-8-ANEPPS, to stably label the plasma membrane of live BeWo trophoblast cells. Compared to the methods currently available to quantify trophoblast fusion, our new fluorescent labeling method is simple, economical, robust and versatile, enabling quick and accurate quantification of fusion index in living cells.

1. Introduction

Trophoblast fusion is a critical process in placental development and physiology [1–3]. Even though various factors such as fusogenic proteins, proteases and cytoskeletal proteins as well as cytokines, hormones and transcription factors have been implicated in trophoblast fusion [1–3], the mechanisms of cell-cell fusion are still unclear. Accurate quantification of fusion indexes, a parameter of fusion efficiency, is a key to understand trophoblast fusion mechanisms *in vitro*.

One of the prevailing methods to quantify fusion indexes is utilizing E-cadherin staining of fixed trophoblasts [4]. Nevertheless, marked reduction of E-cadherin expression in fused trophoblasts significantly reduces the staining signal on cell surfaces [5], making identification of syncytiotrophoblasts and quantification of fusion indexes a challenging task. Other quantification methods require expression of fluorescent proteins through transfection or viral infection [4], a process that could complicate trophoblast fusion. Therefore, a simple and robust live cell imaging method is urgently needed to evaluate trophoblast fusion efficiency. Here we evaluated several fluorescent membrane probes to label the plasma membrane of live trophoblasts, and identified that the voltage sensing dye Di-8-ANEPPS has superior membrane labeling and stability, enabling quick and accurate assessment of fusion index.

2. Materials and methods

2.1. Cell culture

BeWo cells (ATCC-#CCL98) were cultured in Gibco Dulbecco's Modified Eagle Medium-Hams F12 (DMEM/F12) medium,

supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Placental cytotrophoblast cells from human term placentas (IRB# PRO00014627) were prepared using a modified Kliman method [6] as described previously [7,8].

2.2. Forskolin-induced fusion and membrane labeling

BeWo cell syncytialisation was induced by forskolin as previously described [9]. Briefly, BeWo cells were treated with 60 μM forskolin (Cell Signaling Technology, #3828s) for 48 h to induce cell fusion and the culture medium with 60 μM forskolin was replaced every 24 h. After treatments, BeWo cells were incubated with Hoechst (Invitrogen™, #H3570, 1:1500) and various fluorescent membrane dyes: 2 μM Di-8-ANEPPS (Invitrogen™, #D3167), 5.0 μg/mL Wheat Germ Agglutinin-Alexa Fluor™-488 Conjugate (Invitrogen™, #W11261), 1:200 Cell-Brite™-Orange Cytoplasmic Membrane Dye (Biotium, #30022), and 50 μg/mL Concanavalin A-Alexa Fluor™-647 Conjugate (Invitrogen, #C21421) in 5% CO_2 at 37 °C for 15–20 min. Di-8-ANEPPS stained cells can be directly imaged without extra rinsing steps. The other membrane markers need to be washed twice with the dye-free medium at room temperature.

2.3. Fusion index quantification

Fluorescent images of six random fields of view were captured with a Zeiss-780 inverted confocal microscope using a 63x/1.4 NA oil Plan-Apochromat DIC objective. To avoid complication from dividing cells, only cells containing more than two nuclei were defined as fused cells. To quantify cell fusion, fusion index (FI) of each sample was calculated
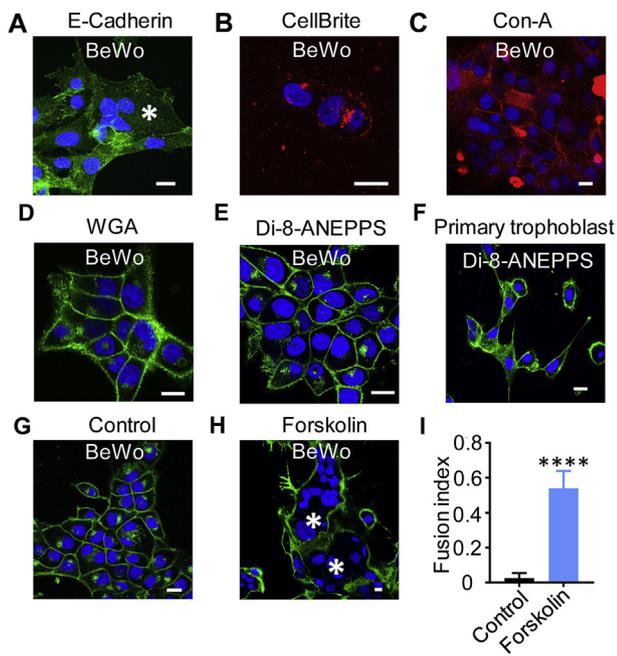

