CHAPTER FOURTEEN

REAL TIME QUALITATIVE AND QUANTITATIVE GLUT4 TRANSLOCATION ASSAY

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Abstract
Insulin-stimulated glucose transporter 4 (GLUT4) translocation promoting glucose uptake is vital to glucose homeostasis and is a defined target of antidiabetic drug research. Existing functional assays to detect the process of GLUT4 translocation are hampered due to assay variability and low sensitivity, thus slowing down the progress towards the development of preferred alternative to insulin. This chapter describes a real time, visual, cell-based qualitative GLUT4 translocation assay suitable for screening insulin mimetics. The basic strategy consists of establishment of insulin-sensitive CHO-HIRc-myc-GLUT4eGFP cells those stably express myc and eGFP-tagged GLUT4 in addition to human insulin receptor (HIRc). GLUT4 translocation is visualized by tracking the movement of GLUT4 associated GFP fluorescence from perinuclear space to the plasma membrane by employing cooled charge-coupled device (CCD) camera.

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ISSN 0076-6879, DOI: 10.1016/B978-0-12-388448-0.00022-X All rights reserved.

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attached to a simple fluorescent microscope. This video imaging method and further quantitative analysis of GLUT4 on the cell membrane provides rapid and fool-proof visual evidence suitable for screening GLUT4 translocation modulators. This assay is further validated by complementary assays.

**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>CCD</td>
<td>cooled charge-coupled device</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter 4</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HIRc</td>
<td>human insulin receptor</td>
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<td>LCI</td>
<td>live cell imaging</td>
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<td>PM</td>
<td>plasma membrane</td>
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**1. Introduction**

It is well established that insulin action promoting glucose uptake by adipocytes and muscle cells through glucose transporter 4 (GLUT4) is a major mechanism for clearance of excess exogenous glucose in blood and hence is the rate limiting step in diabetes (Pessin and Saltiel, 2000). GLUT4 is 1 of 13 sugar transporter proteins (GLUT1-12, and a proton-coupled myoinositol transporter [HMIT]) that facilitate transport of sugar moiety across cell membrane (Joost and Thorens, 2001). These transporters differ in respective hexose specificities and concentrations. Skeletal muscle, adipose tissue, and cardiac cells primarily express GLUT4 in addition to a distinct set of other transporters. GLUT4 has 12 transmembrane domains with unique sequences in NH$_2$ and COOH terminal domains that navigate its membrane trafficking ability. It has a specific substrate binding domain and a domain for cytochalasin, a known inhibitor of receptor transport (Huang and Czech, 2007). Exofacial loop of GLUT4 is a site for insertion of epitope (either myc or HA [hemagglutinin]) that would assist in tracking of insulin-induced translocation. Under basal or nonstimulated conditions, more than 95% GLUT4 is localized to the perinuclear region or in cytoplasm close to the plasma membrane (PM). Insulin stimulation shifts the balance in favor of localization of GLUT4 to the PM which is associated with increase in glucose transport (Suzuki and Kono, 1980). All these factors make GLUT4 a most promising target of antidiabetic drug research.
2. GLUT4 Translocation Assay

2.1. Rational for the development of real time GLUT4 translocation assay

Clinical studies have shown that diabetic patients have normal level of GLUT4 with intact translocation machinery (Garvey et al., 1992). Therefore, it is likely that abrogation in GLUT4 translocation is due to defects in the insulin signaling transduction cascade. Considering the fact that exercise induced glucose uptake via GLUT4 in skeletal muscle is facilitated by circumventing defects in insulin signaling pathway, GLUT4 could well be a potential target for drug development (Kishi et al., 1998). However, an oral hypoglycemic agent that offers a needle less alternative to insulin therapy is yet to be fulfilled target of pharmaceutical research. With no limit of natural products and synthetic compounds, or derived products available for antidiabetic drug research, the need of the hour is efficient *in vitro* systems and methods for GLUT4 translocation assay which will facilitate in rapid screening. General GLUT4 translocation assays employ indirect methods such as western blot analysis of PM fractions, photoaffinity labeling, binding assay using cytochalasin B and qualitative assessment by immunofluorescence or immuno-electron microscopy (Kozka et al., 1991; Smith et al., 1991). All these assays are not only relatively less sensitive and time consuming but also cost intensive.

