Nuclear import mechanism of EGFR in breast cancer cells

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By

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DECLARATIONS

I declare that the experiences and results described in this thesis were carried out by myself in the School of Biology and Biomedical Sciences, Durham University, under the supervision of Dr Martin W. Goldberg. This thesis has been composed by myself and in record of work that has not been submitted previously for a higher degree. Work cited other than my own is clearly indicated by reference to the relevant workers or their publications

Elie Ngandu Mpoyi

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Dedications

This thesis is dedicated to my most lovely family who have supported me during this period and since I was born; Pierrot Tshibuyi, Regine Kabisa, Francine Masengu, Henoc Bangana, Rachel Miandabu, Pierrette Kabisa, Joel Mukanya, Nathan Lukusa and my cherished grandmother Jeanne Masengu mua Mpoyi in Canada. Their love, generosity and everlasting support was more of a fuel to keep me going. I am really grateful to them for emotional, moral and financial support. Thank you to my entire family, friends and relatives for accepting me in their world.

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Abstract

Receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR) are internalised from the plasma membrane by endocytosis and may be transported to the nucleus. EGFR, a receptor for EGF and other RTKs, HER-2 and HER-4 has an important role in signalling; it contains transactivational activity and can function as a transcription co-factor to activate gene promoters. High nuclear accumulation of imported full length EGFR is associated with an increased tumour proliferation and a reduced survival in cancer patients. However, little is known about the mechanism by which membrane-bound proteins, such as EGFR, translocate from the cell surface into the cell nucleus; how nuclear membrane proteins cross through the NPC to reach the INM. The mechanism of translocation for soluble proteins is also presently unclear. EGFR nuclear import is mediated by importin α/β. And it is exported from the nucleus by the exportin CRM1. Sec61β which may reside in the inner nuclear membrane (INM) is required for the release of EGFR from the INM into the nucleus. Nuclear transport involves binding of nuclear localisation sequences (NLSs) within the cargo to a transport receptor (karyopherins or importin). Karyopherins interact with certain nuclear pore complex (NPC) proteins (nucleoporins). Membrane proteins can access the INM through the NPC membrane: by diffusion, using classical nuclear transport factors (the importin/Ran system); or by an ATP dependent mechanism. EGFR may use the former mechanism. This work concentrates to show by electron microscopy and by Immuno-Fluorescence that upon EGF treatment, the biotinylated cell surface EGFR is trafficked to the INM through the NPC, yet remaining a membrane-bound protein. We also confirm that importin regulates EGFR nuclear transport to the INM and in addition, Sec61β is required for EGFR release to the nucleoplasm.

Altogether, this study of the mechanism of EGFR nuclear-cytoplasmic import in breast cancer cells, further confirms previous reports and provides an understanding of the nature and regulation of the nuclear EGFR pathway and the mechanism by which cell-surface EGFR is shuttled in the cell cytoplasm and channelled through the Golgi and Endoplasmic Reticulum (ER) compartments and into the nucleus through the NPC.

Keywords: EGFR, NPC, Breast cancer, importin

Abbreviations

EGFR	Epidermal Growth Factor Receptor
CPD	Critical Point Drying
EGF	Epidermal Growth Factor
INM	Inner Nuclear Membrane
ONM	Outer Nuclear Membrane
RTKs	Receptor Tyrosine Kinases
HER	Human Epidermal Growth Factor Receptor
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediaminetetraacetic acid
min	minute
ml	millilitre
NE	nuclear envelope
ICM	Intracellular membrane
ECM	Extracellular membrane
INM	Inner nuclear membrane
ONM	outer nuclear membrane
NPCs	Nuclear Pore Complexes
NE	Nuclear Envelop
ATP	Adenosine Triphosphate
CRM1	Chromatin Region Maintenance, Exportin-1
Nups	Nucleoporins
NP	Nucleoplasm
kDa	Kilodalton
kb	kilobase
TGF	Tumour Growth Factor
FGFR	Fibroblast Growth Factor Receptors

FGF	Fibroblast Growth Factor
IL1,2,5	Interleukin
FS	Freeze-substitution
cNLS	classical Nuclear Localisation Sequences
siRNA	small interference Ribonucleic Acid
DNA	Deoxyribonucleic Acid
Cy	Cytosol
Nu	Nucleus
NM	Nuclear Membrane
ER	Endoplasmic Reticulum
FG	Phenylalanine-Glycine
IFNs	Interferons
ECD	Extracellular Ligand-Binding Domain
TMD	Transmembrane Domain
CTK	Cytoplasmic Tyrosine Kinase
IF:	ImmunoFluorescence
EM	Electron Microscopy
WB	Western Blots
COOH	carboxyl-terminus, C-terminal
NH2	amino-terminus, N-terminal
Imp	Importinβ
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
PBG	Phosphate Buffer glucose
PFA	Paraformaldehyde
RTKs	Receptor tyrosine kinases
WT	Wild Type

Aims of the study

Receptor tyrosine kinases are transported from the cell surface into the cytoplasm by various pathways; EGFR being one of the RTKs. Among the epidermal growth factor receptor (EGFR) signalling pathways are (I) the traditional or classical EGFR pathway that involves transduction of mitogenic signals through activation of multiple signalling cascades and (II) the recent novel direct pathway in which activated EGFR undergoes nuclear translocalization and subsequently regulates gene expression and potentially mediates other cellular processes. The EGFR signalling pathway can be triggered by ligand binding and exposure to vitamin D, radiation, cisplatin, heat and H_2O_2; which in turn initiates both the traditional/classical pathway and the novel direct pathway. There are accumulating suggestions indicating that other RTKs of the ErbB family members such as ErbB-2 may be using similar nuclear transport pathways as EGFR. So far, significant advances have been made towards the knowledge of the mode of EGFR signalling pathway. The pathways have been characterised; nevertheless, the detailed mechanism of the nuclear EGFR pathways is yet to be clarified. Nuclear transport of EGFR involves interaction with different proteins, Importinβ, Sec61β. It is documented that EGFR interacts with Importinβ and Sec61β during its nuclear transport; yet it is not clear when and where EGFR recruits Importinβ and Sec61β. More has been discovered but our current knowledge raises questions. Many studies have shown by IF the translocation of EGFR to the nucleus and to the INM; nonetheless few studies have shown in details EGFR import by EM. For that reason, the aim of this work was set to observe by EM and by IF microscopy the interaction of EGFR/Importinβ, EGFR/Sec61β and that EGFR resides in the INM.

As nuclear EGFR has been implicated in cancer, in particular in breast cancer, this study of the EGFR pathway, has been carried out in breast cancer cell lines because there are many supporting data suggesting that EGFR might be a key agent that is invaluable in the signalling pathway of some cancer types, therefore we believe that understanding of its particular different pathways might be of therapeutically advantages. A long term goal would be to identify therapeutic targets. Indeed, the nuclear transport mechanism of EGFR can also be used as a model for other cell-surface receptors.

This is what we know about EGFR nuclear transport, but more details are needed to clarify the models presented.

Chapter 1: General Introduction

Cancer

Cancer, medically known as a malignant neoplasm, is a broad group of various diseases caused by an uncontrolled growth of normal cells that form a lump called a "tumour" (De Vita, Hellman et al. 2008, Perry 2008). Cells divide and grow uncontrollably, developing malignant tumours that invade surrounding parts of the affected tissue or part. What affects one body tissue may not affect another; for instance, overexposure to the sun could cause melanoma cancer of the skin, not lung cancer which could be caused by smoking (De Vita, Hellman et al. 2008, Perry 2008). All tumours are not cancerous, benign tumours do not grow uncontrollably; they do not invade neighbouring tissues or spread throughout the body. There are currently over 200 different forms of cancer known to develop in humans as there are over 200 different types of body cells grouped into epithelial tissue cells, connective tissue cells and cells of the blood and lymphatic system (Perry, 2008). Cells that make up the lungs can cause a lung cancer; yet there are different cells in the lungs, so these may cause different types of lung cancer. The most common forms of cancer to cite are bowel cancer, breast cancer, lung cancer, prostate cancer; all these are of a significant social and economic burden to our society (De Vita, Hellman et al. 2008, Perry, 2008). Bowel cancer is more common in developed countries and preeminent in people over the age of 65; in the UK, it is the third most common cancer in women after breast and lung cancer, and the third most common in men after prostate and lung cancer. Breast cancer is most common in women with the greatest incidence in more-developed countries, accounting for 11,762 deaths in the UK in 2011. Lung cancer, 9 in 10 lung cases occur in people over 60; rates in Scotland are among the highest in the world, reflecting the history of high smoking. Prostate cancer, develops generally in men over 50 with around 40,000 men in the UK diagnosed each year; http://www.cancerresearchuk.org/cancer-info/cancerstats/keyfacts/worldwide/. All types of cancer, each presenting both stereotypical and unique phenotypes, involve unregulated cell growth; (Dalerba and Clarke 2007, De Vita, Hellman et al. 2008, Perry 2008, Bansal and Banerjee 2009, Weiner, Murray et al. 2012).

Cancer research is going on all over the world and determining what causes cancer and how to treat and prevent it is complex. A single research paper cannot give the whole picture about research into a particular cancer. Most cancers (about 85%) are derived from epithelial cells, and are called carcinomas. The commonest epithelial carcinoma is breast cancer, with around 50,000 diagnoses in the UK each year, of which only ~300 are men. Breast cancer is the most common cancer in women (De Vita, Hellman et al. 2008, Perry 2008, Weiner, Murray et al. 2012), and for that reason, funds

are raised for research to understand its cause and process and to discover possible therapies to treat and prevent this frightening disease.

Researchers have shown much evidence suggesting that cancer onset is multifactorial. Environment, genetic predispositions and life style are among many other factors that could cause cancer. All these factors have a universal link, alterations or damage to genetic material. Genetic factors mean that some individuals have an increased chance of contracting the disease. Cancer is caused by gene alterations or deletions/losses that lead to unregulated cell growth, (De Vita, Hellman et al. 2008, Perry 2008, Weiner, Murray et al. 2012). Genes are coded messages; one gene 'codes' for one protein. Proteins are building blocks of a cell. Some proteins act as 'on and off switches' that control cell behaviour. For example, EGFR (Epidermal Growth Factor Receptor, a 170 kDa glycoprotein encoded by a gene located on chromosome 7p12 is a therapeutic target for many cancers, (Shaib, Mahajan et al 2013)) and its family members when activated stimulate a complex cascade of signal transduction pathways, Ras/Raf/MEK/ERK and PI3K/AKT pathways (Figure 3) (Oda et al 2005; Wang, Lien et al. 2004). The cellular response depends on which pathways are activated and the duration of activation. This is partly dictated by the activating ligand and composition of the receptor dimer (Wells and Marti 2002, Wang, Lien et al. 2004).