Fig. 1. Di-8-ANEPPS enables a simple and accurate method to quantify trophoblast fusion. A) Immunofluorescence staining of E-cadherin in paraformaldehyde fixed BeWo cells after forskolin treatment. E-cadherin expression decreased in the fused BeWo cell (*), making identification of syncytial cells difficult. B-E) Membrane staining of live BeWo cells with four plasma membrane markers: CellBrite Orange (red, B), Concanavalin-A (Con-A, red, C), Wheat germ agglutinin (WGA, green, D), Di-8-ANEPPS (green, E). F) Membrane staining of live human primary trophoblasts with Di-8-ANEPPS (green). G-H) Di-8-ANEPPS (green) labeled BeWo cell boundaries before (G) and after (H) 60 μM forskolin treatment for 48 h. Asterisks indicate fused BeWo cells. I) Fusion indexes quantified using the Di-8-ANEPPS membrane labeling. Error bars represent standard deviation (s.d.). ****: p < 0.0001. All fluorescence images were the representatives of at least three biological replicates. Nuclei were labeled with Hoechst (blue). The scale bars represent 20 μm.

as:

$$FI = \frac{\sum_1^6 fi}{6} = \frac{\sum_1^6 \left(\frac{\sum n_f}{N} \right)}{6}$$

where fi is fusion index of each individual random field; n_f is the number of nuclei from fused cells and the N is the total number of nuclei in each field.

2.4. Immunofluorescence staining

Bewo cells were fixed with 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.2% triton X-100, and blocked with 10% goat serum prior to antibody staining. Anti-E-cadherin primary antibody (Thermo Fisher, Cat#710161) was added at 1:200 dilution overnight at 4 °C. Fluorescent staining was developed using the goat anti-rabbit IgG (H + L) secondary antibody-DyLight 488 (Thermo Fisher, Cat#35552).

3. Results and discussion

Consistent with previous reports that E-cadherin transcription is dynamically downregulated in fusing trophoblasts [5], the faint E-cadherin immunostaining of the multi-nucleated BeWo cells shown in Fig. 1A demonstrated that E-cadherin is not an ideal membrane marker to quality trophoblast fusion index. To identify a better membrane marker and develop a convenient method to quantify fusion index without fixation, we labeled BeWo cells with four different fluorescent

membrane probes that are widely used in live cell imaging. Cytoplasmic membrane labeling with CellBrite Orange, a lipophilic carbocyanine dye based on DiI [10], was poor in BeWo cells (Fig. 1B). Concanavalin A (Con-A) is a lectin that binds glycoproteins and other sugar-containing entities on cell surface [11]. Nevertheless, Con-A suffered from severe internalization, which made cell boundaries difficult to identify due to strong intracellular fluorescence (Fig. 1C). Wheat germ agglutinin (WGA), another lectin that also binds to the glycoproteins on cell membranes [12], can reliably label BeWo cell surface with less internalization (Fig. 1D). However, the unbound WGA fluorescent conjugates induce strong background fluorescence and thus thorough washing steps are required before imaging.

Next, we examined a widely used fluorescent membrane potential dye, Di-8-ANEPPS [13]. Due to its two eight-carbon acyl chains, Di-8-ANEPPS is lipophilic and is mainly retained in the plasma membranes of live BeWo cells with more photostablity and thermostability, less phototoxicity and minimal internalization (Fig. 1E). Moreover, Di-8-ANEPPS fluorescence is environmentally sensitive. Di-8-ANEPPS is essentially non-fluorescent in water and becomes strongly fluorescent upon binding to cell membranes. The property therefore allows direct imaging without extensive washing steps. Remarkably, Di-8-ANEPPS also reliably labeled the plasma membrane of human primary trophoblasts from a term placenta (Fig. 1F).