Direct visualization of GLUT4 trafficking by tagging of GLUT4 with GFP (green fluorescent protein) and its expression in cells have made detection of GLUT4 movement by various microscopy techniques relatively easy (Dobson et al., 1996). Also, addition of specific tags within first exofacial loop of GLUT4 is an added advantage for immunofluorescence detection, or ELISA for the PM-associated GLUT4 distribution in fixed and nonpermeabilized cells (Dawson et al., 2001; Kanai et al., 1993). These tags aid in differentiating between GLUT4 on the PM and those retained close to the PM in certain cell lines. Similar to the endogenous GLUT4, the overexpressed GLUT4 chimera is also localized to perinuclear region and is responsive to insulin treatment (Fig. 14.1A and B). Since epitope-tagged GLUT4 helps in detecting cell-surface expression with GFP chimeras revealing total expression of GLUT4 (Fig. 14.1C and D), it is better to have fusion chimeras between GFP and GLUT4 with an HA or myc tag in their exofacial loops that will not alter its function. The presence of an exofacial tag and GFP in a chimera provides an additional tool to normalize cell-surface expression levels to total expression levels in a single cell. Chimera is shown to remains intact, and is not susceptible to proteolysis which generates native GFP (Dobson et al., 1996). Such a model system would be of immense use in screening antidiabetic drugs or natural products for GLUT4 translocation modulators.
2.2. Rational for the development of insulin sensitive cell line

One of the arduous tasks in the antidiabetic drug development is screening enormous numbers of lead compounds for an appropriate biochemical or cellular outcome. The ways of making this screening faster, more effective and less expensive can revolutionize antidiabetic drug screening and are being relentlessly pursued and developed. Cell or tissue culture models are being used to understand the cellular and molecular processes involved in the disease and also to elucidate the cellular or molecular actions of a lead compound in advanced stages of drug development. 3T3-L1, L6, and C2C12 cells are generally used in vitro models for GLUT4 translocation assays. Yet, they are far from insulin sensitive muscle and adipose cells as a reliable in vitro model. Prerequisite of differentiation and the presence of GLUT1 in these cell lines leave ambiguity in tedious pharmaceutical drugs screening (Liu et al., 2009; Mehra et al., 2007). In addition, traditionally these cells are least competent for transfection and hence the establishment of stable cell line expressing GLUT4 chimera with tags is difficult. We were also not successful in stably transfecting 3T3-L1 cells with GLUT4 chimera, as GFP displayed considerably lower levels of fluorescence and it was eventually lost over a period of time.
Due to high transfection efficiency, a number of laboratories use Chinese hamster ovary (CHO) cell line which is known to exhibit maximum intensity of GFP fluorescence. This is attributed to its characteristic redox potential, cytoplasmic pH, oxygen tension, and expression of heat shock proteins (Dobson et al., 1996). Since, insulin signal transduction machinery essential for GLUT4 translocation is incomplete in CHO cells, it is essential to manipulate these cells by overexpressing insulin receptor (IR) and GLUT4 to make them responsive to insulin (Kanai et al., 1993; Lampson et al., 2001; Perfetti et al., 1997; Quon et al., 1994). To establish suitable insulin sensitive CHO cells generally clones expressing IR and tagged GLUT4 chimera are selected. The most preferred chimera of GLUT4 with tag is mycGLUT4eGFP or HAGLUT4eGFP (Dawson et al., 2001; Jiang et al., 2002). CHO cells expressing IR and mycGLUT4eGFP or HAGLUT4GFP are a strong *in vitro* model for the development of screening assays to test antidiabetic drugs. Insulin-stimulated translocation of these chimera to the PM can be visualized by live cell imaging (LCI) based on the movement of GFP fluorescence (Oatey et al., 1997, Vijayakumar et al., 2010).

