

GLUT4 Dispersal at the Plasma Membrane of Adipocytes: A Super-Resolved Journey

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ABSTRACT

In adipose tissue, insulin stimulates glucose uptake by mediating the translocation of GLUT4 from intracellular vesicles *to* the plasma membrane. In 2010, insulin was revealed to also have a fundamental impact on the spatial distribution of GLUT4 *within* the plasma membrane, with the existence of two GLUT4 populations at the plasma membrane being defined: 1) as stationary clusters and 2) as diffusible monomers. In this model, in the absence of insulin, plasma membrane-fused GLUT4 are found to behave as clusters. These clusters are thought to arise from an exocytic event that retains GLUT4 at the fusion site; this has been proposed to function as an intermediate hub between GLUT4 exocytosis and re-internalisation. By contrast, insulin stimulation induces the dispersal of GLUT4 clusters into monomers and favors a distinct type of GLUT4-vesicle fusion event, known as fusion-with-release exocytosis. Here, we review how super-resolution microscopy approaches have allowed investigation of the characteristics of plasma membrane-fused GLUT4 and further discuss regulatory step(s) involved in the GLUT4 dispersal machinery, introducing the scaffold protein EFR3 which facilitates localisation of phosphatidylinositol 4-kinase type III α (PI4KIII α) to the cell surface. We consider how dispersal may be linked to the control of transporter activity, consider whether macro-organisation may be a widely used phenomenon to control proteins within the plasma membrane, and speculate on the origin of different forms of GLUT4-vesicle exocytosis.

INTRODUCTION

GLUT4 Trafficking

Glucose plays a pivotal metabolic role in most mammalian cells. Its diffusion from the blood stream into cells relies on a family of fourteen glucose transporters, known as GLUTs, that are expressed in a tissue-specific manner **(1)**. Following isolation of GLUT1 cDNA in 1985 **(2)**, a further thirteen members of the GLUT family were identified: GLUT2 to 12, HMIT, and GLUT14 **(1, 3-6)**.

In 1939, Einar Lundsgaard first observed an insulin-mediated increase in glucose uptake into cat skeletal muscle **(7, 8)**. This phenomenon was subsequently shown, first in adipocytes **(9-11)** and later in muscle cells **(12, 13)**, to rely on a specific trafficking system that involves the insulin-dependent translocation of glucose transporters - subsequently identified as GLUT4 - from intracellular compartments, now defined as GLUT4 storage compartments (GSC) **(14)**, to the plasma membrane (PM) (Figure 1). In both adipose and muscle tissues, GLUT4 is the main glucose transporter to undergo insulin-stimulated translocation **(15-19)**.

In adipocytes, it is now established that in the absence of insulin 95% of cellular GLUT4 transporters accumulate in GSC, which includes the *trans*-Golgi network (TGN), endosomes, and 'insulin responsive vesicles' (IRV) **(20-22)**. Upon insulin stimulation, up to 70% of the sequestered GLUT4 undergoes exocytosis to the PM **(20, 23, 24)**. IRV are transported along cytoskeletal elements, facilitating their final tethering, docking, and fusion to the PM. As a consequence, GLUT4 levels at the PM increase with a concomitant increase in glucose entry into the cells **(21, 22, 25, 26)**. Upon insulin removal, most of the PM-localised GLUT4 are re-internalised into IRV **(25)** (Figure 1). Failure of GLUT4 to undergo insulin-stimulated translocation to the PM represents a major manifestation of insulin resistance in type-2-diabetes **(25)**. This has in turn driven extensive efforts to understand the itinerary of GLUT4 trafficking within cells, and to understand how its delivery to and from the PM is regulated. The complexity of GLUT4 trafficking is exemplified by the number of proteins identified as regulators of different aspects of intracellular GLUT4 storage, the levels of many changing in type-2-diabetes, and by the difficulties in unravelling the pathways involved in its endocytotic trafficking **(27)**. These areas have been the subject of many excellent reviews **(21, 22, 26, 28-32)**.

GLUT4 Trafficking Revealed by GFP

The discovery and subsequent use of green fluorescent proteins (GFP) as localisation markers for sub-cellular structures **(33, 34)** fundamentally altered the scope of questions which could be addressed by cell biologists. In 1996, GLUT4-GFP was expressed in 3T3-L1 adipocytes, allowing for real-time observation of GLUT4 insulin-mediated translocation to the PM for the first time **(35)**. Subsequently, a hemagglutinin (HA)-tag was added to the first exofacial loop of the GLUT4-GFP construct and expressed in primary rat adipocytes. Using confocal microscopy, this allowed distinction of intracellular and PM-fused GLUT4 populations **(36)**. These studies laid the foundations for many future projects using fluorescence microscopy to investigate GLUT4 dynamics, and the HA-GLUT4-GFP construct became widely used in studies of GLUT4 traffic.

The main site of GLUT4 function is at the PM. While the steps of GLUT4 trafficking to the PM have been dissected and insulin established as the main regulator, less is known about the dynamics of GLUT4 *within* the PM. 30% of human genes encode membrane proteins **(37)** and given that one of the most fundamental functions of the PM is the transport of materials/signals into and out of the

cell, complex cellular mechanisms likely regulate the activity and location of membrane proteins such as GLUT4. To study the behaviour and regulatory mechanisms of individual or group of molecules within the PM, structural and dynamic information is required on length scales, below the resolving power of traditional light (optical) microscopy.

The ability of optical microscopes to distinguish individual structures from one another is indeed physically limited, as first described in 1873 by Ernst Karl Abbe **(38)**. Due to its wave nature, the light collected through an objective lens aperture which emanates from a single point source, such as a fluorophore, ultimately produces an image that is broadened into a series of concentric rings and lacks the sharpness of the original details within the illuminated specimen (Figure 2).

Following on from Abbe's work, it was established that light microscopes cannot distinguish between two objects closer than $\lambda/2NA$, where λ is the wavelength of light used to image the specimen and NA is the numerical aperture of the imaging lens. More specifically, NA is defined as $n \cdot \sin\theta$, where n is the refractive index of the medium in which the light propagates between the point source and the objective (Air: $n = 1$, Immersion Oil: $n = 1.52$) and θ is the half-angle of the maximum cone of light that can enter the objective lens aperture (Figure 2). Accordingly, the only way to increase the resolving power of an imaging system is through some combination of reducing λ or increasing NA, both of which have physical limitations.

Microscopy techniques which do not rely on illumination with light, such as electron microscopy, do not suffer from the limit outlined above. However, while electron microscopes can offer molecular and even atomic resolution **(24)**, the need for specimen fixation and destructive mounting means that biologists still favour fluorescence microscopy, despite the limit on resolution, which allows live imaging of specimens in atmospheric conditions and non-invasively. In the past 30 years, there has been a concerted effort to surpass the diffraction limit of light, resulting in a new family of fluorescence imaging, known as super-resolution microscopy. After an overview of these approaches, we consider in this review their impact on our understanding of GLUT4 (Figure 3).