To determine if Di-8-ANEPPS could be used to quantify fusion index, we applied 60 μM forskolin to induce BeWo cell fusion and then directly applied Di-8-ANEPPS to label BeWo cell membranes. As shown in Fig. 1G and H, the cell boundaries of both non-fused and fused BeWo cells were clearly demarcated by Di-8-ANEPPS and the numbers of nuclei can be reliably counted within each individual fused BeWo cells. The fusion index measured using Di-8-ANEPPS was 0.54 ± 0.10 (Fig. 1I), which is similar to the reported fusion indexes measured with anti-E-cadherin [14].

In summary, the superior plasma membrane staining using voltage-sensing dye Di-8-ANEPPS enables a simple, robust, economic and accurate method to quantify cell fusion index.

Conflicts of interest

The authors declare no conflicts of interest in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.01.016.

References

[1] A.J. Potgens, U. Schmitz, P. Bose, A. Versmold, P. Kaufmann, H.G. Frank, Mechanisms of syncytial fusion: a review, Placenta 23 (Suppl A) (2002) S107–S113.

[2] P. Gerbaud, G. Pidoux, Review: an overview of molecular events occurring in human trophoblast fusion, Placenta 36 (Suppl 1) (2015) S35–S42.

[3] B. Huppertz, M. Gauster, Trophoblast fusion, Adv. Exp. Med. Biol. 713 (2011) 81–95.

[4] S. Drewlo, D. Baczyk, C. Dunk, J. Kingdom, Fusion assays and models for the trophoblast, Methods Mol. Biol. 475 (2008) 363–382.

[5] C. Coutifaris, L.C. Kao, H.M. Sehdev, U. Chin, G.O. Babalola, O.W. Blaschuk, J.F. Strauss 3rd, E-cadherin expression during the differentiation of human trophoblasts, Development 113 (3) (1991) 767–777.

[6] H.J. Kliman, J.E. Nestler, E. Sermasi, J.M. Sanger, J.F. Strauss 3rd, Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae, Endocrinology 118 (4) (1986) 1567–1582.

[7] D.M. Nelson, R.D. Johnson, S.D. Smith, E.Y. Anteby, Y. Sadovsky, Hypoxia limits differentiation and up-regulates expression and activity of prostaglandin H synthase 2 in cultured trophoblast from term human placenta, Am. J. Obstet. Gynecol. 180 (4) (1999) 896–902.

[8] H. Clabault, L. Laurent, J.T. Sanderson, C. Vaillancourt, Isolation and purification of villous cytotrophoblast cells from term human placenta, Methods Mol. Biol. 1710 (2018) 219–231.

[9] B. Wice, D. Menton, H. Geuze, A.L. Schwartz, Modulators of cyclic AMP metabolism induce syncytiotrophoblast formation in vitro, Exp. Cell Res. 186 (2) (1990) 306–316.

[10] M.G. Honig, R.I. Hume, Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures, J. Cell Biol. 103 (1) (1986) 171–187.

[11] H.G. Schiefer, H. Krauss, H. Brunner, U. Gerhardt, Ultrastructural visualization of surface carbohydrate structures on mycoplasma membranes by concanavalin A, J. Bacteriol. 124 (3) (1975) 1598–1600.

[12] B. Chazotte, Labeling membrane glycoproteins or glycolipids with fluorescent wheat germ agglutinin, Cold Spring Harb. Protoc. (5) (2011) (2011) pdb prot5623.

[13] R.S. Bedlack Jr., M. Wei, L.M. Loew, Localized membrane depolarizations and localized calcium influx during electric field-guided neurite growth, Neuron 9 (3) (1992) 393–403.

[14] U. Nadeem, G. Ye, M. Salem, C. Peng, MicroRNA-378a-5p targets cyclin G2 to inhibit fusion and differentiation in BeWo cells, Biol. Reprod. 91 (3) (2014) 76.