### 3. Methods and Procedures

In this part, we provide methods and procedures for the establishment of a stable insulin sensitive CHO cell line, its application in GLUT4 translocation assay and validation by different approaches. The protocol involves sequential transfection of cells with IR and GLUT4 chimera expression vectors. This is followed by selection and screening of ideal clones for insulin sensitivity, real time assay for GLUT4 translocation and further validation using complementary experiments.

#### 3.1. Reagents

Ham’sF12 medium, penicillin, streptomycin, LipofectAMINE 2000 (LF-2000), fetal bovine serum (US origin), and trypsin–EDTA are available from Invitrogen (Carlsbad, CA, USA). Tissue culture wares such as 35 and 60 mm petridishes, 24-well plates, and chambered slide with cover can be procured from Nunc (Rochester, NY, USA). Fine chemicals and reagents including 2-deoxy glucose (2-DG), cytochalasin B, paraformaldehyde, sodium dodecyl sulfate, and bovine insulin are available from Sigma-Aldrich (St. Louis, MO, USA). 14C-2DG radiolabeled glucose can be purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Monoclonal anti-myc antibody, polyclonal anti-IR antibody and rhodamine conjugated goat antimouse secondary antibody are available from
Santa Cruz Biotechnology (Santa Cruz, CA, USA). Stock solution of insulin (100 μM) is prepared by dissolving 5.7 mg insulin in 10 ml of acidified water

3.2. Methodological considerations

Development of vectors suitable for transient or stable expression of specific proteins tagged with a fluorescent moiety for visualization in desired cell lines have revolutionized studies on cellular receptor dynamics and analysis. CHO cells stably overexpressing IR and GLUT4 have several advantages in antidiabetic drug screening as they avoid the need for expensive and repeated transient transfections and also provide a nearly uniform expression. CHO cells are tolerant to exogenously expressed proteins. In addition, stable cell line will ensure perinuclear localization of GLUT4 and their response to insulin or insulin mimetics by translocation. To establish an insulin sensitive cell line, CHO cells are to be transfected with a eukaryotic expression vector containing the cDNA for B isoform (exon 11) of wild-type human insulin receptor (HIRc). Most of the established laboratories generously provide the construct and its map upon mutual understanding or after signing material transfer agreement. However, it is important to verify the construct before utilizing by standard restriction digestion analysis taking clue from the map or by sequencing. The commonly used plasmid that carries human IR is pCVSVHIRc (Ullrich et al., 1985). This plasmid contains the origin of replication and the ampicillin-resistance gene of the Escherichia coli plasmid pBR322, and an insert containing the human IR coding sequence under the control of SV40 early promoter (SVE).