Cancer therapies and EGFR

Almost all cells express EGFR, and several cancer cell types are found to over-express EGFR (Offterdinger, Schofer et al. 2002, Wang, Lien et al. 2004). High nuclear accumulation of full length EGFR is associated with an increased tumour proliferation as shown by increased expression of cyclin D1 and Ki-67 and a reduced survival in cancer patients (Stachowiak, Maher et al. 1997, Lo, Xia et al. 2005, Psyrri, Kassar et al. 2005, Hanada, Lo et al. 2006). The role of cell surface receptors in the nucleus (such as EGFR) is suggested to be in growth stimulation yet; there are still doubts about their function. These cell surface receptors interact with specific DNA sequences on the promoters of cyclin D1/inducible nitric oxide synthase and cyclooxygenase-2, stimulating genes activation. These gene products are involved in tumorigenesis and tumour progression, (Lo, Xia et al. 2005). EGFR is suggested to be involved in transcriptional regulation, cell proliferation, DNA repair, DNA replication, and chemo and radio resistance. If mutations happen in specific proteins in the EGFR signalling pathway, for instance PI-3K, MAPK, STATs…, the pleiotropic effects of EGFR signalling above might not function properly, thus, could contribute to uncontrolled growth, (Figure 3); and many studies on cancer have demonstrated various mutations in EGFR pathways. These studies have also evidently proved that nuclear EGFR is associated with poor clinical

prognosis for breast cancer, ovarian cancer, etc (Stachowiak, Maher et al. 1997, Lo, Xia et al. 2005, Psyrri, Kassar et al. 2005, Hanada, Lo et al. 2006).

Characteristics of cancer phenotypes are different (De Vita, Hellman et al. 2008, Perry 2008). Some are aggressive others benign. Detecting the onset or process of cancer is challenging despite current methods. Scientists are now using different approaches to improve cancer survival. Research focuses on developing new detection methods that can identify degenerating cancerous cells prior to metastasis. Prominent cancerous cells frequently express various surface markers that are not found on normal healthy cells (De Vita, Hellman et al. 2008, Perry 2008). These markers have alterations to protein composition. For ovarian and pancreatic cancer, a change in CD44 level is a known marker (Li, Heidt et al. 2007, Zhang, Lei et al. 2009). For most breast cancers, oestrogen receptor over-expression and HER2 are markers (Weigel et al 1995). These markers nevertheless only tell part of the story. More elements need to be identified to predict with more certainty the developmental stage, the nature of aggressiveness and invasion of the cancer. Hence in this study, we intended to investigate how EGFR plays a role in breast cancer.

There are many researches on cancer therapy (De Vita, Hellman et al. 2008, Perry 2008). The immune system alone is very unlikely to fight off an established cancer completely without help from conventional cancer treatment. Cancer is currently treated by various ways depending on the cancer type, stage, grade and the general health. Among the many therapies are chemotherapy, radiotherapy, surgery, hormone therapy, biological therapy and stress management. For biological therapy or immunotherapy, interferon, interleukin 2 (IL2), monoclonal antibodies are used for treatment (Perry, 2008, (De Vita, Hellman et al. 2008, Weiner et al., 2012). Antibodies invoke tumour cell death by blocking ligand-receptor growth and survival pathways by targeting surface antigens differentially expressed in cancer called markers. Rituximab targets CD20 in non-Hodgkin B cell lymphoma, Cetuximab targets EGFR in colorectal cancer, Trastuzumab targets HER2 in in certain breast cancer and Cetuximab targets EGFR in colorectal cancer, (De Vita, Hellman et al. 2008, Perry, 2008, Weiner et al., 2012).

EGFR and its downstream signalling pathway play a crucial role in normal cell growth and differentiation and are involved in tumour proliferation and survival. Aberrant expression or activation of EGFR and its downstream signalling proteins are frequently observed in cancer cells. It is believed that EGFR might be a key agent that is invaluable in the signalling pathway of some cancer types, and understanding of its particular different pathways might be clinically validated targets for cancer therapy (Lin, Song et al. 2008). Research has been carried out on EGFR in

different cell lines for various aims. This study aims to gain more detailed information on the import pathway of EGFR from the cell surface membrane to the INM in different cancer cell lines, MCF-7, MDA-MB-231. We examine whether EGFR nuclear-transport in these cell lines is different as we know for instance that, MCF-7 cells, less aggressive Luminal A subtype of breast carcinomas cell lines are treatable with Tamoxifen, whereas, MDA-MB-231 cells, highly aggressive basal subtype of breast carcinomas cell lines are not treatable with any of the current chemotherapies (Harari 2004, De Vita, Hellman et al. 2008, Perry 2008 , Weiner, Murray et al. 2012). Breast cancer is the most common cancer in the UK and the most common cancer in women. Breast cancer is not one single disease. There are several types of breast cancer, most of which have been found to over-express EGFR, (De Vita, Hellman et al. 2008, Perry 2008), therefore, more research is needed to gain more understanding of one of the factors that is believed to trigger its development (Luo, Solimini et al. 2009, Haber, Gray et al. 2011).

The Plasma Membrane

The Plasma membrane (PM) is composed of a bilayer of lipids and integrated proteins, whose interactions as one body enable it to receive, remember, process, and relay information along and across it (Grecco, Schmick et al. 2011; Sorkin and Von Zastrow 2002; van Meer, Voelker et al. 2008). These interactions form a signal transduction hierarchy of interconnected time- and length scales bridging more than three orders of magnitude, from nanometer-sized proteins to the micrometer scale of the cell (Sorkin and Von Zastrow 2002). The PM is not just a static barrier but a complex, dynamic organelle that integrates the cell with both its intracellular and extracellular environments (Grecco, Schmick et al. 2011; van Meer, Voelker et al. 2008). It functions in a dual way; isolating the cell from the extracellular environment while at the same time integrating the cell with its surroundings by transferring messenger molecules or initiating reaction cascades within it (Sorkin and Von Zastrow 2002, Grecco, Schmick et al. 2011). The PM is continuously renewed to preserve its non-equilibrium state. Its lipid composition is dynamically maintained by a combination of lipid synthesis and chemical conversion, vesicular fusion and fission events that tie into intracellular transport and sorting processes (van Meer, Voelker et al. 2008). The lipids in the PM do not only have structural function; they are subject to chemical modification and can thereby relay signals and allow for bidirectional information transfer (van Meer, Voelker et al. 2008). Lipids also provide a fluid matrix in which proteins reside and diffuse laterally; making up more than 50% of the cross-sectional area of the membrane and providing the machinery for most of the plasma membrane's dynamic properties (Grecco, Schmick et al. 2011; van Meer, Voelker et al. 2008).

The Nucleus

The nucleus is the largest membrane-enclosed organelle found in eukaryotic cells. The nucleus contains most of the cell's genetic material and it where DNA replication and RNA synthesis occur. It communicates with the cell through nuclear pores, exchanging proteins and RNA, for a variety of nuclear and cytoplasmic processes (Miao and Schulten 2010; Yamada, Phillips et al. 2010). The nucleus requires protecting and isolating the genetic information; therefore it is enclosed by a double membrane system, the nuclear envelope (NE); (Hetzer, 2010).

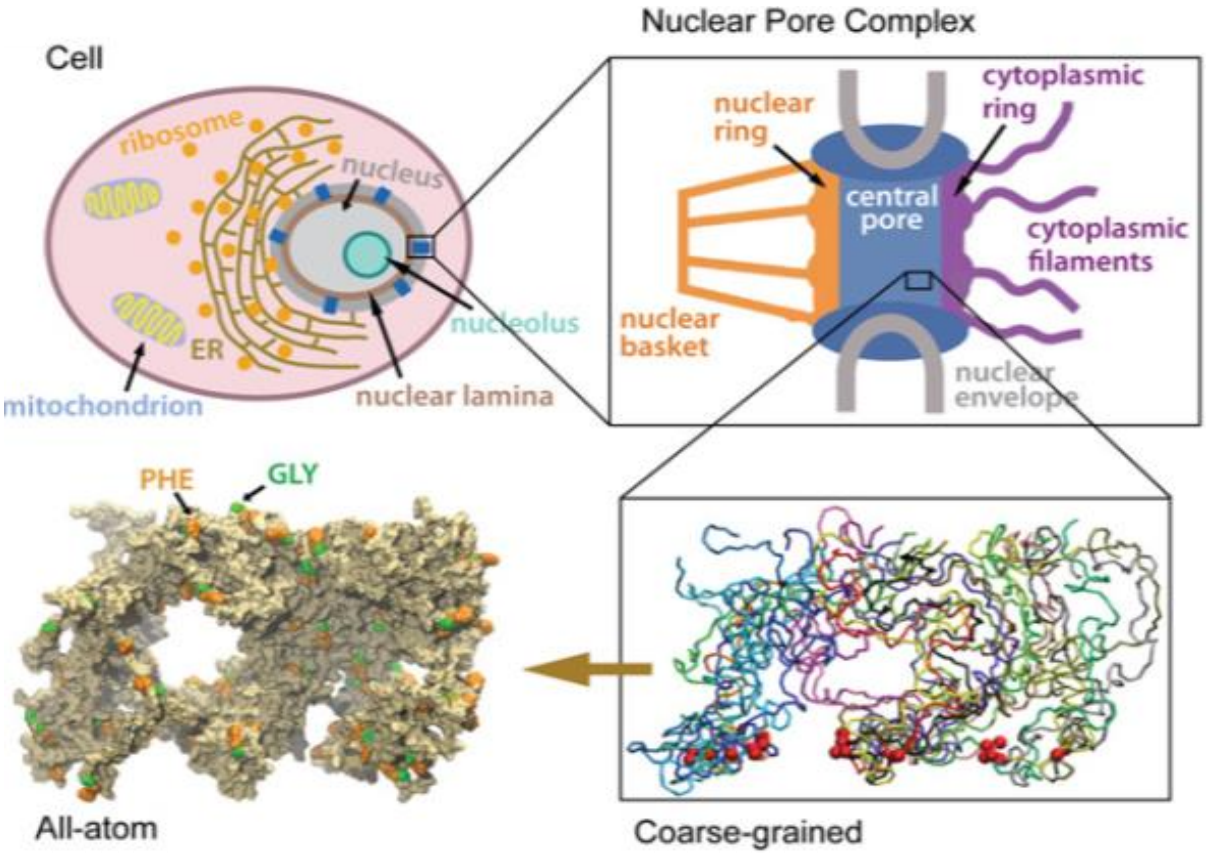


Figure 1. **The cell**. The top two figures show schematically the cross sections of a typical eukaryotic cell and of the nuclear pore complex. The wild-type bushlike structure of FG-nups adopted in two simulations (ntfm1 and exom6) is shown (bottom right, licorice representation) with each segment depicted in a different colour. (Bottom left) The same structure is rendered in surface representation. Adapted with permission from Miao & Schulten, 2010.