SUPER-RESOLUTION MICROSCOPY - A NEW ERA OF MICROSCOPY

Super-resolution microscopy, or nanoscopy, bypasses the diffraction limit imposed by the physical properties of light by utilising phenomena in optical physics and molecular chemistry to resolve structures previously only observable with electron or atomic force microscopy, but without the rigorous and often destructive sample preparation required by these methods. The range of light (optical) techniques which fall under the umbrella of super-resolution microscopy is extensive, and ever-growing. However, there are several useful review articles which enable researchers to informatively select the most appropriate super-resolution method for their purposes **(39-44)**. Broadly, the field of super-resolution microscopy can be split into two categories: techniques which rely on shaped illumination, and localisation-based methods.

Shaped Illumination Techniques

The first category of super-resolution microscopy approaches includes techniques which rely on altering the excitation beam path and therefore how it is projected onto the studied specimen. The oldest approach within this family is termed Total Internal Reflection Fluorescence (TIRF) microscopy. This technique improves the axial resolution of diffraction limited systems by employing the evanescent byproduct of a laser beam undergoing Total Internal Reflection, to excite exclusively fluorophores within a few hundred nanometres of the specimen-substrate interface **(45)**. As will be briefly discussed below, this approach is widely used to study GLUT4-vesicles dynamics (Figure 3).

A further technique is Structured Illumination Microscopy (SIM), which includes 2D-SIM, a method where typically nine spatially altered illumination grating patterns are sequentially applied into the excitation beam path. Subsequent computational processing of images acquired using this approach allows for a two-fold improvement in lateral resolution, beyond the diffraction limit of the imaging objective lens **(46)**. To extend this concept to volumetric imaging, SIM can also be combined with LightSheet Microscopy (LSM). While LSM is a diffraction-limited technique, it has provided great biological insight over the years **(47)**. By decoupling excitation and illumination paths, this multi-objective, high-resolution shaped illumination method allows fine optical sectioning of thick biological samples without the need for physical sectioning. Super-resolution LSM-SIM, also known as Lattice LSM, therefore uses the diffraction grating of SIM, alongside a lightsheet illumination source to achieve the spatial resolution of SIM through depth **(47)**.

Finally, a much improved lateral spatial resolution, on the order of 30-50 nm, can be achieved using STimulated Emission Depletion (STED) microscopy **(48, 49)**. This method relies on having two laser sources, a conventional Gaussian excitation beam and a longer wavelength masked 'donut-shaped' depletion beam which overlap, and force emitting fluorophores within the profile of the depletion beam into a dark ground state. Therefore, only fluorophores within the unmasked region of the excitation beam are detected, giving rise to sub-diffraction limit resolution. Consequently, this approach is considered as somewhat less gentle.

Single Molecule Localisation Microscopy (SMLM) Techniques

The second family of super-resolution techniques, namely Single Molecule Localisation Microscopy (SMLM) methods, was developed on the basis that the precise localisation of a single fluorophore within a studied image can be established by mapping its intensity to a Gaussian function, the peak of that function being directly related to the localisation where the most photons are

produced. However, as previously explained, due to the diffraction limit of light, when viewed under a microscope, the size of the image, as well as the intensity distribution, of the fluorophore will appear larger than its true size, limiting the spatial resolution of a conventional light microscope with a high NA to around 250 nm laterally and 550 nm in the axial direction, depending on the used λ (40). This signifies that, if positioned at a lower distance from each other than the wavelength of the light used to image them (<250 nm), closely labelled proteins will appear as a single fluorescent entity when viewed through a microscope, leaving their precise localisation undetectable (Figure 2). SMLM methods therefore approach visualising structures below the diffraction limit by iteratively switching on and off fluorescent molecules, such that only a sparse population of single molecules are detected at any one time.

The first methods for achieving SMLM were shown with Photo-Activated Localisation Microscopy (PALM), where the specimen was imaged and subsequently bleached through depth (50), and STochastic Orientation Reconstruction Microscopy (STORM), which used photo-switchable fluorophores to image single molecules over several thousand image frames (51). Subsequently, the use of conventional cyanine dyes with specialised buffer solutions became increasingly popular (52), allowing for direct STORM (dSTORM), the direct nature coming from the fact that fluorescent antibody pairs are not required (53, 54). More recently, fluorescent transiently-binding single DNA strands have re-emerged as a versatile tool, allowing for improved single molecule localisation with the development of Point Accumulation In Nanoscale Topography (DNA-PAINT) (55).

One big family

Truly, both super-resolution families do not have to remain as separate entities. An efficient means of improving the localisation precision of SMLM has been demonstrated by the concurrent use of shaped illumination methods, with TIRF microscopy lending itself well as the most popular illumination method for SMLM. Its thin, tunable depth optical range significantly reducing background fluorescence and thus increasing localisation precision. A further technique, termed ModLoc, was developed to employ structured modulation patterns, allowing for a uniform 6.8 nm axial localisation precision through several microns of a specimen (56). The principle of STED and SMLM have also been combined to achieve isotropic resolution on the order of 1-3 nm in a technique called MINFLUX (57).

A variety of methods have also been developed as purely computational and post-processing in nature, such as Super-resolution Optical Fluctuation Imaging (SOFI) (58) and Super-Resolution Radial Fluctuations (SRRF) (59), allowing for super-resolution to be achieved using diffraction-limited systems.

Collectively, these tools open the range of structures and biological processes that can be studied. Below we consider the impact of some of these on GLUT4 biology (Figure 3).

GLUT4 DYNAMICS NEAR AND WITHIN THE PLASMA MEMBRANE

The emergence of new fluorescence microscopy approaches permits investigations beyond the diffraction limit of light and have allowed further study of GLUT4 behaviour *near* (approaching and adjacent to the PM) and *within* the PM (i.e., post fusion) (Figure 3). Some key studies performed to date are reviewed here. We use the term 'GLUT4-vesicles' to reflect a level of ambiguity/controversy regarding which sub-population of the GSC is being studied **(22)**.

Insulin-Stimulated GLUT4-Vesicle Fusion

In 2004, using EGFP-fused GLUT4, Li et al. were the first to apply TIRF microscopy to investigate the three-dimensional mobility of GLUT4-vesicles approaching the PM in live 3T3-L1 adipocytes. Tracking GLUT4-vesicles using TIRF and single-particle analysis revealed the restricted movement of GLUT4-vesicles within a mean radius of 160 nm from the PM, suggesting the presence of an intracellular tethering matrix **(60)**. GLUT4-vesicles were found to exhibit a smooth continuum of 3D diffusional coefficients, indicating that despite there being several potential routes for GLUT4-vesicles to reach the PM, they appear to be organised in a continuous range of mobility and do not reflect distinct 'classes' of GLUT4-vesicles (see below) **(60)**. In the absence of insulin, similar behaviour of GLUT4-vesicles rapidly moving along the PM, periodically stopping and loosely tethering to the membrane, were also observed by applying time-lapse TIRF microscopy in primary rat adipocytes **(61)**.

