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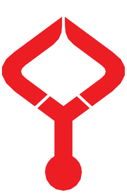

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Affordable and simple protocol for immunofluorescence staining of insulin secreting iGL cell line 2D and 3D

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Abstract
Immunofluorescence staining is a widely used technique to visualize the localization of specific proteins in cells. This protocol provides an affordable and simple method for immunofluorescence staining of the Insulin-GLase (iGL) cell line in both 2D and 3D culture conditions. We describe cost-effective alternatives for reagents and materials without compromising the quality of the results, such as the use of Bovine Serum Albumin (BSA) 3% as a blocking buffer instead of normal serum. Immunofluorescence staining on the iGL cell line is a valuable technique for visualizing the distribution and localization of target biomolecules. With careful consideration of cost-effective strategies and an understanding of the unique characteristics of the iGL cell line, it is possible to achieve reliable and informative immunofluorescence results even with budget constraints.

Keywords: cell culture, immunofluorescence, iGL, 2D and 3D culture

1. Introduction

Immunofluorescence staining is a powerful technique to visualize the distribution and localization of specific biomolecules within biological samples, such as cells and tissues. It involves using antibodies linked to fluorophores, which emit fluorescence when excited with specific wavelengths of light. Immunofluorescence can provide detailed information about the localization, expression levels, and distribution of target proteins within a sample (1-3). Insulin-GLase (iGL) is a cell line developed for studying insulin production and regulation. This cell line is derived from INS-1E rat insulinoma cells and MIN6 cells, which are known to display beta-cell characteristics and secrete insulin in response to glucose (4). It is important to understand the characteristics of the iGL cell line to optimize the immunofluorescence staining protocol effectively.

The iGL cell line is widely used for studying the expression and localization of specific biomolecules within the cells. Its unique features and growth patterns make it a valuable model for immunofluorescence studies. The immunofluorescence analysis of the iGL cell line allows researchers to visualize and quantify the expression of specific proteins, including insulin, within the cells (3).

Immunofluorescence was performed in the iGL cell line using various techniques, such as co-transfection with plasmids expressing FLAG-tagged ChREBP and Mlx and staining for insulin using specific antibodies (5-6). The immunofluorescence analysis of the iGL cell line revealed robust glucose-stimulated insulin secretion and the expression of ChREBP, a transcription factor involved in regulating insulin production. Furthermore, the iGL cell line displayed a uniform pattern of insulin immunofluorescence that closely resembled normal islets within the pancreas (7). Overall, immunofluorescence analysis of the iGL cell line provided valuable insights into the expression and regulation of insulin (5, 8-10).

This study can provide valuable insights into an affordable and simple protocol for immunofluorescence staining of the iGL cell line. Using immunofluorescence analysis, researchers can confirm the successful differentiation of iGL cells into functional beta cells that produce insulin. Additionally, immunofluorescence analysis can be used to evaluate the response of iGL cells to glucose stimulation. For example, if the iGL cell line exhibits robust glucose-stimulated insulin secretion, this suggests that the cells are responsive to changes in glucose levels and capable of producing insulin in response to physiological demand.

2. Materials and Methods

2.1. iGL cell 2D and 3D culture system

iGL cells derived from Cosmo Bio, IGL01C, Japan were thawed and cultured in complete media. The complete media

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was produced using the following recipe:

1. RPMI1640 (containing L-glutamine, phenol red, and HEPES) as the basal media (Gibco, 11875093,USA)

   Additives :

2. 5% of FBS (Fetal Bovine Serum) (PAN Biotech, P303306, South America)
3. 1 mM Sodium Pyruvate (Gibco, 11360070, USA)
4. 500 µM monothioglycerol (Sigma-Aldrich, 96275, USA)
5. 200 µg/mL G-418 (Sigma-Aldrich, 108321422, USA)