3.3. Transfection procedure to establish IR overexpressing CHO cells

1. Exponentially growing cells are plated at 60–80% confluency in 35 mm dish 24 h before transfection.
2. Cotransfect CHO cells with plasmid pCVSVHIRc (4 μg) and a plasmid (pSVNeoe) coding for neomycin–resistance gene under control of SVE (1 μg) by LF–2000 method as described by the manufacturer. For this, dilute pCVSVHIRc and pSVNeoe plasmids to 200 μl with F12 medium and incubate for 5 min. Dilute 5 μl of LF–2000 to 200 μl with F12 medium separately and incubate for 5 min.
3. Mix the solutions of diluted plasmids and LF–2000 by gentle repeated pipetting and incubate for 30–45 min at room temperature to allow DNA–liposome complex formation.
4. Add this complex to the cells in culture plates incubated in 1.6 ml of fresh F12 media with serum for 1 h. Incubate the cells for further 24 h before the medium was changed with fresh F12 medium with serum.
5. Twenty-four to 48 h posttransfection, the cells are split 1:3 or 1:5 into separate, preferably 60 mm, dishes.
6. After a recovery period of 24 h, add 800 µg/ml of neomycin analog G418 to the medium to select cells expressing neomycin-resistance gene contained in the pSVEneo plasmid. Selection medium is replaced every second or third day. Selection is continued generally for 2–3 weeks.
7. Once the majority of cells are dead, surviving cells tend to form colonies which are monitored and these are tracked by marking at the bottom of the dish.
8. When the colony size is approximately 1–3 mm in diameter, they are detached with 2–5 µl of trypsin and placed directly into wells of a 24-well plate and cultured in presence of G418 (200 µg/ml) to be propagated as cell line.
9. The IR expression levels in each clone should be verified by standard western blotting procedure. The clones those express IR levels 5–10 folds higher than control CHO cells should be selected and amplified in tissue culture. It is of utmost importance to cryopreserve cells in liquid nitrogen as soon as the expression levels are verified. We have observed that any kind of stress due to undesired pH of the medium and low density (culturing less than $2.5 \times 10^5$ cells in 35 mm plate or $5 \times 10^5$ cells in T-25 flask) causes decrease in IR expression. The level of IR expression can also be determined by insulin binding assay (White et al., 1987).

3.4. Development of CHO-HIRc-mycGLUT4eGFP cells

The backbone of the plasmid construct used to establish this stable cell line is modified pGreen Lantern Vector with EcoRI and XbaI sites and the cDNA coding region for mycGLUT4eGFP (Jiang et al., 2002). DNA sequence for myc epitope is between the 66th and 67th amino acid of GLUT4 and GFP encoding sequences is at C-terminal of myc-GLUT4. The protein, mycGLUT4eGFP expressed constitutively under cytomegalovirus (CMV) promoter is shown to be translocated to membrane upon insulin stimulation.

3.4.1. Transfection procedure to establish CHO-HIRc-mycGLUT4eGFP cells involves following steps

1. CHO-HIRc cells were cotransfected with 4 µg of pGreen Lantern mycGLUT4eGFP and 1 µg pTk-Hyg plasmid (Clontech, Mountain View, CA, USA) using LF-2000 method as mentioned earlier except that selection was done using 200 µg/ml hygromycin B. GFP clones should be picked up and maintained separately.
2. Before proceeding on to drug screens, the stably expressed GLUT4 chimera should be evaluated for its translocation efficiency in response to insulin. It is also important to establish the response of chimera to a
broad range of insulin concentration (1–1000 nM) to measure the dose responsiveness of the translocation event. Specificity of insulin sensitivity can be further verified by pretreating the cells with pharmacological inhibitors of insulin action (wortmannin, cytochalasin B, genestein, etc.) followed by insulin treatment (Vijayakumar et al., 2005, 2010).

3.4.2. Simple protocol for verification of insulin sensitivity

1. Plates 5000–10,000 cells per well in a chambered slide with cover and allow cells to adhere and grow for a day.
2. Wash cells with serum free medium and serum starve further in Ham’s F12 medium (100 μl) containing 1 mg/ml BSA for 3 h. It is recommended to have BSA present in the medium if experiments are performed for more than 5–10 min.
3. Remove 50 μl media, and add 2× concentration of 50 μl insulin diluted in prewarmed F12 medium containing 1 mg/ml BSA at 37°C, so that final volume of 100 μl and desired concentration is achieved. To verify the specificity of insulin-induced GLUT4 translocation, pharmacological inhibitor of insulin action to be added to incubating media.
4. GLUT4 translocation based on increase in membrane GFP fluorescence upon stimulation with different concentrations of insulin (10–1000 nM) for 1–20 min can be visualized under a fluorescent microscope (Olympus, Shinjuku-ku, Tokyo, Japan). Alternatively, following insulin treatment, cells can be fixed with 3% paraformaldehyde in PBS pH 7.4, for 10 min at room temperature, rinsed with PBS three times. Cells are then quenched with 1% glycine for 5 min followed by washing with PBS and can be observed under a fluorescent microscope. We have observed that fixed cells give better resolution as far as images are concerned.
5. Clones in which GLUT4 chimera translocated to the PM upon insulin stimulation and the level of membrane associated GFP fluorescence was distinct from that in cytoplasm should be selected.