The use of a dual-coloured probe to follow GLUT4-vesicle trafficking in TIRF mode also represented a further advance, allowing quantification of fusion events as well as vesicular location. Insulin was observed to induce a 40-fold increase in the fusion of GLUT4-vesicles with the PM in 3T3-L1 adipocytes compared to basal conditions. However, it was observed that a fraction (~15%) of GLUT4-vesicle fusion to the PM exhibited a fusion-with-retention or 'kiss-and-run' type event, where PM-fused GLUT4 molecules are retained at their sites of fusion before being re-internalised from the PM **(62)**. Lizunov et al. similarly showed that insulin regulates immobilisation, tethering, and fusion of GLUT4-vesicles with the PM in primary rat adipocytes **(63)**.

TIRF microscopy, as well as SIM, STED, and TIRF-SIM, have all been deployed to study aspects of GLUT4 intracellular trafficking and delivery to the cell surfaces; see for example **(60-68)**.

Insulin-Regulated GLUT4 Dispersal Within the Plasma Membrane

While the studies outlined above confirmed that insulin affects intracellular GLUT4 trafficking and fusion to the PM, in 2010, it was suggested that insulin also has an impact on the spatial distribution of GLUT4 within the PM **(69)**. Using multi-colour TIRF microscopy (Figure 3), the existence of GLUT4 molecules present as stationary 'clusters' at the cell surface was revealed. It was further suggested that a portion of GLUT4 in the PM existed as monomers. Stenkula et al. posited that these monomers derive from the exocytosis of GLUT4-vesicles involved in fusion-with-release type events, where GLUT4 transporters are shown to rapidly spread out into the PM, away from their sites of fusion. Experimentally, the authors showed that in the basal state, fusion-with-retention events represent 95 % of all fusion events (Figure 1). At this point, the amounts of clustered and monomeric GLUT4 at the PM were found to be broadly similar. Insulin was observed to increase the total fusion rate of GLUT4-vesicles with the PM from 0.03 events/ $\mu\text{m}^2/\text{min}$ to 0.15 events/ $\mu\text{m}^2/\text{min}$ after only 2 minutes of stimulation. This increase was shown to be the result of

the differential stimulation of both previously described modes of exocytosis **(62)**, with a 60-fold increase on the number of fusion-with-release events against a 2-fold increase in fusion-with-retention. These boosts in fusion events could account for observed increases in both total PM-fused GLUT4 as well as clustered and dispersed GLUT4, respectively (Figure 1). Hence, suggesting that in the basal state, fusion-with-retention is the primary GLUT4 provider of the PM in the form of clusters. Insulin stimulation then triggers fusion-with-release events where a significant amount of GLUT4 is delivered to, and dispersed into, the PM. Overall, these observations demonstrated that GLUT4 proteins are distributed non-homogeneously within the PM. Nevertheless, it was established that upon insulin-stimulation the total amount of translocated GLUT4 corresponded broadly to a cellular increase in glucose uptake, suggesting that both GLUT4 populations at the PM are functional glucose transporters **(69)**.

Considering the above data, Stenkula et al. proposed a new kinetic model to explain GLUT4 behaviour among GSC and the PM, which they based on the inclusion of specific parameters. The first being that, at the PM, insulin only affects the dispersal of GLUT4 (i.e., not its activity), and the second that the amount of GLUT4 in all GSC, apart from IRV, remains relatively constant upon insulin stimulation. This extends previous models **(70)** by adding GLUT4 clusters as a new quasi-compartment. Under basal conditions, PM-fused GLUT4 are mostly found to localise in this compartment, which function as intermediate hubs between GLUT4 exocytosis and re-internalisation. Upon insulin stimulation, this model considers a rapid increase in GLUT4 monomers at the PM primarily due to an increase in GLUT4-vesicle fusion, more specifically fusion-with-release events (Figure 1). Based on this new kinetic model, Stenkula et al. provided evidence for a hitherto unidentified step to GLUT4 trafficking system: insulin increases GLUT4 dispersal within the PM, from clusters to monomers, and glucose uptake becomes more efficient **(69)**.

The GLUT4 dispersal model is readily amenable to probe using super-resolution microscopy, and different techniques have been developed to further investigate its dynamics within the PM in the presence of insulin. By transfecting adipocytes with HA-GLUT4-EOS probe and using a TIRF-PALM microscopy system (Figure 3), Lizunov et al. confirmed that insulin contributes to the transfer of GLUT4 from a clustered to a monomeric state (Figure 1). Insulin increases GLUT4 monomer dissociation from clusters, as well as decreasing the rate of GLUT4 endocytosis **(71)**. The spatial arrangement of HA-GLUT4-GFP within the PM of 3T3-L1 adipocytes has been studied at a single molecule level using dSTORM **(72, 73)** (Figure 3). The studies reinforced the idea that insulin stimulates GLUT4 dispersal *within* the PM, after fusion of GLUT4-vesicles, highlighting a novel facet of GLUT4 biology with potentially important consequences. Further, our group assessed whether GLUT4 dispersal operates in cell types other than adipocytes. We ectopically expressed HA-GLUT4-GFP in either induced pluripotent stem cell-derived cardiomyocytes or HeLa cells, and in both cell-types observed insulin-stimulated GLUT4 dispersal **(74)**. Of note, insulin-stimulated dispersal of the transferrin receptor (TfR), was not observed in HeLa cells, supporting the hypothesis that this mechanism is unique to GLUT4; however, we will return to this point below.

Impaired GLUT4 Dispersal in Insulin Resistance

In 2017, Gao et al. used dSTORM to image HA-GLUT4-GFP at the PM of basal and maximally insulin-stimulated 3T3-L1 adipocytes **(72)**. Consistent with data from TIRF microscopy, in the presence of insulin, the authors observed a shift in the distribution of GLUT4 at the PM to a more dispersed configuration. Interestingly, experimental insulin resistance was also observed to increase GLUT4 clustering, suggesting a link between the clustering ability of GLUT4 and insulin

sensitivity **(72)**. Similar results were observed by our group **(74)**. This link was further revealed when considered how GLUT4 dispersal is correlated with cell size **(74, 75)**. Adipocytes of different sizes have been shown to exhibit distinct metabolic properties: larger adipocytes exhibit reduced insulin-stimulated glucose transport compared to smaller cells and are associated with adverse metabolic outcomes **(76)**. Strikingly, we observed that GLUT4 dispersal is reduced in larger cells, supporting the hypothesis that larger adipocytes are refractory to insulin challenge and further suggesting that GLUT4 dispersal is an integral facet of the cellular response to insulin. Such studies emphasise a need to understand the mechanism(s) which underpin this dispersal.