First, the base media was prepared: RPMI1640 containing L-glutamine, phenol red, and HEPES (Gibco, 11875093,USA). The media was then supplemented with 5% of FBS (Fetal Bovine Serum) (PAN Biotech, P303306, South America), 1 mM pyruvic acid (Gibco, 11360070,USA) 500 µM monothioglycerol (Sigma-Aldrich, 96275, USA), 200 µg/mL G-418 (Sigma-Aldrich, 108321422, USA) into the base media (Cosmo Bio, IGLM, Japan). After that, frozen cells were thawed in vials in a water bath at 37 °C for two minutes. (Cosmo Bio, IGLM, Japan). We added 1 mL of complete media into the vials, then transferred the cell suspension into 15 mL tubes (Corning, 430791, USA) with 9 mL of complete media. Next, the cells were centrifuged at a speed of 300 g for five minutes, and then the supernatant was discarded. Pellets of cells were resuspended with 1 mL of medium and counted with an automatic cell counter (LUNA Automated Cell Counter, Logos Biosystem, 0300119, South Korea). The cell suspension was seeded into a 100-mm dish (Corning, 430167, USA) and incubated at 37 °C in 5% CO_2. It was replaced with fresh medium every three to four days. After reaching 70-90% of confluency, the cells were passaged. (11)

After cell propagation, cells were collected and divided into two culture systems. The first is a 2D or monolayer culture which was conducted in 6-well culture plate (SPL, 13485, South Korea) with 50.000 cells per well confluency. The other is a 3D culture system using the hanging drop method. The cell suspension drops were formed by placing 25 µL cell suspension on the inner part of the bacterial petri dish lid of a 100×20 mm bacterial petri dish (SPL,10101, South Korea). The cell suspension drops were placed in a relatively identical distance at the inner part of the bacterial petri dish lid. Forty drops were prepared for each cell concentration performed in one petri dish. Once all the drops were completed, the lid was flipped quickly and carefully to maintain the drops. The petri dishes were placed in the incubator at 37 °C with 5% of CO_2 for two days. After the 3D culture system was harvested, we then continued with the 3D iGL cell line immunofluorescence method. (11)

2.2. 2D iGL cell line immunofluorescence method

After cell propagation, the cells were passaged up to the 9th passage. Subsequently, the cells were harvested, and cell staining was initiated. For iGL cell line immunofluorescence analysis in 2D, the following procedure was followed. After the cells were seeded and harvested in a 24-well cell culture plate (Corning, 3524, China), the iGL cells were fixed with glass coverslips (coverslips are optional). Next, they were rinsed once with warm PBS (Gibco, 18912014, USA) and allowed to sit briefly, and then the PBS solution was carefully discarded using a pipette. After that, the cells were covered to a 2–3 mm depth with a warm fixation solution of 10% Neutral Buffered Formalin (Leica Biosystem, 3800698, USA) and fixed for 15 minutes at room temperature.

After incubating for 15 minutes at room temperature, the fixation solution is carefully discarded using a pipette. The cells are rinsed three times in 1X PBS (Gibco, 18912014, USA) 300 µl per well for five minutes each time. Permeabilization solution 0.3% Triton X-100 is added in PBS (Invitrogen, HFHF10, USA) at 100 µl per well and incubated for five minutes at room temperature. Then, it is washed once with Milli-Q/aquabidest, 300 µl per well, and after that 200 µl per well of blocking buffer, Bovine Serum Albumin (BSA) 3% (Sigma-Aldrich, 1003269866, USA) is added to the chamber sections and incubated for one hour at room temperature. After incubating for one hour, the blocking buffer is discarded and the primary antibody solution is added in the amount of 200 µl per well to the chamber slides and incubated at room temperature or overnight at 4 °C. The recommended concentrations on the datasheet in the antibody dilution buffer are 1: 200 (PBS = 995 µl + connexin 36 = 5 µl), 1: 100 (PBS = 990 µl + connexin 36 = 10 µl), 1: 50 (PBS = 980 µl + connexin 36 = 20 µl), 1: 10 (PBS = 900 µl + connexin 36 = 100 µl). After being incubated overnight at 4 °C, it is washed three times in PBS (Gibco, 18912014, USA) 300 µl per well.

A fluorochrome-conjugated secondary antibody, goat anti-Rabbit IgG (H+L) Secondary Antibody, FITC (Invitrogen, 656111, USA), is prepared and diluted and added at 200 µl per well. The secondary antibody dilution consisting of (1: 1000; PBS = 999 µl + FITC 1 µl) is then incubated for two hours at room temperature in the dark. Next, it is washed three times with Milli-Q/aquabidest, 300 µl per well. Then, DAPI (Thermo Scientific™, 62248, USA) is added, diluted 200 µl per well, in the dark. The DAPI dilution consisting of (1: 1000; PBS = 999 µl + DAPI 1 µl) is then incubated for 10 minutes at room temperature in the dark, or the coverslips are mounted onto microscope slides using mounting media with a nuclear stain, such as DAPI, to visualize the cell nuclei.