The Nuclear Envelope

The nucleus is separated from the cytoplasm by a double membrane structure, the nuclear envelope (NE). The NE, acts as a barrier that envelope the contents of the nucleoplasm (Goldberg and Allen, 1996). The NE contains over 100 transmembrane proteins that allow communication with the rest

of the cell, exchanging proteins and RNA, for a variety of nuclear and cytoplasmic processes which act in concert (Hetzer, 2010). The transmembrane proteins exchange with the ER and move within the nuclear membranes. It has two membranes that have different protein composition, an inner (INM) and outer nuclear membrane (ONM) linked by the pore membrane at locations where the nuclear pore complexes (NPCs) are inserted; (Figure 1) (Goldberg and Allen 1996, Hetzer, 2010, Miao and Schulten 2010, Wente and Rout 2010, Meinema, Laba et al. 2011). The ONM is continuous with the ER and the INM is associated with the underlying chromatin and lamins; (Batrakou et al. 2009, Hetzer, 2010, Schirmer and Gerace 2005, Wang, Wang et al. 2010). The space between the INM and ONM known as Perinuclear space is also continuous with ER space. NPCs are aqueous channels embedded in the NE that regulate the bidirectional trafficking of facilitated transport for macromolecules (>40 kDa) and passive diffusion for ions and small molecules (≤40 kDa) (Gorlich and Kutay 1999, Keminer and Peters 1999); however, some proteins that are less than 40kD are actively transported – e.g. histones. The Perinuclear space can fill with newly synthesized proteins just as the ER does (Wang et al., 2010). It is conceived that the exchange progress between the ONM and INM usually occurs where the NPCs are inserted in the membrane, although it is not fully understood (Goldberg and Allen 1996, Gorlich and Kutay 1999).

The nuclear pore complexes

The nuclear pore complexes (NPCs), possibly the largest protein complexes in the cell are composed of approximately 500 individual polypeptides representing multiple copies of about 30 different nuclear pore proteins (nucleoporins, Nups) that are present in multiple copies according to the 8-fold symmetry of the assembled structure; (de Las Heras et al., 2013; Goldberg and Allen, 1996; Labokha and Fassati, 2013). NPCs are formed at sites where the INM and ONM join and appear as if the two membranes (INM and ONM) are pinched at that site; (Figure 1) (Goldberg and Allen, 1996, Goldberg et al., 2000). They are composed of 8 subunits that "clamp" over the region of the INM and ONM where they join, forming a ring of subunits (Meinema et al., 2011). The subunit projects a spoke-like unit into the centre. The projected spoke is directed towards the central "plug' or granule. Nups are proteins that make up the NPC, (Goldberg and Allen, 1996). NPCs are made of a membrane-anchored scaffold that stabilises a cylindrical central channel, in which Nups with unfolded phenylalanine-glycine (FG)–rich regions provide the selectivity barrier; (Figure 1) (Meinema et al., 2011, Miao & Schulten, 2010, Yamada, Phillips et al. 2010). The pore serves as a channel and has a maximum diameter for passive diffusion of around 10 nm. Transport in and out

of the nucleus can occur in several ways, diffusion and active transport (Miao & Schulten, 2010, Yamada, Phillips et al. 2010; Ribbeck and Gorlich, 2001). Being the sole gates for regulated exchange, NPCs allow communication with the rest of the cell, exchanging proteins and other molecules; (Fiserova, Richards et al. 2010; Lo and Hung 2006; Ribbeck and Gorlich, 2001).

Epidermal Growth Factor Receptor

EGFR (Waterfield, Mayes et al. 1982) is a prototypical RTK among many other RTKs, such as fibroblast growth factor receptor (FGFR), insulin receptor, and TGF-β type I receptor that undergo nuclear transport. It is a member of the ErbB family of receptors, a subfamily of four closely related RTKs: EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4); (Jorissen, Walker et al. 2003, Bazley and Gullick 2005, Lo and Hung 2006, Wang, Yamaguchi et al. 2010). These receptors have functions and transactivational activity as transcription co-factors and exist as intact proteins in the nucleus. Cytokine receptor ligands including EGF, FGF, IL-1, IL-5, IFN-γ, TGF-α/β also exist in the nucleus and are involved in activation of EGFR and other nuclear receptors (Jans and Hassan 1998, Reilly and Maher 2001, Grasl-Kraupp, Schausberger et al. 2002).

EGFR (erbB-1) is a 170 kDa transmembrane protein with three main functional domains: a glycosylated extracellular ligand-binding domain (ECD), a hydrophobic transmembrane domain (TMD) and a cytoplasmic tyrosine kinase domain (CTK) (Figure 2A) (Bazley and Gullick 2005; Wang, Wang et al. 2010). When a ligand, i.e. EGF, binds to the extra-cellular domain, it triggers the formation of receptor homodimers and/or heterodimers (Figure 2B) and internalisation by endocytosis. Dimerization leads to activation of the intrinsic RTK domain and autophosphorylation of tyrosine residues inside the EGFR C-terminal tail in the cytoplasm, (Figure 2A) (Jorissen, Walker et al. 2003, Harari 2004, Bazley and Gullick 2005). This site-specific phosphorylation functions as docking sites for a range of adaptor proteins or enzymes, which in collaboration activate a cascade of intracellular signalling pathways; (Figure 3) (Jorissen, Walker et al. 2003, Bazley and Gullick 2005).

EGFR regulates fundamental biological processes such as cell proliferation, growth, invasiveness and development, (Figure 3) (Citri and Yarden 2006). Pleiotropic effects of EGFR signalling include apoptosis, mitogenesis, lysosomal degradation, transformed cellular motility, protein secretion and dedifferentiation or differentiation (Harari 2004, Bazley and Gullick 2005). There are currently two modes of EGFR signalling pathways. The classical pathway, which involves activation of multiple cascades and a new novel pathway in which growth signals are directly

transmitted to the nucleus, via EGFR nuclear transport; (Figure 3) (Carpenter 2003, Lo and Hung 2006, Wells and Marti 2002). EGFR is found on various normal cells including fibroblasts and a variety of epithelia. To date it is well established that cell-surface EGFR translocates into the nucleus. Nevertheless, the mechanism of transport is not well understood and remains to be investigated, (Lo and Hung 2006).

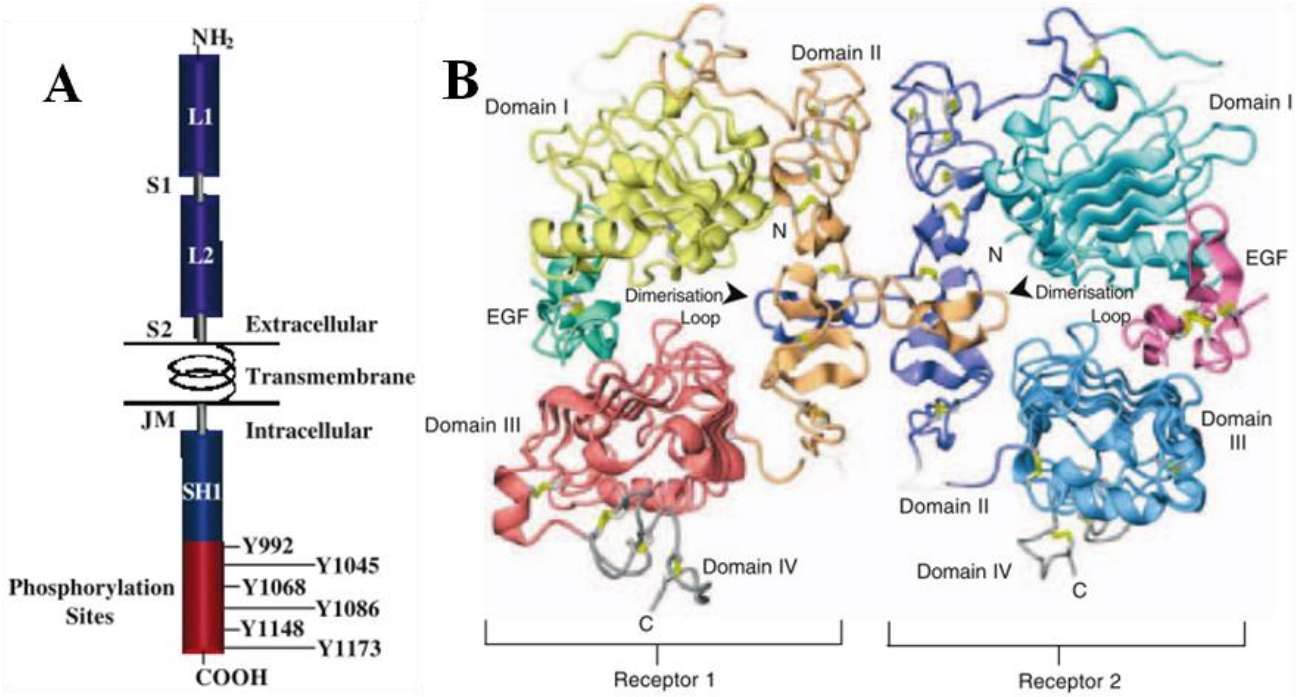


Figure 2. **Schematic representation of EGFR**. **A**, The EGFR monomer possesses an extracellular domain consisting of two ligand- binding subdomains (L1 and L2) and two cysteine-rich domains (S1 and S2), of which S1 permits EGFR dimerization with a second ErbB receptor. SH1 is the protein tyrosine kinase domain and resides in the cytoplasmic domain above the six tyrosine residues available for transphosphorylation. The transmembrane domain and juxtamembrane domain (JM) are required for the targeting of EGFR to caveolae. **B**, Ribbon diagram of the crystal structure of an EGFR homodimer in complex with two EGF ligands. The four subdomains I, II, III and IV of one receptor are coloured yellow, orange, red and grey respectively, while the corresponding domains of the second receptor are coloured cyan, dark blue, pale blue and grey. Held between domains I and III of each receptor are the EGF ligands (green and pink, held by receptors 1 and 2 respectively). Each dimerization loop (indicated by arrowheads) is formed by a b-hairpin and interacts with domain II of the other receptor via the seven residues at the tip of the loop. It can be seen that this structure depicts a 'receptor-mediated' dimerization mechanism by virtue of each receptor binding the other directly and one EGF molecule binding exclusively to one receptor. With permission from Jorissen et al., 2003 and Bazley & Gullick, 2005

When EGFR family members are activated, this stimulates a complex cascade of signal transduction pathways. The cellular response depends on which pathways are activated and the duration of activation. This is partly dictated by the activating ligand and composition of the receptor dimer.