GLUT4 DISPERSAL MACHINERY

It is now well established that many molecular mechanisms that regulate membrane traffic are conserved through evolution, from single cell organisms such as yeast to metazoans, and many seminal findings in the GLUT4 field have initially been made using genetically tractable systems such as *S. cerevisiae*, *D. melanogaster* and *C. elegans*. When heterologously expressed in *S. cerevisiae*, human GLUT4 is sequestered intracellularly, as observed in adipose and muscle cells (77). Such observations indicate that the pathway followed by GLUT4 in insulin-sensitive cells is conserved through evolution from yeast to mammals and has allowed the use of yeast as a model system to study GLUT4 trafficking and thus potentially identify key genes involved in aspects of GLUT4 biology.

Wieczorke et al. used a strain of *S. cerevisiae* engineered to lack hexose transporters (Δ hxt) and therefore unable to grow on media supplemented with hexoses as carbon source. Interestingly, the expression of mammalian (rat and human) GLUT4 in this mutant did not rescue growth on glucose (78). Previous studies showed that, when heterogeneously expressed in *S. cerevisiae* with partially deleted endogenous glucose transporters, rat GLUT4 are retained intracellularly and do not contribute to glucose uptake (79). However, Wieczorke et al. observed a clear redistribution of ectopically expressed human GLUT4 toward the PM in their Δ hxt yeast strain (78), supporting the idea that regulatory machinery of GLUT4 *within* the PM may play an important role in glucose cellular uptake. A genetic screen to generate mutants of the Δ hxt strain that enable the expressed GLUT4 to support glucose uptake identified the recessive mutant *fgy1-1* allele (78, 80). This allele encodes a mutant version of *EFR3*. Efr3 is a membrane protein, essential for the assembly of Stt4-containing patches at the PM. Stt4 is the yeast ortholog of the human phosphatidylinositol 4-kinase type III α (PI4K-III α), which catalyses the synthesis of phosphatidylinositol 4-phosphate (PI4P), an essential signalling lipid at the PM (81, 82).

EFR3 and PI4K-III α Regulate GLUT4 Dispersal in Adipocytes

Our group tested the hypothesis that EFR3 and PI4K-III α play key roles in the insulin-stimulated regulation of GLUT4 at the PM, in adipocytes (73). With a 62% sequence identity, EFR3 was shown to be evolutionarily conserved from mammals to yeast and in higher eukaryotes to have two paralogs: EFR3A and EFR3B. Both proteins contain a N-terminal cysteine rich region that encodes palmitoylation sites, allowing anchoring at the PM. Adjacent, armadillo-repeats permit protein-protein interactions (83). In 3T3-L1 adipocytes, EFR3A was shown to have a higher expression, with mRNA levels >500-fold higher than EFR3B (73).

SiRNA knockdown of EFR3A or PI4K-III α significantly reduced insulin-stimulated glucose transport in 3T3-L1 adipocytes (73), and in as yet unpublished data, we have further shown that the PI4K-III α selective inhibitor C7 (Ximbio, P/N 153579) (84, 85) also significantly reduced insulin-stimulated glucose transport (Angéline Geiser, unpublished work). Collectively, these data support the hypothesis that that EFR3A and PI4K-III α play a role in insulin-stimulated glucose transport in adipocytes. Given the location of EFR3A and PI4K-III α at the PM, we asked whether depletion of EFR3 might affect GLUT4 dispersal. We used 3T3-L1 adipocytes expressing HA-GLUT4-GFP and adopted a dSTORM approach (Figure 3) to assess the effects of EFR3A knockdown on GLUT4 dispersal in the presence and absence of insulin. siRNA-mediated knockdown of EFR3A significantly inhibited the ability of insulin to promote GLUT4 dispersal, suggesting that depletion of EFR3A is required for the transition of GLUT4 from clustered to dispersed monomers in the PM (73).

Our data are consistent with the hypothesis that EFR3A and PI4K-III α are critical components of a newly identified mechanism that controls insulin-stimulated GLUT4 dispersal at the surface of adipocytes. Future challenges will be to understand how this relates to GLUT4 dynamics in the PM, how the insulin signalling machinery interacts with EFR3A and PI4K-III α , and the role of specific phosphoinositide species in controlling dispersal.

UNANSWERED QUESTIONS, LIMITATIONS AND SPECULATIONS

With the advent of both ultra-fast imaging (image capture rates of up to 10 kHz have recently been described; **(86)**) and super-resolution microscopy (Figure 3), the macro-organisation of membrane proteins within the PM, specifically has received much attention, enhancing understanding of the regulation of oligomerisation (**87-89**), the assembly of mobile and non-mobile structures (**88-91**), the role of lipid rafts (**92**), and the concept of transient confinement zones (**92, 93**). Hence, there is an increasing understanding that the regulation of proteins within the PM can have significant implications on their function, as has been suggested by studies on GLUT4 dispersal described above. Increased understanding inevitably leads to more focussed questions: how widespread is this phenomenon of dispersal, does dispersal act to regulate the activity of glucose transporters, and how are the different modes of GLUT4 exocytosis rationalised within existing models? Some studies have begun to shed light on these questions.

Dispersal and Clustering of GLUTs - Size of Clusters and Effects on Activity

Using dSTORM, Yan et al. studied the behaviour of GLUT1 at the cell surface HeLa cells (**94**). Results showed that GLUT1 forms clusters, suggested to be regulated by lipid rafts, the actin cytoskeleton, as well as by glycosylation of GLUT1. Clusters were observed to possess an average diameter of 250 nm on the apical membrane (i.e. medium-exposed membrane) and 137 nm on the basal surface of cells (i.e. facing the coverslip) (**94**). Estimations of the number of GLUT1 in these structures revealed that the majority contained between two and four molecules, and that around 35% of these clusters were associated with lipid rafts. While these data provide clear evidence in favour of GLUT1 adopting a clustered state, it should be noted that these estimates are subject to the linkage error of labelling antibodies (see below) (Figure 3). Hence, the precise stoichiometry of these clusters remains uncertain. It is important to clearly state that our studies using dSTORM are subject to similar limitations, thus our reluctance to quantify numbers of GLUT4 molecules per clusters (**73, 74**).

Nevertheless, Yan et al. considered how GLUT1 clustering may be related to its activity. Both methyl-cyclodextrin (a cholesterol-depletion agent) and sodium azide (a metabolic poison) are known to mediate a small increase in glucose transport in HeLa cells. dSTORM analysis suggested that upon treatment with these agents, the number of GLUT1 monomers increased, while the apparent diameter of clusters decreased. These results further suggest that the activation of GLUT1 might correlate with changes in the molecular organisation of its clusters (**94**).