Verified clones may be designated as CHO-HIR.c-mycGLUT4eGFP with appropriate numbering and can be cryopreserved for further usage. During the selection process of transfected cells with hygromycin, we had obtained eight clones that expressed GLUT4 chimera and these were designated as CHO-HIRc-mycGLUT4eGFP clone 1–8. However, only clone 4 and 6 exhibited concentration dependent sensitivity to insulin. Details mentioned in this chapter are experiments done with clone 4.

3.4.3. Real time GLUT4 translocation assay

Major goal of a biologist is to understand the molecular processes that happen in cells in real time. LCI with the aid of microscope and digital technologies helps in monitoring cellular dynamics in real time under high
resolution and has great benefit in basic research as well as in drug screening. Real time microscopy experiments using GFP fusion protein helps in unraveling many aspects of cells including complex motion of cellular machinery unexplorable by in vitro biochemical methods. Fluorescence microscopy is the most proficient technique for studying the dynamic behavior in LCI. It distinguishes GFP chimera with a high degree of specificity from nonfluorescing matters in a live cell, and hence the dynamics of individual cellular components can be analyzed in real time. By incorporating a monochrome or color chip video camera on a microscope, time-lapse images of cells could be recorded onto magnetic storage media (Fig. 14.2A–F). Video imaging and analysis is affordable and less complex, with the added advantage that the molecular process of interest can be analyzed in real time. Video cameras can detect contrast differences invisible to the human eye, and these differences can be electronically amplified. One of the limitations of these imaging technologies is the

Figure 14.2 A model setup for live cell imaging and monitoring of GLUT4 translocation. (A) Microscope body, (B) color chip CCD camera, (C) DP-30 monochrome CCD camera, (D) mercury burner, (E) computer, and (F) software to monitor GLUT4 translocation. Video files are extracted to obtain frames using Image Pro Plus 5.0 software for qualitative analysis of GLUT4 translocation. (G and H) Software displays image at 1 frame/min and aids in visualizing translocation of mycGLUT4eGFP vesicles to the plasma membrane, upon insulin stimulation based on the movement of GLUT4-associated GFP fluorescence. Sequence tool bar of the software aids in selecting the frame number.
accumulation of huge amount of digital image data. Therefore, to extract reproducible quantitative information, computer-based image analysis is required. In this chapter, we briefly outline the method of LCI and quantitative analysis of GLUT4 translocation. This assay is based on the principles of GLUT4 chimera movement monitored directly by GFP.