Endocytosis of EGFR

Endocytosis in the majority of eukaryotic cells is a highly essential and well regulated process. It is required for trafficking proteins and recycling of PM lipids, and for uptake or down-regulation of cell-surface receptors such as EGFR, studied in this project. During endocytosis, the PM invaginates into the cell, forming vesicles that are then able to fuse with endosomes and enter the endolysosomal membrane system; (Smaczynska-de, Allwood et al. 2010). This process is known to involve more than 50 proteins, which assemble transiently at sites on the plasma membrane. Dynamins are essential to the endocytic progress in mammalian cells; although dynamins-like proteins in yeast are known to only have peripheral functions in endocytosis; (Smaczynska-de, Allwood et al. 2010). It is responsible for carrying out the invagination or scission of the membrane. As well as having roles in endocytosis, it is also believed that dynamins are involved the trans-Golgi network, endosomes and podosomes; (Smaczynska-de, Allwood et al. 2010).

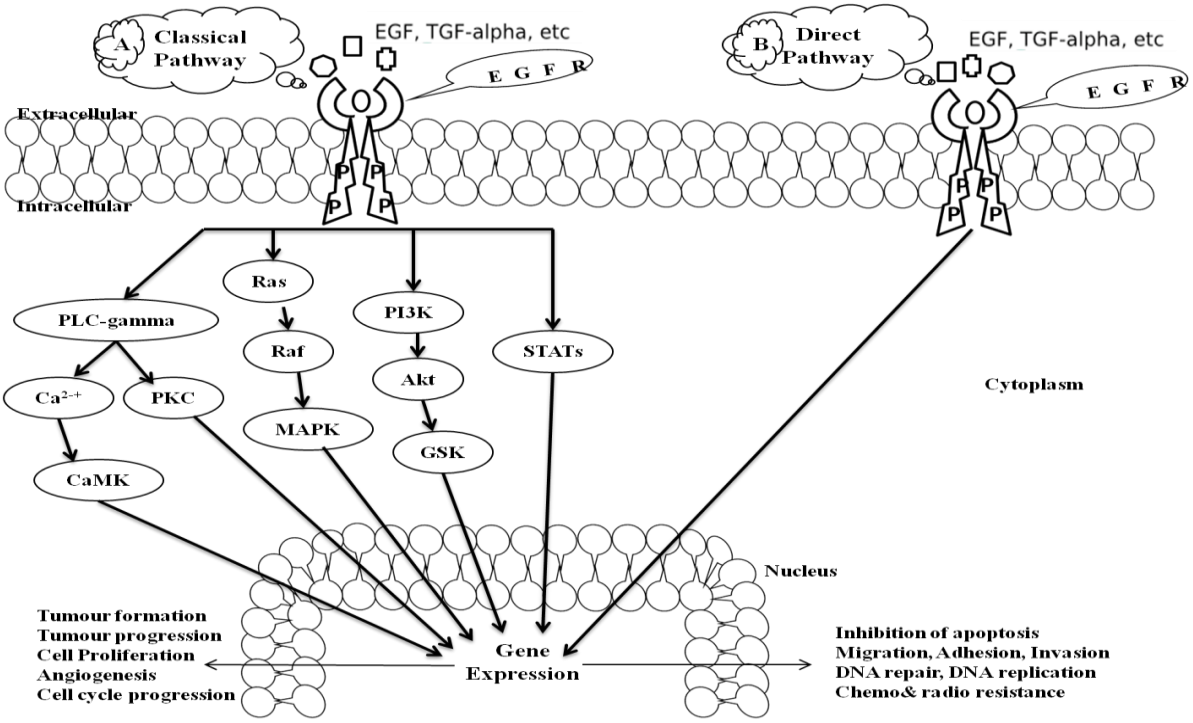


Figure 3. **The EGFR signalling pathway**. EGF, binds to the extra-cellular domain of EGFR, it triggers the formation of receptor homo/hetero-dimers. (**A**) The classical EGFR pathway consists of several key transduction cascades, namely, those involving PLC-Ca^{2+}-CaMK/PKC, Ras-Raf-MAPK, PI-3K-Akt-GSK, and STATs. Each of these pathways involves in transduction of growth factor signals from the cytoplasmic membrane, via activation of cascades of signalling molecules, to specific cytoplasmic targets and into the nucleus. These signalling cascades, once deregulated, lead to malignant transformation, increased proliferation rate, tumour progression and/or chemo-resistance. (**B**) The novel direct EGFR pathway involves EGF-activated nuclear translocalization of cell-surface EGFR and transcriptional regulation of target genes. Nuclear accumulation of EGFR is associated with increased tumour proliferation and poor patient survival. Its role in tumorigenesis, tumour progression and chemo-resistance remains un-investigated. Adapted with permission from different sources.

Nuclear transport of RTKs is mediated by the mechanisms that involve endocytosis and endosomal sorting; although the precise mechanisms remain obscure. EGFR translocation to the nucleus involves the endosomal sorting machinery; and studies suggest that for cell surface EGFR to enter the nucleus it might require EGF mediated internalisation; endocytic vesicles serving as carriers; (Lo and Hung 2006, Wang, Yamaguchi et al. 2010). However, after endocytosis/ internalisation, it is still unclear which pathway directs EGFR protein to the nucleus. This mechanism still raises many questions: (i) are there any vesicles that go directly from the cell surface to the nucleus; (ii) Why is EGFR trafficking through the Golgi and ER; (iii) How do RTKs embedded in the endosomal membrane translocate into the nucleus through NPCs and exist as nonmembrane - bound receptors in the nucleus? (iv) How does the membrane-bound EGFR traffic from the ONM to the INM?

EGFR is imported into the nucleus

Many studies have been done to understand how EGFR is transported from the plasma membrane into the nucleus. Although much effort has been put to this study, it remains unclear how full-length receptor proteins embedded in an endosomal membrane pass through the NPCs and function as non-membrane-bound receptors in the nucleus. Two important events takes place during the EGFR nuclear-cytoplasmic shuttling that is, receptor internalisation and EGFR/importin-β interaction, (Figure 6) (Wang, Wang et al. 2010, Wang, Yamaguchi et al. 2010, Wang, Yamaguchi et al. 2010).

Various groups have demonstrated by different methods the mechanism by which EGFR reaches the nucleus; Biochemical methods, Immunoblotting analysis of cell fractions, confocal-immunofluorescence and real time confocal imaging. All these techniques indicated that EGFR moves from the cell surface to the nucleus trough the NPC, and also localises in the INM; (Figure 4) (Wang, Wang et al. 2010). In this study, we tested these methods to confirm the claims. For instance, one of the experiments showed that emerin detection indicated the localization of EGFR in the INM. ER markers calnexin and calregulin, cell surface protein CD44, early endosome protein Rab5, late endosome protein LAMP1, and nuclear protein Sp1 absence in (Figure 4B, lanes 5 and 6) evidently showed undetectable cross-contamination; (Lo and Hung 2006, Wang, Yamaguchi et al. 2010). In the INM portions after EGF stimulation, the biotinylated EGFR precipitation increased significantly using streptavidin-agarose beads (Figure 4C, lane 2 versus lane 1), and similar results

were obtained using anti-EGFR antibodies to immunoprecipitate EGFR (Figure 4C, lane 4 versus lane 3).

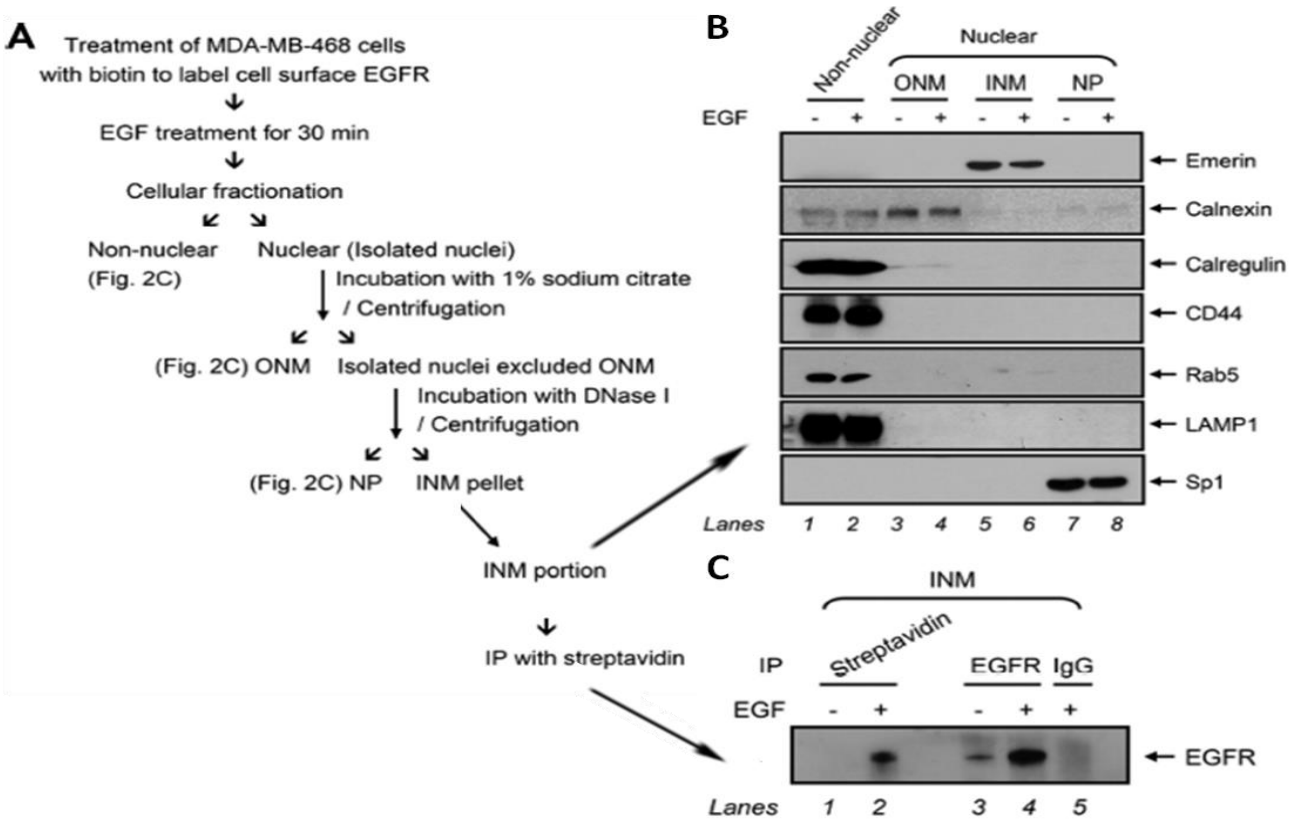


Figure 4. EGFR is imported to the INM in response to EGF. A, Schematic description of cellular fractionation of biotinylated cell surface proteins in MDA-MB-231 cells. IP, immunoprecipitation. **B**, INM portions of MDA-MB-231 cells cellular fractionation showing undetectable cross-contamination. Biotinylated cell surface proteins were isolated using cellular fractionation as described in A and subjected to immunoblotting with antibodies. **C**, cell surface EGFR was translocated to the INM upon EGF stimulation. The purified INM portions in C were immunoprecipitated using streptavidin-agarose beads and anti-EGFR. IgG was used as a negative control. Adapted with permission from (Y.-N. Wang et al., 2010)

The results from these experiments clearly suggested that EGF induced the translocation of EGFR from the cell surface to the INM; (Wang, Wang et al. 2010). Different biochemical methods have also been used to assess the first endocytic steps for EGFR. Small interference RNA (siRNA) is used to knockdown clathrin, which blocks translocation of EGFR to the nucleus. Transient expression of a dominant dynamin mutant (i.e. dynamin II/K44A) has also been used (De Angelis Campos, Rodrigues et al. 2011). Labelled EGFR nuclear internalization/translocation is Dynamin and clathrin – dependent as they are important in formation of clathrin-coated pits (Damke, Baba et al. 1994, Henley, Krueger et al. 1998).