While correlative, these observations provide a further potential link between GLUT dispersal and increased activity. Using classical kinetic approaches, GLUT1 oligomerisation has been implicated in the control of transport activity, but how this oligomerisation relates to clusters reported by dSTORM remains uncertain (**95-97**). A driver for further consideration of this point has been provided by recent data indicating that some mutations of GLUT1-deficiency syndrome, involving reduced expression or loss of function of GLUT1 in the brain, may impact GLUT1 oligomerisation (**98**). This is an exciting area of research worthy of further investigations.

Some comparison with data on GLUT4 on this point is informative. Using our dSTORM datasets for control and EFR3 knockdown adipocytes (**73**), we used a recently described ImageJ plug-in (**99**) to quantify the amount of GLUT4 molecules present at the surface of those same cells. While the knockdown of EFR3A significantly decreased GLUT4 dispersal at the PM, total levels of GLUT4 in both control and EFR3A-knockdown cells appeared to be similar, suggesting that EFR3A is not

engaged in the translocation of GLUT4 to the PM (**73**). Under the same conditions however, insulin-stimulated glucose transport is impaired upon EFR3A knockdown. Hence, similarly to GLUT1, these (correlative) findings further suggest that regulation of dispersal may represent a physiological control mechanism for this group of transport proteins. Further studies are needed to evaluate this hypothesis and are presently on-going in our laboratory.

Dispersal and Clustering - A Behaviour of PM-Resident Proteins?

The question of whether other PM-localised proteins exhibit clustering/dispersal abilities is also worthy of further consideration. This was to some extent addressed by comparing the behaviour of GLUT4 and the TfR in insulin-stimulated HeLa cells (**74**). While effects of insulin on clustering of GLUT4 were small (but still clearly evident), data revealed that the TfR did not appear to exhibit insulin-dependent dispersal. However, it would be wise to replicate these studies in physiologically insulin sensitive cells.

Related to this point, a fascinating recent study has provided evidence for insulin receptor incorporation into 'dynamic clusters' at the PM of hepatocytes and adipocytes (**100**), revealing a dynamic redistribution of insulin receptors into so-called 'condensates'. Biomolecular condensates are proposed to be compartments within cells into which proteins can be concentrated without being delimited by a membrane. First observed in T-cells upon activation of the T-cell receptor (**101**), these condensates been widely reported to act as a foci for organising signalling platforms. Studies using covalently linked probes to monitor the properties of the membrane environment around integral membrane proteins have revealed that insulin drives a significant change in the fluidity of the PM in the region of the receptor, while exerting minimal effects on the average PM fluidity (**102**). These data suggest that changes in the lipid microenvironment may underscore changes in clustering behaviour. How these relate to GLUT4, and other protein clusters remains to be revealed.

Dispersal and Clustering - Solving the Antibody Linkage Issue

As previously described, super-resolution microscopy has allowed to gain substantial understanding of GLUT4 *within* the PM of adipocytes (Figure 3), with the identification of new GLUT4 dispersal regulatory actors. However, in this review, we also highlight multiple unanswered questions about the clustering nature of GLUT4, and how these uncertainties relate to the limitations of certain super-resolution microscopy techniques. For instance, while dSTORM generates consistent imaging datasets, the requirement of fluorescently labelled antibodies decreases the quantitative localisation accuracy of targeted molecules due to their size. As a result, each recorded localisation corresponds to much larger structures than the initial proteins of interest, artificially scaling-up molecular clustering measurements. The application of labelling antibodies therefore automatically introduce a localisation offset from the imaged protein of around ~10 nm (**103**), a phenomenon known as the antibody 'linkage error'.

This error can be somewhat minimised in post-processing analysis (**73**), by using smaller labelling nanobodies, or obviated by using different imaging systems. For instance, DNA-PAINT (**55**) (Figure 3) employs designed pairs of oligonucleotides, where fluorescently conjugated *imaging* stands controllably and transiently bind selected protein target previously-labelled with complementary *docking* strands, with sub-10 nm localisation precision. In this instance, the transient binding of *imaging* stands represents blinking events that can be recorded over several thousand imaging

frames, protected from photobleaching and representative of smaller structures when compared to both direct and indirect immunofluorescence.

In recent years, several adaptations to the DNA-PAINT system have been introduced. While some allow for brighter and faster DNA-PAINT imaging through the design of novel transient probes **(104)**, others have improved the nanometric localisation accuracy of DNA-PAINT into the Ångström domain **(105)**. Employing either variation of DNA-PAINT for the study of GLUT4 at the PM would allow for a circumvention of the antibody linkage error seen in other SMLM imaging systems, with the added benefits of improved localisation accuracy and acquisition speed. Work is underway in our group to address this.

Clusters or Oligomers? Same or Different?

Protein structural databases posit that many, if not most, proteins can form homo-oligomers **(106)**. Verification of this hypothesis is limited due to the challenging nature of the experimental approaches required. For instance, energy transfer methods such as fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) have been employed to argue the close proximity of proteins **(107, 108)**. However, these methods are limited as they cannot differentiate between true oligomers and molecules that are only 'very close' to each other **(109)**, a limitation that is similarly applicable to all previously described super-resolution microscopy techniques (see above). Other microscopy based methods, to determine the oligomeric state of proteins, focus on the analysis of the temporal fluctuation of fluorescence intensity of a fluorescent protein of interest **(110, 111)**. These approaches are challenging in the confines of PM-localised proteins.

G-protein coupled receptors (GPCRs) are an example of a family of proteins that exist in different oligomeric states, which affects their function and in some cases even their location within the cell **(109, 112)**. The dynamics of GPCR interactions have been studied using various approaches, including Spatial Intensity Distribution Analysis (SpIDA). In SpIDA, the protein of interest is tagged with a fluorescent protein and imaged using conventional confocal microscopy, in either live cells or fixed samples. From these images, the fluorescence intensity of the fluorescent protein is measured. On a conventional laser-scanning confocal microscope, this is usually achieved using an analogue photomultiplier tube that counts the number of collected photoelectrons **(113)**. The intensity of all pixels in a region of interest on the acquired image is then plotted as an intensity histogram over which a Poissonian distribution is fitted. This distribution will vary from monomeric, oligomeric, to a mixed populations and is used to calculate the quantal brightness ϵ of the fluorophore **(114)**. ϵ is defined as the mean intensity within a point spread function of a fluorescent unit **(115)**. The quantal brightness ϵ of a primarily monomeric population is defined as ϵ_0 . By reason, for a dimeric population ϵ will equal $2 \cdot \epsilon_0$, $3 \cdot \epsilon_0$ for a trimeric population, and so on. This approach has provided a powerful insight into the quaternary structure of different GPCRs **(116-119)**, and has for example revealed the antagonist-dependent oligomerisation behaviour of M_1 muscarinic acetylcholine receptors **(118)**.