3.4.4. Simple protocol for real time GLUT4 translocation assay

1. Plate the cells in 35 mm dishes and allow them to attach and to grow for a day.
2. Wash the cells with serum free medium and then serum starve further in Ham’s F12 medium (1 ml) containing 1 mg/ml BSA for 3 h.
3. Remove 500 µl medium and place the plate on a fluorescent microscope.
4. Focus cells under 40× objective as image at this magnification increases the area of the field that provides reliable and collective information about significantly large number of cells ($n > 25$), a most desired parameter in any form of research.
5. Add 500 µl of prewarmed reagents at 2× concentration. With a quick final adjustment of focus, capture images at room temperature at the rate of 1 frame/min for 10–30 min using cooled charge-coupled device (CCD), 1.4 megapixel, 12 bit camera (Olympus) attached to fluorescent microscope.
6. It is necessary to adjust exposure time (50–100 ms) to keep the background minimum to obtain maximum signal. Importantly, exposure time should be kept constant within each experiment to avoid variability in quantitation. Video files are saved in “avi” format and can be played with windows media player, VLC media player, quick time movie player, etc.
7. Analysis of images and conversion of videos to individual frames can be done (Fig. 14.2G and H) using Image-Pro Plus AMS software (MediaCybernetics, Silver Spring, MD, USA) or any compatible software (Metamorph image-processing software, Molecular Devices Corp, Sunnyvale, CA, USA). This aids to visualize kinetics of GLUT4 translocation under normal as well as stimulated conditions.
8. Open video files and define the area of the cell membrane for analysis. The software quantifies GLUT4 translocation based on increase in mean GFP fluorescence intensity on a defined area of the PM of cells in the video frame (Fig. 14.3). We have observed that monochrome images are sharp and would give better read out.
9. The mean intensity data obtained versus frame (1 frame/min) can be used to calculate fold GLUT4 translocation per minute. Repeat the analysis for 10–20 cells per experiment to obtain statistically significant values. This provides the optimum time at which GLUT4 translocation
was maximum. Standardization of dose and time point is advantageous in further molecular studies of suitable GLUT4 translocation modulators.

10. Alternatively, using the above stated method, measure the background fluorescence and subtract these values from specific signals for each individual myc-GLUT4-GFP-expressing cell. Plot the intensity versus frame/time.

3.5. Immunofluorescence microscopy

Data obtained by LCI on GLUT4 translocation in CHO-HIRc-mycGLUT4eGFP cells can be further validated by immunofluorescence detection of GLUT4. Since direct immunological detection of GLUT4 on the cell surface with an anti-GLUT4 antibody being difficult due to conformational changes accompanying stimulation or unexposure of domains in the membrane, most of the investigators rely on detection of inserted epitope (myc or HA) on expressed GLUT4 chimera by binding of anti-myc antibody. This assay detects the proportion of mycGLUT4eGFP in the PM of
nonpermeabilized cells, and aid in comparing the amounts of GLUT4 in the PM under basal and insulin-stimulated states. In a cell-by-cell analysis, the amount of anti-myc antibody bound to the PM (surface mycGLUT4eGFP) is normalized to the amount of GFP expressed in the cell (total mycGLUT4eGFP). This normalization corrects for differences in the expression levels of mycGLUT4eGFP among the cells.

3.5.1. Protocol for detection of myc epitope

1. Plate and treat the cells as mentioned in the Section 3.4.2.
2. Wash the cells quickly with ice cold PBS and fix by treating with 3% paraformaldehyde in PBS pH 7.4, for 10 min at room temperature. Rinse the cells with PBS three times and quench with PBS containing 1% glycine for 5 min followed by washing with PBS three times.
3. Block the cells with PBS containing 5% BSA and 5% FBS for 1 h at room temperature.
4. Incubate the cells with anti-myc (9E10) antibody or respective isotype control for overnight at 4°C or 2 h at room temperature at a dilution of 1:50 (4 μg/ml) in PBS containing 2.5% FBS.
5. Wash the cells five times at 2 min interval with PBS to eliminate unbound antibodies.
6. Incubate the cells with a rhodamine conjugated goat antimouse secondary antibody at a dilution of 1:100 (2 μg/ml) for additional 1 h, at room temperature. Wash again five times with PBS at 2 min interval and mount using Vectashield (Vector Laboratories, Burlingame, CA, USA) for visualization by immunofluorescence or confocal microscopy (LSM510, Carl Zeiss Heidelberg, Germany). Treatment with GLUT4 modulators like insulin increases immunoreactive chimera on the PM in parallel with increased GFP fluorescence (Fig. 14.4).