After the staining steps are complete, we visualize and quantify the marker expression and localization within the iGL cell line using fluorescence microscopy (Nikon, Ts2 Ph Fl, Japan) integrated with a camera (Nikon, DS-Fi3, Japan).

2.3. 3D iGL cell line immunofluorescence method

After the cells were harvested (hanging drop method) in a 100×20 mm bacterial petri dish (SPL, 10101, South Korea) the first step in immunofluorescence staining for the 3D iGL cell line is to prepare a pap pen circle onto object glass. Then, the

spheroid area is carefully transferred using a micropipette (Appendorf, USA) inside the pap pen and incubated for 30 minutes at room temperature. After that, cells are covered to 2–3 mm depth with a warm fixation solution of 10% Neutral Buffered Formalin (Leica Biosystem, 3800698, USA) and fixed for one hour at room temperature. After incubating one hour at room temperature, the fixation solution is discarded. The cells are then rinsed three times in 1X PBS (Gibco, 18912014, USA) for five minutes each time. Permeabilization solution 0.3% Triton X-100 in PBS (Invitrogen, HFHF10, USA) is added, 300 µl per object glass (pap pen area) and incubated for five minutes at room temperature. Then, the cells are washed once with Milli-Q/aquabidest, and then 200 µl blocking buffer, Bovine Serum Albumin (BSA) 3% (Sigma-Aldrich, 1003269866, USA), is added to the chamber sections and incubated for one hour at room temperature.

After incubating for one hour, the blocking buffer is discarded and the primary antibody solution is added in the amount of 200 µl to the chamber slides and incubated at room temperature or overnight at 4 °C. The recommended concentrations on the datasheet in the antibody dilution buffer are 1: 200 (PBS = 995 µl + connexin 36 = 5 µl), 1: 100 (PBS = 990 µl + connexin 36 = 10 µl), 1: 50 (PBS = 980 µl + connexin 36 = 20 µl), 1: 10 (PBS = 900 µl + connexin 36 = 100 µl). After incubating overnight at 4 °C, wash three times in PBS (Gibco, 18912014, USA). A fluorochrome-conjugated secondary antibody, goat anti-Rabbit IgG (H+L) Secondary Antibody, FITC (Invitrogen, 656111, USA), is prepared and diluted and 200 µl is added per object glass (pap pen area).

The secondary antibody dilution consisting of (1: 1000; PBS = 999 µl + FITC 1 µl) is then incubated for two hours at room temperature in the dark. Next the cells are washed three times with Milli-Q/aquabidest. Then, DAPI (Thermo Scientific™, 62248, USA) is added, diluted, 200 µl per object glass (pap pen area). This is done it in the dark. The DAPI dilution consisting of (1: 1000; PBS = 999 µl + DAPI 1 µl) is then incubated for 10 minutes at room temperature in the dark, or the coverslips are mounted onto microscope slides using mounting media with a nuclear stain, such as DAPI, to visualize the cell nuclei. After the staining steps are complete, the marker expression and localization within the iGL cell line are visualized and quantified using fluorescence microscopy (Nikon, Ts2 Ph Fl, Japan) integrated with a camera (Nikon, DS-Fi3, Japan).

3. Results

3.1. Microscopic observation results of 2D and 3D cultures of iGL cells

The monolayer culture of iGL cells reached more than 70% confluency after two days of incubation (Fig. 1). This type of adherent cells aggregated to each other as per typical characteristics in natural conditions. In the 3D culture system, the aggregated cells formed a compact round-shaped structure called a spheroid (Fig. 2). The spheroid began to form from the first day after cell seeding with average size.