We attempted to use this approach to examine the behaviour of GLUT4 tagged with EGFP **(Silke Morris, unpublished work)**. While a trend towards a reduction in the oligomeric state of GLUT4 was observed, this did not reach significance. In addition, this analysis cannot distinguish between GLUT4 within the PM from GLUT4 within vesicles adjacent to the PM, hampering the utility of this approach.

Fusion-with-Retention and Fusion-with-Release - Some Speculations

A final question concerns what mechanism(s) may underscore the different GLUT4-vesicle exocytosis events alluded to above? The notion of a specialised subset of GLUT4-vesicles that fuses with the PM specifically in response to insulin has received a good deal of attention and experimental support as discussed above. However, as we and others have noted, the ability of insulin to promote increased levels of GLUT4 at the cell surface is found in a broad range of cells (including HeLa cells **(120)**, podocytes **(121)** and neurones **(122)**), hinting toward more of a ubiquity in GLUT4 translocation machinery than initially thought **(123)**.

A recent study may offer some clues. By combining multiple types of quantitative analyses based on dual-colour live-cell imaging and super-resolution microscopy approaches (STED and TIRF-SIM) (Figure 3), Hatakeyama et al. recently provided new insights into GLUT4 trafficking events and suggested an alternative to the differential GLUT4-vesicle delivery model discussed above **(67, 124, 125)**. These studies focus on both GLUT4 and the TfR, a well-known recycling protein that, while insulin-responsive, exhibits a reduced extent of translocation compared to GLUT4. In 3T3-L1 adipocytes, Hatakeyama et al. documented anchoring mechanisms statically retaining GLUT4 in their storage compartments, close to TGN. Insulin was then shown to mediate the ‘heterotypic endosomal fusion’ of these static GLUT4-containing vesicles with mobile TfR-containing endosomes, followed by GLUT4 ‘liberation’, or individual movement, within these new compartments (Figure 4). As a result, the authors propose endosomal GLUT4 trafficking, rather than differential GLUT4-vesicle delivery as described previously, to the PM as a new insulin-responsive GLUT4 translocation paradigm **(67, 124, 125)**. This reflects to our earlier decision of using the term ‘GLUT4-vesicles’ rather than specific sub-population of GSC, highlighting the uncertainties surrounding the balance of these two mechanisms operating at any given point *in vivo*.

These models need not to be incompatible. It is entirely possible that in specialised cells, such as adipocytes and muscle cells, a portion of IRV which localise near the PM, are rapidly mobilised in response to insulin, as previously suggested **(126)**. After this initial ‘bolus’ of GLUT4 delivery, it is conceivable that other delivery mechanisms, such as those outlined in Figure 4 predominate. This is likely to be important as endocytosis will seek to re-internalise GLUT4 from the PM, unless it is rapidly re-exocytosed. It is also important to note that the experimental systems’ extremes (no insulin or maximal insulin concentrations) do not represent physiological situations, and thus may bias interpretation and analysis of different mechanisms. The balance between these two delivery ‘pathways’ may perhaps underlie the proposed distinction in fusion-with-retention and fusion-with-release observations. Further work over a range of kinetic periods is clearly warranted.

CONCLUSION

Understanding the dispersal mechanisms which control proteins within the plasma membrane represents a new frontier for cell biology and exemplifies the importance of interdisciplinary research. While this review highlights evidence in favour of GLUT4 dispersal in response to insulin, understanding the biological relevance of this mechanism and its operation in a range of cells, other proteins and disease, offers the potential for fascinating new cellular insight and potential new therapies.

Author Contribution

Geiser - Conceptualisation, writing and editing; literature survey; figure preparation. **Foylan** - Writing and editing of original draft; literature survey. **Tinning** - Writing and editing. **Bryant** - Conceptualisation and editing; funding acquisition. **Gould** - Conceptualisation, writing and editing; funding acquisition.

Acknowledgements

Work in the Gould lab is supported by grants from BBSRC (BB/X005178/1 and BB/V019643/1), EPSRC (EP/V048031/1 and studentship EP/T517938/1) and Diabetes UK (18/0005847 and 18/0005905; both held jointly with NJB).