Imported EGFR goes through the Golgi/ER

Researches confirm that upon EGF treatment, EGFR redistributes to intracellular organelles such as the Golgi/ER and nuclear membrane; (Wang and Hung, 2012). Full-length EGFR anchors to the membranes of these intracellular organelles where its NH2-terminus resides within the lumen of Golgi/ER, whereas COOH-terminus is exposed to the cytoplasm; (Wang and Hung, 2012).

Tests were carried by the Wang group to find if the translocation of membrane-bound EGFR to the INM used a similar pathway, based on the INTERNET (integral trafficking from the ER to the NE transport) pathway used by some large INM proteins. The INTERNET model involves the nuclear transport of integral INM proteins and is thought to also include other integral membrane proteins such as cell surface EGFR. In this model, integral INM proteins are primarily inserted into the ER membrane, in which the NLSs present in the extralumenal domains bind to Kaps, and the proteins are then targeted to the INM of the NE through the ONM and NPC; (Wang, Yamaguchi et al. 2010). Analysis of the EGF-dependent kinetics of EGFR translocation from the ER-INM to the NP confirmed the order of ER-to-INM-to-NP for the EGF-induced EGFR nuclear translocation. After internalisation into the cytoplasm of the cell, EGFR is thought to translocate though the ER, and assumingly also in the Golgi before reaching the ER, then it moves to the ONM and the INM to finally end up in the NP. The problem still remains to understand why the imported EGFR needs to pass through the Golgi. It could be that it gets some important modification in order to be able to pass through the NPC and interact with other NPC structural proteins. EGFR might need some alteration to its composition to facilitate the interaction with the translocon Sec61β, as the Golgi's function is to modify proteins going through (Osborne, Rapoport et al. 2005, Wang, Yamaguchi et al. 2010). There is some evidence suggesting that EGFR goes through the ER and Golgi, yet so far there is not solid proof of the modifications that EGFR might encounter to support this suggestion. It is important to mention that Sec61β translocon resides in the ER, and therefore, it would interact with EGFR that passing though the ER; more evidences of EGFR/ Sec61β interactions are also need to confirm this claim.

Passage through the NPC

Eukaryotic cells transport a myriad of molecules between the nucleus and cytoplasm and have evolved a number of related biochemical pathways to achieve this, many of which have been elucidated in recent years. One central and common component to all the pathways is the NPC. It regulates the transport across the nuclear envelope. Its components appear to play vital roles in

transport and the NPC is structurally dynamic, but whether its role is as a facilitator, a controller or both is yet to be decided and awaits further analysis on the role of individual components in specific pathways. The NPC contains a central structure called the central channel, which is about 30 nm in diameter and allows the transport, by passive diffusion, of ions and small molecules, including proteins with a molecular mass up to 40 kDa (Ribbeck and Gorlich, 2001). Energy dependent active transport mechanisms mediated by nuclear transport receptors (NTRs; also called Karyopherins) are required for the traffic of larger molecules through the NPCs. NTRs have the privilege of facilitated NPC passage (Goldberg and Allen 1996; Gorlich and Kutay, 1999; Labokha and Fassati, 2013). NTRs are classed into importins or exportins based on the directionality of the transport process. Importins recognise classical and non-classical nuclear localisation signals (NLSs) on cargo molecules and enable their translocation from the cytoplasm into the nucleus (Labokha and Fassati, 2013). A series of interactions between nuclear transport factors (NTFs)-cargo complexes and NPC Nups, specifically those rich in FG residues is required for the movement of NTFs through pores; (Cairo et al., 2013).

Import of membrane proteins carrying a NLS for the transport factor karyopherin-α required at least a 120-residue-long intrinsically unfolded linker. Binding to karyopherin α/β is crucial to pass the NPC and reach the INM (Meinema et al., 2011). Transport of soluble proteins implicates that transport factors shuttle cargo through the NPC by binding to FG domains of the central channel nucleoporins (Batrakou et al 2009; Schirmer and Gerace 2005). A mechanism for membrane protein transport has been proposed in which the disordered linker slices through the NPC scaffold enabling binding between the transport factor and the FG domains in the centre of the NPC. In order for a membrane protein to pass through the NPC, its transmembrane domain needs to pass through the pore membrane, and its extraluminal soluble domain(s) must pass through the NPC by yet obscure mechanisms (Freitas and Cunha 2009, Wang, Yamaguchi et al. 2010).

The rate of translocation through NPCs is estimated at around 1000 molecules per second, (Freitas and Cunha, 2009; Ribbeck and Gorlich, 2001); yet more is to be clarified for different cell types. The nucleus of human cells and mature Xenopus oocytes may contain $5x10^3$ – $5x10^7$ NPCs per nucleus while yeast cells usually contains about 200 NPCs; (Freitas and Cunha, 2009).

Nucleo-cytoplasmic transport of EGFR through the NPCs heavily depends on Karyopherins α and β.

Importin regulates EGFR nuclear transport

The translocation of most cargos through the NPC occurs in a specific way: (i) binding of a cargo molecule to its cognate nuclear transport receptors (NTR); (ii) docking of NTR-cargo complex to the NPC; (iii) translocation through the nuclear pore; and (iv) cargo release on the opposite side of the nuclear envelope. Nuclear import is facilitated by karyopherins (Kaps). Kaps bind to nuclear localization signals (NLS) or nuclear export signals (NES) targeting sequences of cargo molecules. After binding to the cargo, Kaps are able to overcome the diffusion barrier of the NPC by unknown mechanism. It is thought that Kaps bound to the cargo though NLS or NES interact with FG Nups, and this interaction is found to be vital for translocation into the nucleus. Movement of molecules in and out of the NE is typically by diffusion and active transport; (Miao and Schulten 2010, Yamada, Phillips et al. 2010, Meinema, Laba et al. 2011). As it has been mentioned above, the nucleo-cytoplasmic transport of the majority of macromolecules through the NPCs is an energy-dependent process mediated by β-karyopherins (Freitas and Cunha 2009). These soluble transport receptors mediate both the import and export of all proteins displaying dimensions over and the size exclusion limit, (and also some that are less the 40kD) for simple diffusion through the NPCs. Import or export complex formation is dependent on the interaction of β-Karyopherins with small peptide motifs present in protein cargos. These motifs are designated NLS or NES as mentioned further; (Freitas and Cunha, 2009). Importin α binds to the EGFR-NLS and importinβ interacts with importin α/cargo complex directing them though the nuclear pore. importinβ is characterized by the ability to directly interact with both the Ran GTPase and the FG domains of Nups; (Freitas and Cunha, 2009). Interaction of transport receptors with the FG motifs of nucleoporins has been found to be of an essential step for translocation through NPCs. FG binding motifs of importin-β is required for the import pathways mediated by this transport receptor (Bednenko et al., 2003; Freitas and Cunha, 2009). Importin α, β are acidic proteins with molecular masses ranging from 90 to 145 KDa; (Freitas and Cunha, 2009).

The import of EGFR is assisted by Karyopherins. Importin-β is thought to be responsible for the nuclear import of all proteins that contain a classical NLS; (Reilly and Maher 2001, Ogawa, Miyamoto et al. 2012). Nonetheless, the interaction of importin-β with the amino acid sequence of the NLS occurs indirectly and involves the participation of other proteins which are members of the importin-α family. Importinβ receptor participates in the import pathways of EGFR. So far it is found that EGFR interacts and co-localises with importin-α1/β1; (Figure 5), (Gorlich and Kutay 1999, Pelaez, Fernandez-Garcia et al. 2012). Importin-β regulates EGFR nuclear transport to the

INM in addition to the nucleus/nucleoplasm. Much work has been carried out to find if/how importin-β1 is crucial in EGFR nuclear import. RanGTP gradient across the NE determines the directionality of Import/Export-mediated transport of macromolecules the NE. Ran regulates nuclear transport by modulating soluble nuclear transport factors, karyopherins; (King & Lusk, 2006). Facilitated transport and diffusion take distinct spatial routes through the NPC (Fiserova et al., 2010). 'Classical' nuclear import is activated when the classical lysine-rich nuclear localization signal (cNLS) of a cargo molecule is recognised by the import receptor karyopherin-α (importin-α/Kap60/Srp1) in an interaction that is stabilized by binding of karyopherin-β1 (importin-β1/Kap95) that directs them though the NPC. This ternary complex (importin-β interaction with importin α/NLS cargo complex) traverses the NPC and is disassembled by binding to Ran-GTP within the nucleus (King, Lusk et al. 2006, Lo et al., 2006, Pelaez, Fernandez-Garcia et al. 2012). Clearly, Importin α binds to the EGFR-NLS and importinβ interacts with importin α/cargo complex directing them though the nuclear pore. EGFR's cNLS is crucial for EGFR/importin interaction and EGFR nuclear import as its mutation does not allow association with importin and transport into the nucleus; (Cingolani et al., 2002, Lo et al., 2006,Y.-N. Wang et al., 2010).