3.6. Glucose transport assay

Insulin stimulation of cells expressing GLUT4 results in rapid recruitment of GLUT4 transporter from internal compartment to the PM with resultant increase in the rate of glucose transport. Being the rate limiting step, this process is often impaired in patients with type 2 diabetes. It is assumed that therapies which augment insulin-stimulated GLUT4 translocation should also increase glucose uptake in target tissues, thereby improving insulin sensitivity. Therefore, glucose uptake assay could be considered as another validation assay for real time GLUT4 translocation assay.

3.6.1. Procedure for glucose uptake assay

1. Wash the cells \((1 \times 10^5)\) grown in 24-well plates with serum free medium and then serum starve for 3 h in F12 medium containing 0.1% BSA.
2. Wash the cells twice in KRP buffer (137 mM NaCl, 4.7 mM KCl, 10 mM sodium phosphate pH 7.4, 0.5 mM MgCl2, 1 mM CaCl2, 2 mg/ml BSA), incubate at 37 °C for 30 min and then treat with various concentration of insulin (0.1–1000 nM) or test compounds for additional time. Control cells are to be treated with respective vehicle control.

3. The glucose uptake reaction is initiated by adding cocktail of 0.1 mM 2-DG and 0.5 μCi/ml 14C-2-DG in final volume of 250 μl/well. After incubation at 37 °C for 10 min, the reaction is terminated by keeping the cells on ice, and washing three times with ice-cold PBS containing 20 mM d-glucose.

4. Add 50 μl 0.1% SDS to solubilize cells. Following protein estimation, transfer the lysate to unifilter-96/GFB plates (PerkinElmer, Waltham, MA, USA) and allow to dry at 37 °C for 6 h.

5. Add 20 μl of scintillation fluid (PerkinElmer) per well and radioactivity incorporated into cells is quantified with a top count microplate scintillation counter (Packard, Albertville, MN, USA).

6. Nonspecific uptake, measured in the presence of 10 μM cytochalasin B is to be subtracted from all the values obtained. To examine the specificity of signaling, pretreat the cells with pharmacological inhibitor of insulin signaling activation. This assay aids in establishing a dose response curve (EC-50) of insulin or any GLUT4 modulator. EC-50 of insulin in CHO-HIRc-mycGLUT4eGFP cells is found to be 1 nM.

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**Figure 14.4** Confocal microscopic analysis of GLUT4 translocation in CHO-HIRc-mycGLUT4eGFP cells. GLUT4 translocation was visualized based on GFP fluorescence or immunostaining of myc epitope present in nonpermeabilized cells. As compared to control cells, treatment with GLUT4 modulators like insulin increases immunoreactive chimera on the plasma membrane.
4. CONCLUSIONS AND FUTURE APPLICATIONS

GLUT4 translocation in response to its activation can be monitored and measured by real time GLUT4 translocation assay described here. Being rapid and fool-proof assay, it offers multiple methods for application in antidiabetic drug discovery. Presence of a fluorescent GFP tag aids in automatically monitoring GLUT4 translocation from perinuclear space to the PM by GLUT4 modulators. Considering the fact that several lead compounds await screening and identification for GLUT4 modulation activity, the strategy presented here may augment the antidiabetic drug discovery process.

ACKNOWLEDGEMENTS

We thank Dr. G. C. Mishra, Director, NCCS for being very supportive and giving all the encouragement to carry out this work. We thank Department of Biotechnology, Government of India for providing financial support. We thank Dr. M. P. Czech, University of Massachusetts Medical School, Worcester, MA, USA for generously gifting pGreen Lantern mycGLUT4eGFP construct and Dr. M. Bernier, National Institute of Aging, Baltimore, MD, for kindly providing CHO cells over expressing wild-type insulin receptor (CHO-HIRc). We thank Dr. Amrendra Kumar Ajay for image analysis and Mrs. Aswini Atre for confocal microscopy studies. We thank Journal of Bioscience for allowing us to reproduce from the published article in this chapter.

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