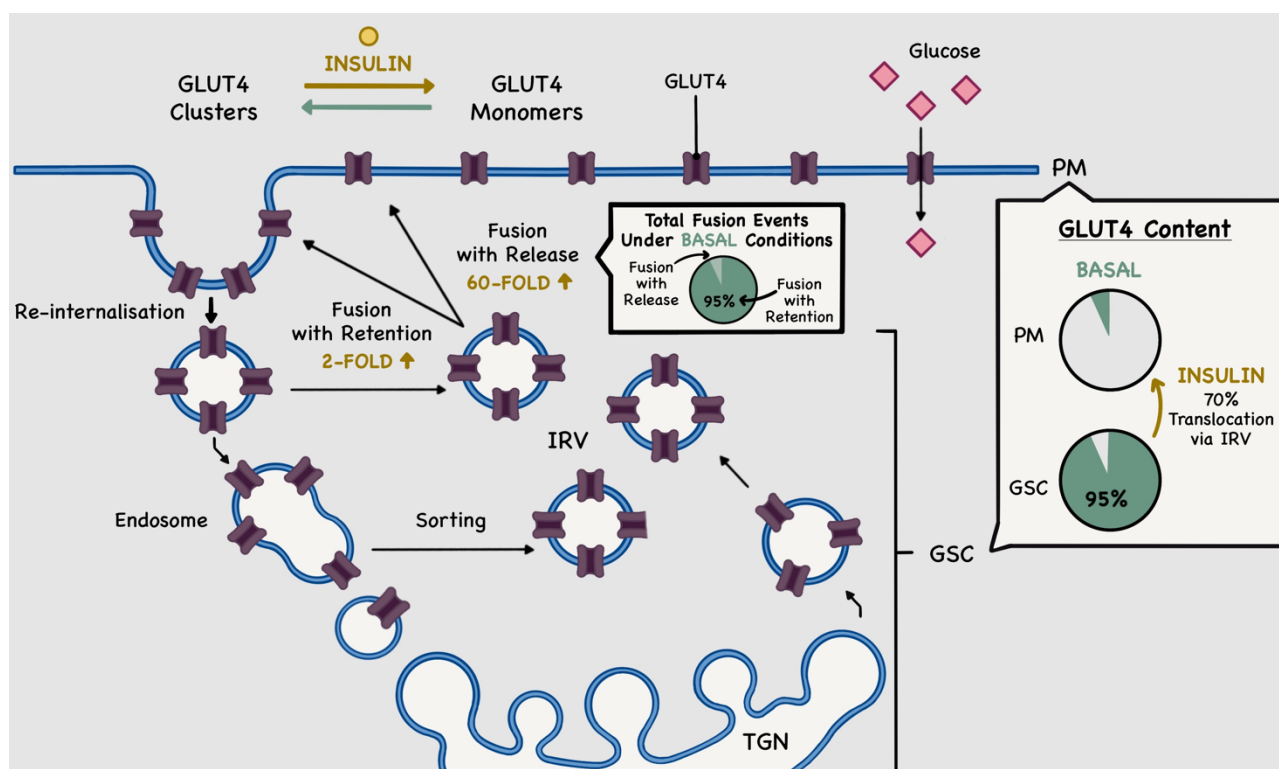


Figure 1. Model of GLUT4 trafficking to, and dispersal within, the plasma membrane in adipocytes. In adipocytes, insulin regulates GLUT4 translocation to, and dispersal within, the plasma membrane (PM). Under basal conditions, 95% of cellular GLUT4 transporters (shown as purple structures) accumulate in GSC, including the *trans*-Golgi network (TGN), endosomes, and insulin responsive vesicles (IRV). Upon insulin stimulation, up to 70% of the sequestered GLUT4, primarily in IRV, translocate to and fuse with the PM. In terms of exocytosis, in the basal state, 95% of all fusion events happen in a fusion-with-retention manner, upon which PM-fused GLUT4 molecules are retained at their site of fusion forming clusters. In the presence of insulin, the number of fusion-with-retention events increases by 2-fold. Fusion-with-release events are increased by 60-fold. GLUT4 transporters therefore primarily undergo exocytosis through the fusion of IRV and release into the PM as monomers, leading to increased and more efficient glucose (pink) uptake into the cell. Upon insulin removal, GLUT4 molecules are re-internalised and recycled back into the GSC. For details, refer to text and (69).

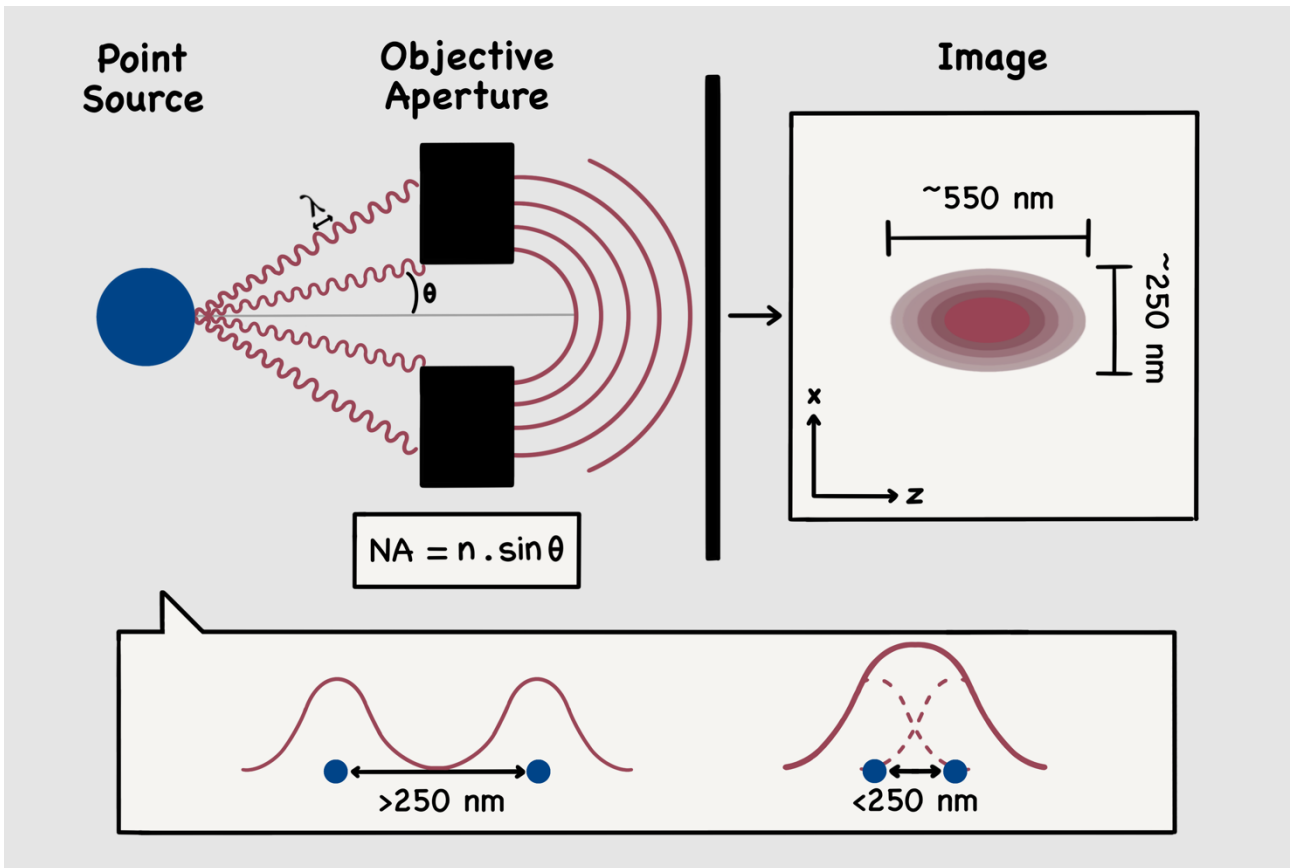


Figure 2. Diffraction-Limited Resolution of Light (Optical) Microscopy.

As it passes through the objective aperture, the emitted light emanating from a single point source (blue) is diffracted into a series of concentric rings, ultimately producing an image that is broadened and lacks the sharpness of the original illuminated details. Using a conventional light microscope with a high numerical aperture (NA), the spatial resolution of the image point source is limited to around 250 nm laterally (x) and 550 nm axially (z), depending on the used wavelength (λ). While multiple point sources separated by a distance larger than this resolution limit appear as two separate entities in the image, if positioned at a closer distance, a single entity will be observed. $NA = n \cdot \sin \theta$, where n is refractive index between the point source and the objective (Air: $n = 1$, Immersion Oil: $n = 1.52$) and θ is the half-angle of the maximum cone of light that can enter the objective lens aperture.