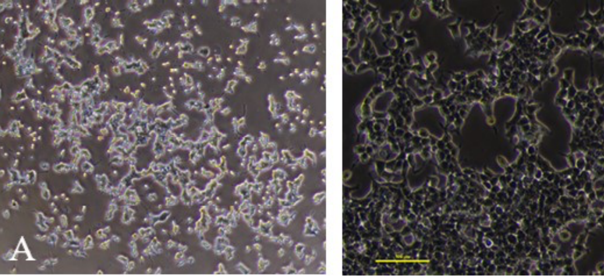


Fig. 1. Monolayer cell culture of iGL cell. Phase contrast microscope, mag. 40x (A) and 100x (B)

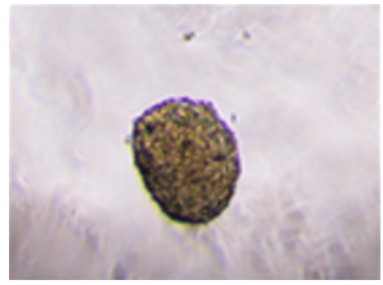


Fig. 2. 3D cell culture of iGL cell. Phase contrast microscope, mag. 40x

3.2. Microscopic observation results of immunofluorescence staining of iGL cell line 2D

Fluorescence microscopy revealed the distribution and localization of the target proteins within the iGL cells. The cells showed positive staining for the target biomolecule, indicating its presence within the cells. This suggests that the immunofluorescence staining technique successfully identified the target biomolecule in the iGL cell line. Representative images show the expression patterns of the markers in the 2D culture condition (Fig. 3). The fluorescence signal was observed in the cytoplasm of the iGL cells, confirming the presence and localization of the target biomolecule.

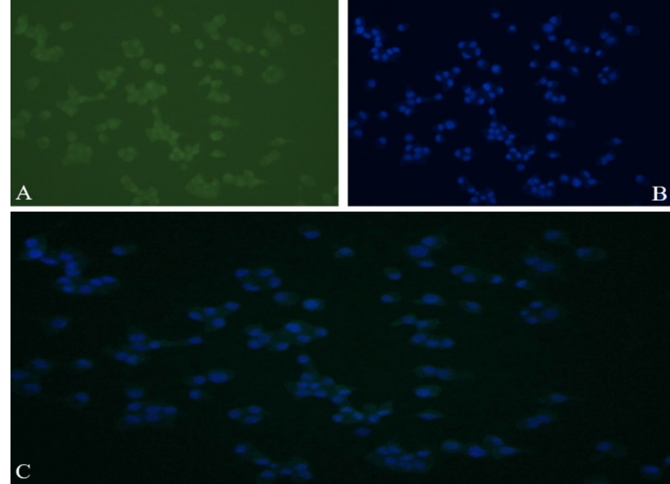


Fig. 3. Immunofluorescence staining of iGL cell line 2D. (A) Cx-36-FITC (green panel), (B) DAPI (blue panel), (C) merged

3.3. Microscopic observation results of immunofluorescence staining of iGL cell line 3D

In the 3D culture condition, immunofluorescence staining revealed the spatial organization and distribution of the target biomolecule within the iGL cells. The fluorescence signal was

observed throughout the 3D structure, indicating widespread expression of the target biomolecule. This suggests that the immunofluorescence staining technique successfully identified and localized the target biomolecule in both 2D and 3D culture conditions, providing valuable insights into its spatial distribution within the iGL cell line. Fluorescence microscopy revealed the distribution and localization of the target proteins within the iGL cells. Images depict the spatial organization and morphology of iGL cells within the 3D matrix. Representative images show the expression patterns of the markers in the 3D culture condition (Fig. 4).

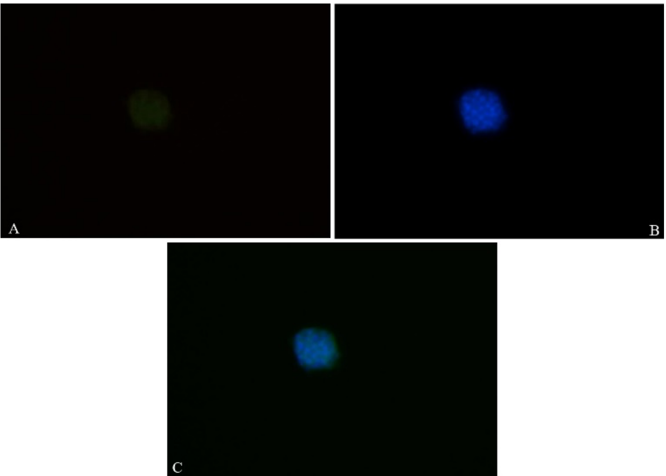


Fig. 4. Immunofluorescence staining of iGL cell line 3D. (A) Cx-36-FITC (green panel), (B) DAPI (blue panel), (C) merged