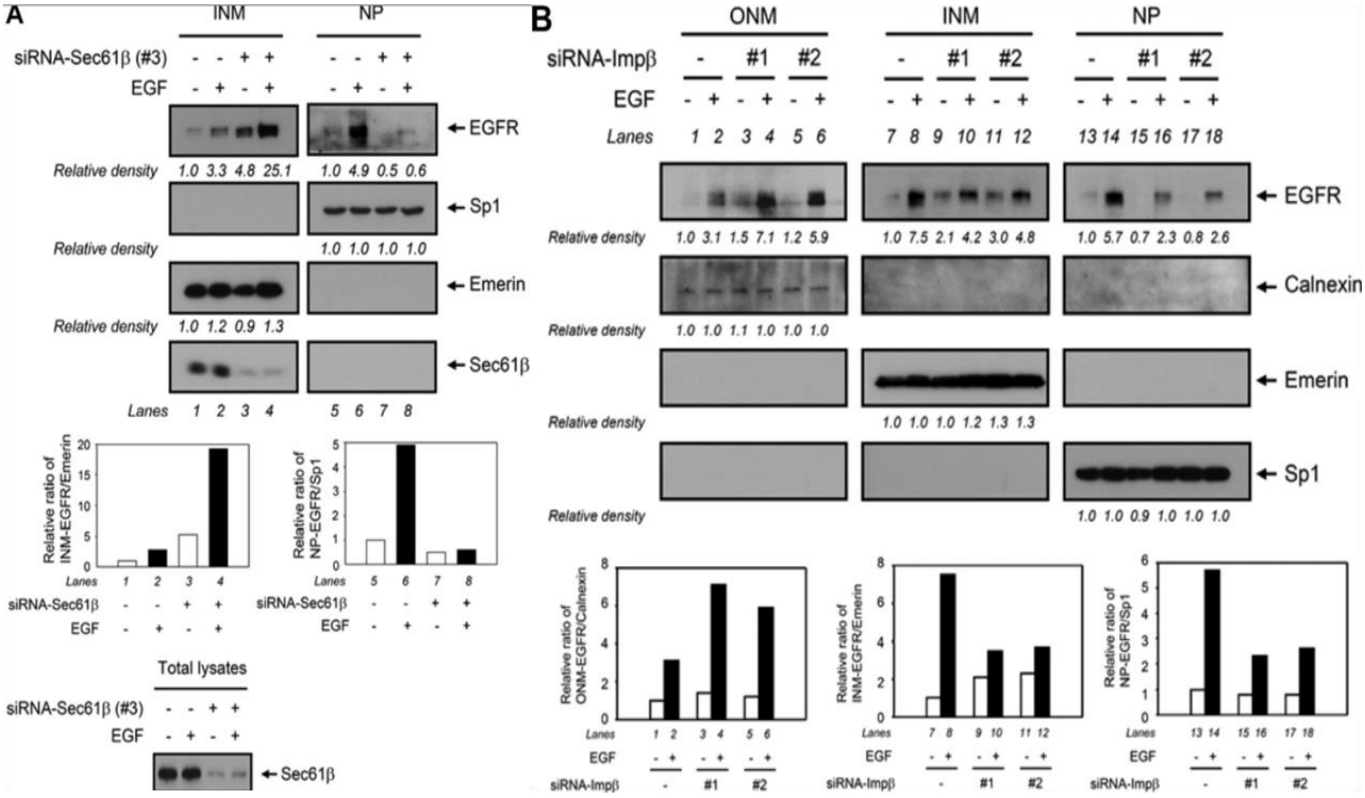


Figure 5. **EGFR interaction with Sec61*β* and with Importin *β*.** Adapted from (Wang, Yamaguchi et al. 2010). (**A**) Association of EGFR with Sec61*β* in the nucleus assists INM-anchored EGFR in releasing to the nucleus. Knockdown of Sec61 prevents EGF-dependent transport of EGFR from the INM to the NP in HeLa cells. Cells transfected with an siRNA targeting Sec61*β* (siRNA-Sec61 *β*-3) (+) or a nonspecific control siRNA (-) using electroporation. Proteins from total lysates, INM and NP by cellular fractionation analysed using immunoblotting with the antibodies as indicated. Emerin and Sp1 used as markers for the INM and NP portions, respectively. (**B**), Knockdown of Importin*β* (Imp*β*) by two individual siRNAs targeting Importin*β* (siRNA-Imp*β*-1 and siRNA-Imp-2) in HeLa cells down-regulates EGF-dependent EGFR translocation to the INM and NP. The relative density by quantification is plotted diagrammatically as shown in the middle panel. Adapted with permission from (Y.-N. Wang et al., 2010)

Importinβ involved in the import of EGFR to the INM through the ER/ONM. During the import of EGFR from the cell surface into the NP of the nucleus through the cytoplasm, it transits through the ER where it is thought to get modified, and then it goes through the ONM, to finally get to the INM and get released into the NP by Sec61β. This was found in a study to confirm whether the down-regulation of Importinβ expression inhibited EGFR nuclear translocation and was consistent with the previous studies. The results showed that knock down of Importinβ expression significantly accumulated EGF-dependent EGFR translocation in the ONM after analysis in the ONM, INM, and NP portions; (Cingolani et al., 2002, Lo et al., 2006,Y.-N. Wang et al., 2010). This study attempts to confirm these previous observations.

In the canonical model of nuclear import, it is strongly suggested that nuclear localization signal (NLS)-bearing molecules form a complex with importin α/β or importinβ alone. Importinβ is responsible for nuclear translocation through NPCs to the INM by directly associating with the nucleoporins. Nevertheless, it is not definite that Importinβ alone is responsible to import EGFR from cell surface to the INM and the NP; (Lo et al., 2006, Ogawa, Miyamoto et al. 2012).
Some reports speculated other proteins to be involved in the traffic of EGFR to the nucleus. It has been suggested that the nuclear pore protein Nup62, a nucleoporin lining the central regions of NPCs and maintain of the structural integrity of NPCs, has also an essential role in nuclear import of EGFR to the INM through the NPCs when interacting with Importinβ as its down-regulation clearly inhibited EGF-dependent EGFR import in the INM and NP; concluding that if Nup62 is knocked-down, the structure of the NPCs gets disrupted, therefore, EGF could not enhance EGFR translocation to the INM; (Stoffler, Fahrenkrog et al. 1999, Lin, Makino et al. 2001, Ogawa, Miyamoto et al. 2012). The problem with this finding is that the experiments used a siRNA approach; it could be that siRNA affect the traffic in other ways that have not been yet discovered. Henceforth, it is important to test different methods in order to have a firm clear understanding of this translocation.

The translocon Sec61β regulates EGFR in the nucleus

Translocation of EGFR from the INM into the nucleus, the nucleoplasm, is also found to be regulated by the endoplasmic reticulum (ER) associated translocon Sec61β; (Lu, Ladinsky et al. 2009, Wang, Yamaguchi et al. 2010). The Sec61β translocon is well known and is found to reside in the INM and interacts with EGFR (Liao and Carpenter 2007). The Sec61 complex is the main element of the protein translocation apparatus of the ER membrane. It is located in both the ER and the Golgi-ER intermediate compartment and is a doughnut shaped pore through the membrane and

consists of 0ligomers composed of three membrane proteins alpha (Sec61α), beta (Sec61 β), and gamma (Sec61γ) that associate to form a heterotrimer; (Osborne, Rapoport et al. 2005, Stefanovic and Hegde 2007). Multiple heterotrimers associate forming the ER translocon (Lu, Ladinsky et al. 2009). The ER translocon is a transmembrane channel where proteins are translocated across and integrated into the ER membrane. The Sec61β subunits are found in the post ER compartment, implying that these proteins can escape the ER and recycle back. Sec61β is found to be closely linked with membrane bound ribosomes, either directly or through adaptor proteins, and is required for assembly of membrane and secretory proteins; (Osborne, Rapoport et al. 2005, Park and Rapoport 2012, Wang, Yamaguchi et al. 2010). The Sec61 translocon complex in the ER has bidirectional functions; (a) protein import: during protein synthesis it inserts transmembrane and secretory proteins into the ER, and (b) protein export: as part of the ER-associated degradation (ERAD) pathway, misfolded proteins are retrotranslocated from the ER to the cytoplasm for degradation (Campbell, Williamson et al. 2006, Wang, Yamaguchi et al. 2010).

To test the function of Sec61β in relation with EGFR and nuclear location, its expression was knocked down, and it was observed that EGFR level in the nucleoplasm reduced and that it accumulated in the INM; (Figure 5) (Wang, Yamaguchi et al. 2010). More than a decade ago, investigators elucidated a role for Sec61 complex to only translocate proteins across the rough ER membrane. This new observation showed that Sec61β translocon also plays an unprecedented unrecognized role in the release of the membrane-anchored EGFR from the lipid bilayer of the INM to the nucleus. The newly identified Sec61β function can provide an alternative pathway for nuclear transport that may be utilised by membrane-embedded proteins such as full-length EGFR; (Wang, Yamaguchi et al. 2010). Immunoblotting analysis, sucrose gradient purification, and ultrastructural studies using immuno-EM methods all together suggested that the ER translocons Sec61β localises in the INM, but not in the NP; (Wang, Yamaguchi et al. 2010). These observations suggest that Sec61β remains in the INM while it releases the membrane-bound EGFR to the NP in the nucleus.

Sec61β interacts with EGFR in the ER and may be involved in its translocation from the ER to the INM/nucleus via the INTERNET model, like the import of INM proteins (Wang, Yamaguchi et al. 2010). However, the mechanism of interaction of EGFR/Sec61β remains to be explored. There is a need to determine whether Sec61β is transported with EGFR or separately and determine the mechanism. If it is transported with EGFR which is in a membrane-bound environment in the nucleoplasm, when does it separate? Where does it go? What are the precise molecular events that

happen, in time and space? This work looks to confirm the localisation of Sec61*β* in the INM and in the nucleoplasm.

Nuclear EGFR import involving importin*β* and Sec61*β*

EGF stimulation transduces its actions by dimerization, autophosphorylation/activation or internalisation of EGFR. It is proposed that upon EGF stimulation, EGF binds to EGFR on the cell surface membrane, the receptor homodimers dimerise, the biotinylated cell surface EGFR is internalised and endocytosed via clathrin mediated endocytosis into early endosomes, trafficked to the cytoplasm where: a portion of endocytosed membrane-bound EGF Receptors is re-transported/recycled to the plasma membrane, another fraction is degraded by lysosomes, and another is transported into the nucleus via the INM (via the Golgi/ER) after binding importin*β* and translocating through the membrane domain of the NPC to the INM where Sec61 appears to release EGFR into the nuclear interior, nucleoplasm (remaining in a membrane-bound environment), where it interacts with transcription factors (STAT3, E2F1, STAT5), (Figure 6) (Gorlich and Kutay 1999, Sorkin and Von Zastrow 2002, Stefanovic and Hegde 2007, Fiserova, Richards et al. 2010, Wang, Yamaguchi et al. 2010).

It is speculated that EGFR that has been transported to the nucleus could be re-transported or recycled back to the cell surface or transferred into endosomes and eventually degraded in lysosomes (Sorkin and Von Zastrow 2002). The concentration of EGFR in the cytoplasm may rise due to the fast rate of internalisation, although some is degraded in the lysosomes (Sorkin and Von Zastrow 2002). Nuclear transports of cell surface receptors involves endocytosis into vesicles and then translocating translocation through the cytoplasm space to reach enter the nucleus and enter in its NP. The route taken by the vesicles is not clear enough to date. It is suggested that the receptors endocytosed in these vesicles interact with certain proteins that aid them in some ways. For instance, it is documented that EGFR interacts with different proteins, Importinβ, Sec61β…, during its translocation. Yet it is not clear when and where EGFR recruits Importinβ and Sec61β. More has been discovered but our current knowledge raises questions. Many studies have demonstrated the expression of EGFR in cancerous cells; IF studies have also shown the translocation of EGFR to the nucleus and to the INM and NP; none-the-less few studies have shown in details EGFR import by EM. For that reason, the aim of this work was set to observe by EM and by IF microscopy the interaction of EGFR/Importinβ, EGFR/Sec61β and that EGFR resides in the INM. EGFR expression is to be confirmed in different cancer cell lines and the translocation of EGFR from the

cell surface in the cytoplasm to the nucleus and into the NP to be examined in details to confirm previous reports.