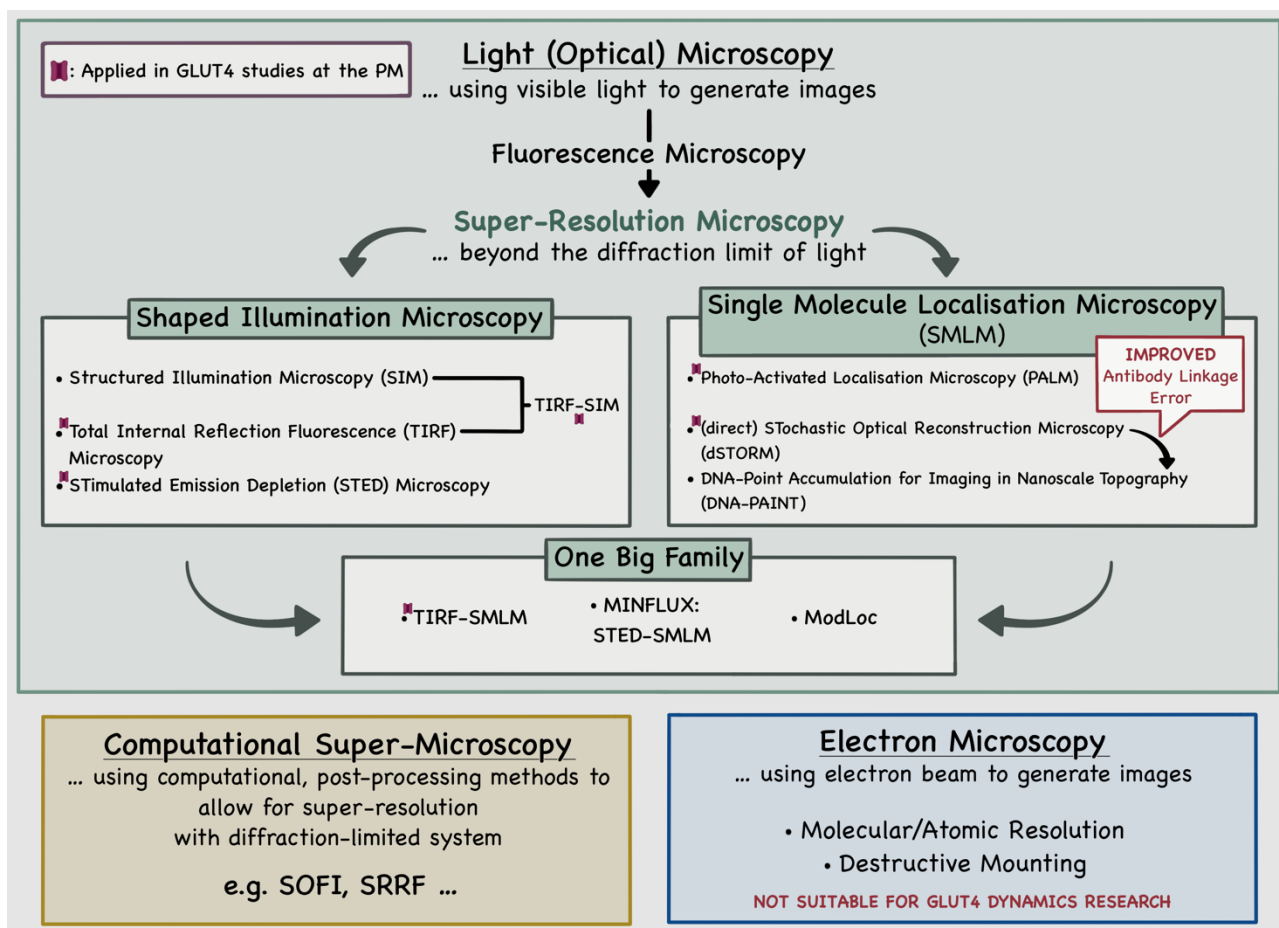


Figure 3. Super-Resolution Microscopy Approaches and GLUT4 Studies at the Plasma Membrane. This figure illustrates an overview of super-resolution light (optical) microscopy in the GLUT4 field and highlights (purple structures; GLUT4) which techniques have been applied to elucidate the mechanism of GLUT4 behaviour *within* the PM: TIRF (69); TIRF-SIM (67, 68) STED (67); PALM & TIRF-SMLM (71); dSTORM (72-74). TIRF, SIM, STED, and TIRF-SIM, have also all been deployed to study aspects of GLUT4 intracellular trafficking and delivery *to* the cell surface; see for example (60-68). Note that in dSTORM, requirement of fluorescently labelled antibodies decreases the quantitative localisation accuracy of targeted molecules due to their size, known as the antibody 'linkage error'. This error is improved (minimised) in DNA-PAINT. Computational super-microscopy is included to highlight that these relatively new approaches offer potential to further enhance our resolution and understanding of GLUT4 dynamics using diffraction-limited systems. The reader is referred to several excellent reviews to informatively select such methodologies for their purposes (39-44). For details, refer to text.

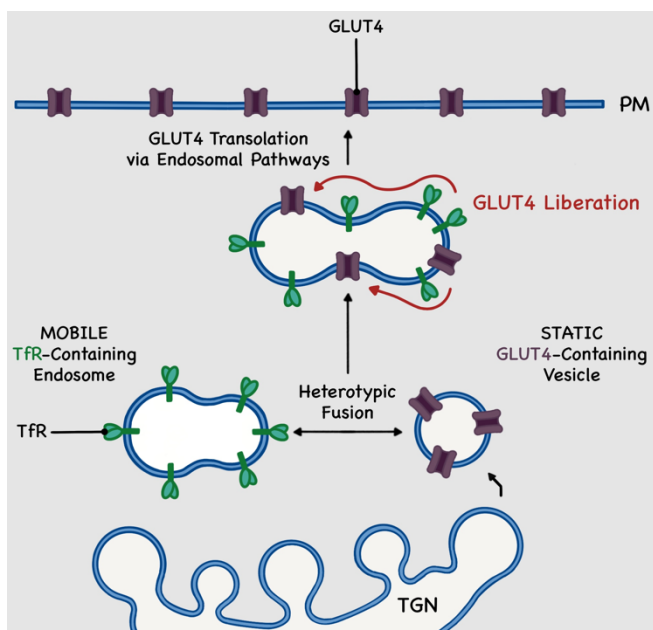


Figure 4. Endosomal-dependant model of insulin-stimulated GLUT4 trafficking to the plasma membrane.

Alternative model of GLUT4 trafficking to the plasma membrane via endosomal pathways. Insulin stimulates the heterotypic fusion of static GLUT4 (purple)-containing vesicles with mobile transferrin receptor (TfR; green)-containing endosomes in the *trans*-Golgi network (TGN) and perinuclear region. Upon entry into TfR-containing endosomes GLUT4 transporters are liberated from their static status and get dispersed amongst TfRs. In this newly formed compartment, GLUT4 translocate to the plasma membrane by taking advantage of the trafficking properties possessed by TfR-positive endosomes. This model differs from that shown in Figure 1 in the nature of the GLUT4 storage compartments and their subsequent mobilisation to the plasma membrane. This figure emphasises on a static intracellular pool of GLUT4 which, in the presence of insulin, reaches the cell surface via an endosomal pathway rather than a direct GLUT4-vesicle delivery. For details, refer to text and (117).

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