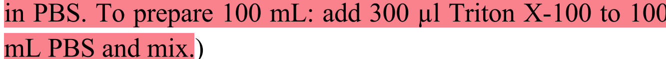
4. Discussion

The iGL cells, in either a monolayer or 3D culture system, were growing well until two days of incubation. This is in accordance with previous research by (11) that showed iGL cells with a stable morphology and viability could be derived from 50-100 cells / µL with two days of incubation. The stable morphology and viability are both critical aspects when considering an immunofluorescence assay. Cell morphology may provide valuable information about the shape, size, and organization of the cell. On the other hand, cell viability proposes the proportion of live and healthy cells within in a population (12).

The distinctive aspect of our study is that the cells are different. The iGL cells used in this study are still new and there are not yet many references using them as a research subject, so it is hoped that this protocol study can serve as an additional reference for knowledge regarding iGL cells, especially regarding methods/protocols for developing these iGL cells.

In the blocking section, the materials used are different. We use BSA, while in the literature normal serum/skim milk is used. The reason BSA is used is that it is less expensive than normal serum/skim milk and can still produce a fairly good fluorescent image.

In the antigen retrieval section, sodium citrate is normally used, but now we use a permeable solution (0.3% Triton X-100


in PBS. To prepare 100 mL: add 300 µl Triton X-100 to 100 mL PBS and mix.)

The similarity of our study to others is that we use viable 3D cell culture samples, that are fixated right away before immunostaining. We also use the same buffer, formaldehyde 10% and antibody dilution. (2)

The advantage of the method in this study is that we use inexpensive, easier materials and goods that are easy to obtain, while the disadvantage of this study is that this method cannot be directly compared with existing methods due to limited studies to date about iGL cells.

For microscopic observation of immunofluorescence staining of iGL cell line in 2D and 3D, images were captured using appropriate filter sets for each fluorophore (FITC for green, DAPI for blue).

To perform immunofluorescence staining on the IGL cell line in an affordable and simple setting, certain tips can be helpful:

1. Utilize cost-effective alternatives for reagents and materials without compromising the quality of the results.

2. Consider optimizing the staining protocol by adjusting the concentrations of antibodies and reagents to minimize wastage.

3. Explore using alternative mounting media and blocking buffers that are affordable and readily available.

4. Take advantage of economical options for fluorescent secondary antibodies without compromising the specificity of the staining.

In addition to the cost-effective alternatives mentioned above, there are further options for conducting immunofluorescence staining on a limited budget. For example, consider exploring local suppliers for discounted reagents and materials, and collaborating with other research groups to share resources and reduce costs. Additionally, optimizing the use of equipment and recycling certain consumables can contribute to cost savings without compromising the quality of the staining results. By leveraging these low-budget options and effectively managing resources, it is possible to achieve successful immunofluorescence staining outcomes while working within budget constraints. By continuing to prioritize meticulous technique and attention to detail, we will be able to generate valuable immunofluorescence data for research on the iGL cell line.

Immunofluorescence staining on the iGL cell line is a valuable technique for visualizing the distribution and localization of target biomolecules. With careful consideration of cost-effective strategies and an understanding of the unique characteristics of the iGL cell line, it is possible to achieve reliable and informative immunofluorescence results even with budget constraints.

Ethical Statement

The ethical approval for this study was obtained from the Ethics Committee of the Faculty of Medicine - Universitas Prof. Dr. HAMKA on June 18, 2021, with the approval number KEPKK/FK/003/07/2021.

Conflict of interest

The authors declare that there is no conflict of interest was declared by the authors.

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Authors' contributions

Concept: E.N.S., S.S.N., Z.N., N.D.P., Design: E.N.S., S.S.N., Z.N., N.D.P., Data Collection or Processing: E.N.S., S.S.N., N.D.P., Analysis or Interpretation: E.N.S., S.S.N., N.D.P., Literature Search: T.Y., W.S., Writing: E.N.S., T.Y., W.S.

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