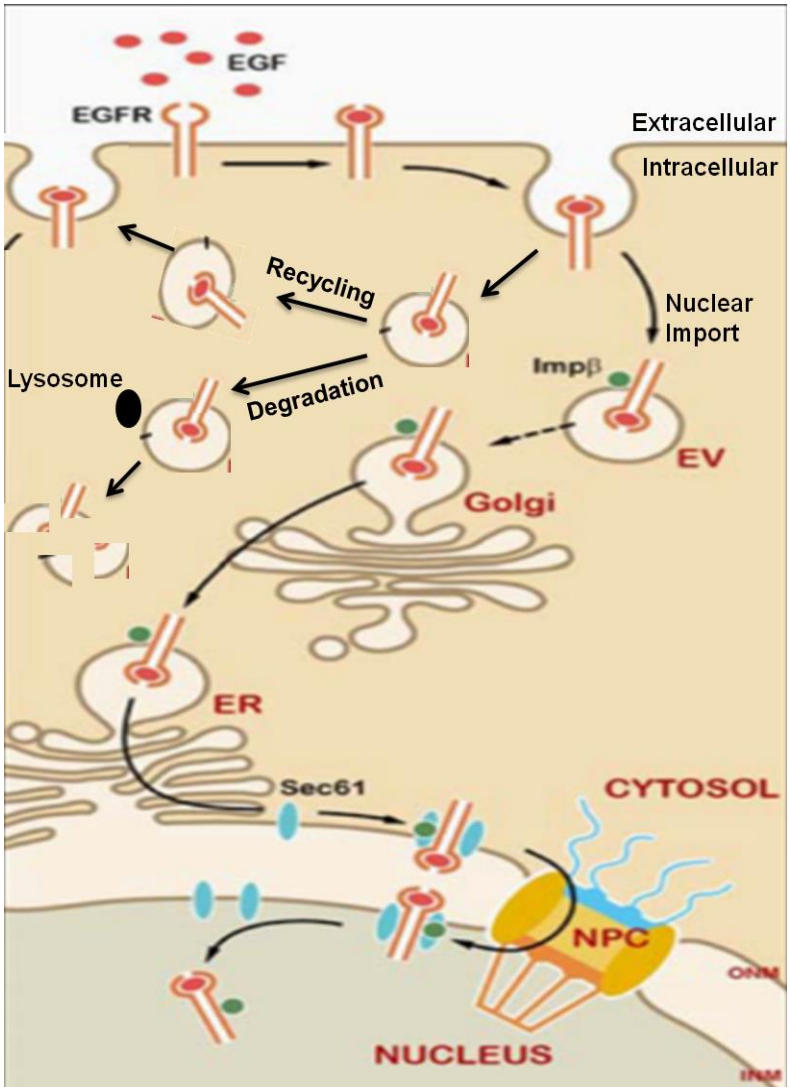


Figure 6. A proposed model of EGFR trafficking. The diagram shows an integral trafficking of EGFR from plasma membrane to (1) the nucleus through the Golgi/ER/NE by EGF treatment, (2) re-transported/recycled back to the cell surface involving CRM1 or (3) degraded by the lysosome. The scale of the diagram does not reflect the relative sizes of different molecules or subcellular structures. EV: endocytic vesicle; Imp β: importin β. The INTERNET model can explain how can be imported from the ER to the nucleus; that is, interaction of membrane-associated EGFR/importinβ and travelling from the ER/ONM to the INM via the NPCs. This insures that EGFR stays embedded in the membrane from the cell surface to the NE in the entire trafficking process. Modified with permission from (Wang, Wang et al. 2010, Wang, Yamaguchi et al. 2010, Wang, Yamaguchi et al. 2010)

After various studies on the nuclear transport of EGFR and ErbB-2, a model for the pathway of EGFR involving importinβ and Sec61β has been proposed by Wang's group,(Wang, Yamaguchi et al. 2010). The model suggests that during the trafficking pathway of the cell surface EGFR to the nucleus in response to EGF binding, EGFR is endocytosed, embedded in endocytic vesicles, fused to the Golgi-ER membrane (Wang, Wang et al. 2010), transported into the nucleus through ER membrane, NPCs, and nuclear envelope, (King, Lusk et al. 2006, Saksena, Summers et al. 2006, Wang, Yamaguchi et al. 2010) and Sec61β releasing it from the lipid bilayer of the INM into the NP; (Figure 6).

Chapter 2: Materials and Methods

2.1: Chemicals

The following antibodies and chemicals were purchased for our study from Sigma-Aldrich, InVitrogen and Dako or other companies unless otherwise noted: Goat anti-Rabbit IgG (InVitrogen), EGF Receptor Rabbit mAb (Cell Signaling), Goat anti-Mouse IgG (InVitrogen), Monoclonal Mouse anti-human wild-type EGFR clone (Dako), Biotin-Egf (Invitrogen), EGF Human (Sigma Aldrich), EM Streptavidin (BB International), EM BSA Gold, Cell mask orange plasma membrane stain (1/100) (Invitrogen), Hoechst 33342 Trihydrochlorine Trihydrate (1/10,000), Importin beta anti NTF97 (Abcam), Anti Se61Beta, mounting medim with DAPI (Vector) , Golgi (1/50), anti-actin (1/100), Phaloidin (1/300), ER Tracker (1/1000) and QDot Strepavidin samples kit (Invitrogen).

2.2: Mammalian cell culture

2.2.1: Cell Lines and Media

For the purpose of this study, the following cell lines were used: MCF-7, MDA-MB-231 human breast carcinoma cells; and HeLa human cervical cancer cell. They were kindly obtained from Dr Kowos Karakesisoglou laboratory (Durham University). These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% FBS (Fetal bovine serum), 0.6% Penicillin/Streptomycin, 0.6% Glutamine, 0.6% Na Pyruvate, 0.6% Non-Essential Amino Acid, in culturing flasks or petri dishes, at 37^{o}C in humidified incubators containing 5% CO_2.

2.2.2: Subculture

Cultures were grown to 70-80% confluence and sub-cultured thereafter into two or three flasks. Briefly, cells were washed in Versene buffer (137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 1.5mM EDTA pH.7.4) and detached by treatment with Versene buffer or PBS containing 10% Trypsin (Sigma) for 3-5 min at 37^{o}C in a humidified incubator. Then, cells were neutralised with fresh DMEM (1:5 ratio) containing 10% FBS and appropriate antibiotics. Thereafter, cells were transferred to a sterile universal tube and centrifuged at 2500g for 5 min in Sigma centrifuge. Cell pellets were diluted in an appropriate volume of fresh DMEM containing 10% FBS and antibiotics and seeded into an appropriate number of flasks.

2.2.3: Cryopreservation of cultures

Cells were routinely cryopreserved at -150^{o}C. Sub-confluent cultures were washed with Versene and detached by trypsinization and pelleted by centrifugation as described above. The cell pellets were re-suspended in 1ml of freezing Media (DMEM containing 5% DMSO, 10% FBS). The cell suspension was transferred to a cryovial and placed in the Cryo 1°C Freezing Containers and in the -70^{0}C freezer overnight before storage into the -150^{o}C freezer. To revive cultures, the cell suspensions were defrosted in 37^{o}C water bath and cells were added quickly to DMEM into a universal tube, centrifuged at 2500g for 5 min in Sigma centrifuge. Cell pellets were diluted in appropriate volume of fresh DMEM and seeded into an appropriate number of flasks. The next day the medium was replaced with new fresh medium.

2.2.4: Proliferation cultures

2.2.4.1: Serum starvation and Re-stimulation

To induce quiescence (transient growth arrest) by serum-starvation, cells were grown for 2 days in complete DMEM (10% serum). On the 3rd day, serum containing media was aspired off, cells were washed with Versene or fresh DMEM (10% serum, FBS); then cells were grown overnight in DMEM (without serum). Serum-starved cells were thereafter processed for immunofluorescence microscopy, biochemical fractionation or immuno-precipitation as described below. In these series of experiments, cells were treated with 100 ng/ml EGF under serum-starved conditions.

2.3: Immuno-fluorescent and Confocal Analysis

In the analyses of the kinetics of EGFR endocytosis, MCF-7 and MDA-MB-231 cells were grown on sterile glass coverslips in 12 or 24-well plates in the presence of DMEM with 10% serum for 1 or 2 days until they reached 70-80% confluence. Then the media was aspired off, cells washed in new media and grown further more in fresh DMEM with or without 10% serum overnight. The next day, cells were incubated at 4^{o}C with or without 100 ng/ml EGF for 1 h and moved to 37^{0}C for 30 min, to initiate endocytosis. Cells were then washed three times 5 min with ice-cold PBS, fixed in 4% paraformaldehyde for 15 min, washed three times for 5 min, and permeabilized using PBS/0.5% Triton X-100 for 10 min, and washed two times 5 min. After the PBS washes after permeabilization, non-specific binding was blocked by incubating in blocking buffer PBG (0.1% Triton X-100, 0.1% cold water fish gelatin (Sigma G-7765) and 1% BSA in PBS) for 1 h at room temperature. Whilst the block solution was being incubated, primary antibodies against the antigens

of interest were prepared at optimised dilutions in PBS (Table 1). The blocking solution was removed, an appropriate volume of the indicated primary antibodies was added, and the cells were then incubated for 1h at room temperature. After incubation, unattached antibodies were removed by washing with PBS three times 10min, and then further incubated with the appropriate secondary antibodies (Table 2) for 1h at room temperature. After antibody labelling, unattached secondary antibody was removed by washing five times 10min. Glass coverslips were mounted using an anti-photobleaching media, Vectashield mounting medium containing the nuclear stain Propidium Iodide (PI) (Vector Laboratories). Then slides were processed for imaging.

2.3.1: Qdot® (Quantum Dot) Strepavidin Conjugate experiments procedures

For experiments done with Qdot® Strepavidin Conjugate 525, 625, cells were processed as follow. Cells were grown as described above on sterile glass coverslips in 12 or 24-well plates in the presence of DMEM with 10% serum for 1 or 2 days until 70-80% confluence. The media was poured off; cells washed in Versene and serum starved in fresh DMEM without serum. The next day, cells were incubated at 4^0C with or without 100 ng/ml Biotin EGF for 30 min, then washed three times 5 min with ice cold Tyrode's buffer (137nM NaCl, 2.7nM KCl, 1nM $MgCl_2$, 0.2nM Na_2HPO_4, 1.8nM CaCl, 12nM $NaHCO_3$, 20nM D-Glucose). Cells were incubated with or without Qdot® Strepavidin Conjugate 525, 625 at dilutions 1/100 and 1/200 for 30 min on ice, and moved to 37^{o}C to initiate endocytosis. Cells were then washed three times 5 min with Tyrode's buffer, fixed at different time point as indicated on Figure legends in 4% paraformaldehyde for 15 min, washed three times for 5 min. Glass coverslips were mounted using an anti-photobleaching media, Vectashield mounting medium containing the nuclear stain Propidium Iodide (PI) (Vector Laboratories). Then slides were processed for imaging.

If labelling with other antibodies, Glass coverslips were processed in the same way as Immuno-fluorescent process described further above.

2.3.2: Image Acquisition

For viewing and imaging cells on coverslips, a Zeiss 510 Meta CLSM, Leica SP5 CLSM FLIM FCCS, Zeiss Apotome or Leica TIRF Microscope microscopes were used equipped with 40X and 63X/1.40 oil immersion lens. See Figure legend for specific details of acquisition.

Collected images were projected as black and white or blue/green/red colour merged micrographs in which DAPI was in blue. Images were processed by ImageJ (version 1.38x; National Institutes of Health) or Adobe Photoshop CS6 (64 Bit) software program.

Antibody Name	Manufacturer	Specificity	Host	Working Dilution
EGF Receptor Rabbit mAb	Cell Signaling	Recognise intracellular domain	Rabbit	1/20 (EM) 1/50 (EM, IF) 1/100(IF) 1/500 (WB) 1/1000 (WB)
Monoclonal Mouse anti-human WT EGFR clone	Dako	Recognise extracellular domain	Mouse	1/20 (EM) 1/50 (EM, IF) 1/100(IF) 1/500 (WB) 1/1000 (WB)
Importin beta anti NTF97	Abcam	Importin beta 1	Mouse	1/30 (EM) 1/200 (IF)
Anti Se61Beta	Molecular	Sec61 beta	Rabbit	1/50 (EM) 1/500 (IF)
Anti actin		Actin	Rabbit	1/50 1/100 (IF)
Golgi		Golgi	Mouse	1/50 1/100 (IF)

Table 1: Primary antibodies and appropriate information

Note: IF, Immuno-fluorescence; EM, Electron Microscopy; WB, Western Blot

Antibody Name	Manufacturer	Working Dilution
Goat anti-Rabbit IgG Alexafluor 594	InVitrogen	1/1000 1/2000 (IF)
Goat anti-Mouse IgG Alexafluor 488	InVitrogen	1/1000 1/2000 (IF)
HRP Donkey Rabbit		1/1000 1/2500 (WB)
HRP Chicken		1/1000 1/2500 (WB)
5, 10 nm Streptavidin Gold	BB International	1/100 (IF)
5, 10 nm EM Streptavidin gold	BB International	1/20 1/30 1/50 (EM)
5, 10 nm BSA Gold	BB International	1/20 1/30 1/50 (EM)

Table 2: Secondary antibodies and appropriate information

Note: IF, Immuno-fluescence; EM, Electron Microscopy; WB, Western Blot

2.4: Electron Microscopy

2.4.1: Transmission Electron Microscopy

Transmission electron microscopy (TEM) cell cultures were processed by the conventional TEM process method or by high pressure freezing then freeze substitution. Cryosectioning & Immuno-labelling was also used to for TEM.

2.4.1.1.: Conventional TEM Processing

2.4.1.1.1: Primary process

Cells were grown in T25 flasks in DMEM for 1 or 2 days until they reached 70-80% confluence at 37^0C in an incubator. Then, they were serum starved overnight by growing them in DMEM without serum at 37^0C. Then next day, cells were washed with Tyrode's buffer, treated with 100ng/ml Biotin-Egf under serum-starved conditions for 30 min and incubated at 4^0C. Cells were washed once with ice cold Tyrode's buffer and incubated on ice for 1 hour with either Qdot® Strepavidin Conjugate 525, Qdot® Strepavidin Conjugate 625, EM Streptavidin 5 or 10 nm Gold at 1:100 dilution, and agitate frequently to cover the whole flask. Then cells were incubated at 37ºC to initiate endocytosis and fixed at different time point (as indicated on the Figure legends) with double strength modified Karnovsky fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M; buffer pH 7.4) for 5 min and with single modified Karnovsky fixative for 30 min.

2.4.1.1.2: Post Fixation & Embedding of scraped cells

Cells were washed with 0.1M sodium cacodylate buffer at room temperature (RT) 3 x 5 minutes. Cells were scraped with a cell scraper and collected into a 1.5 ml Eppendorf tube and spun for 5 minutes at 1000g in an Eppendorf centrifuge. Cells were postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 1 hour at RT in the hood (the pellets were dislodged from the wall of the Eppendorf tube with a cocktail stick to optimize penetration of osmium).

2.4.1.1.3: Dehydration

Cells were washed in 0.1M cacodylate buffer 3 x 5 minutes. The pellets were transferred into glass vials, using a large bore plastic pipette. If the pellets were loose, the samples were left and processed within the eppendorf tube and spun down between dehydration steps. Pellets were dehydrated in the following order: 2 x 5 minutes in 50% ethanol, 2 x 5 minutes in 70% ethanol, and

2 x 5 minutes in 95% ethanol, 3 x 5 minutes in 100% ethanol. Then, pellets were incubated in propylene oxide: 100% ethanol 1:1 mixture 3x 5mins and then propylene oxide 3x 5mins.

2.4.1.1.4: Infiltration and Embedding

The propylene oxide was replaced with a 1: 1 mixture of propylene oxide and Epon (45.3ml fresh Agar 100: 20 ml Agar 100, epoxy resin; 9 ml DDSA, hardener; 12ml MNA, hardener; 1.2 ml BDMA, accelerator) and left on the rotator wheel for 1 hour with the lid of the eppendorf tube opened. It was replaced with pure Epon and left on the rotator wheel for 30mins with lids opened and then replaced with fresh Epon and left overnight on rotator wheel with the lids off. The following morning pellets transferred into labelled moulds filled with fresh Epon and cured in the oven overnight @ 60^{o}C.

2.4.1.1.5: Sectioning and Staining of Semithin sections.

When polymerisation is finished, the resin blocks with sample were trimmed using razor blade. Semithin sections, 0.5µm, of cells in Lowicryl HM20 MonoStep resin blocks were cut using a glass knife on a Leica Reichert Ultracut S Ultramicrotome. Semithin sections were stained for 1min over heat with filtered 1% Toludine Blue (Sigma), before rinsing with distilled water. Slides were blotted with filter paper and imaged on the Nikon Labophot.

2.4.1.1.6: Sectioning and Staining of Ultrathin sections

Ultrathin sections, 50-70 nm for TEM were cut using a glass knife on a Leica Reichert Ultracut S Ultramicrotome. The sections were floated on distilled water, and picked up using formvar coated 200 mesh copper grids. Once on grids, the sections were stained for 10 min with 1% Uranyl acetate in 70% alcohol, washed in water twice, then stained for 10min with Reynolds Lead Citrate, and taken for imaging on the TEM.

2.4.1.2: High Pressure Freezing, Freeze Substitution Processing

2.4.1.2.1: Sample Preparation

Cells were grown on planchettes (gold plated Flat specimen carrier 0.5mm thick, 1.2mm in diameter/200µm deep) in DMEM for 1 or 2 days until they reached 70-80% confluence at 37^{o}C in an incubator. Fresh DMEM with or without 10% serum was added (without 10% serum, is meant to serum-starve cells) and the cells were left to grow overnight at 37^{o}C. Then next day, cells were treated with or without 100 ng/ml EGF or Biotin EGF under serum-starved conditions for 1h at 4^{o}C;

then brought back to 37ºC for 30 min to initiate endocytosis before the planchettes could be processed for high pressure freezing at different time points as pointed in the figures.

2.4.1.2.2: High-pressure freezing of cell cultures

All samples grown on planchettes as described above were cryofixed in the Leica EM PACT high pressure freezer and stored under liquid nitrogen until freeze-substitution. As the quality of the preservation of the specimen is dependent on the loading technique, it was important to work as quickly as possible to preserve the specimen in as near to the normal state as possible. The specimen were loaded and frozen within 30-60 seconds. Samples were kept completely surrounded with liquid to prevent air bubbles.

2.4.1.2.3: Freeze-substitution

The Leica EM AFS freeze substitution unit was used as it offers reproducible temperature course and other features that result in consistent fixation and reliable polymerization of Lowicryl resins. A simple protocol has been used for FS in this study.

- The Leica EM AFS freeze substitution unit was programmed for sample fixation and acetone washes as follows: T1: -90°C 49h; S1: 5°C increment per hour up to -25°C; T2: -25°C 12h; S2 0°C 0h; T3: -25°C 50 h (***see*** **Note 1**).
- FT-capsules were placed into cryovials, half-filed with fixative and frozen in LN2. The specimen carriers were placed under the LN2 on the top of frozen fixative inside the FT-capsules and the lid closed (***see*** **Note 2**); and then placed into the pre-chilled Leica EM AFS freeze substitution chamber and the programme run. The lid of the AFS was closed, the glass plate lowered, the handle unscrewed and lifted away from the glass plate. All subsequent medium changes were carried out by precooling new medium, removing waste medium to a universal container and replacing with the new precooled medium.
- Once the temperature step T2 is finished, acetone washes was performed. FT-capsules were placed into FT-chamber filled with acetone chilled to -25°C and incubate for 15 minutes at -5°C. The acetone washing step was repeated twice by changing the acetone inside the FT-chamber after 15 min of incubation at -25°C.
- Your samples were not removed from the carriers at this point. You have copied and pasted the method for yeast Specimen (which are freely inside FT-capsule placed inside FT-chamber) were infiltrated with Lowicryl by filling the FT-chamber with 50% Lowicryl for 1 hour at -25°C, 66%

Lowicryl for 1 hour at -25°C, 100% Lowicryl for 1 hour at -25°C and 100% Lowicryl overnight at -25°C (***see*** **Note 3 and 4**).

- G-chamber was loaded with G-capsules and placed inside the substitution chamber of Leica EM AFS. G-capsules were filled with 100% Lowicryl and let chill down to -25°C. FT-capsules were placed with specimen inside G-capsules and where needed refill with 100% Lowicryl. G-capsules were closed tightly with spider cover and removed from G-chamber using cryomanipulator and placed on the top of stem holder for spider cover.
- The Leica EM AFS unit was set for resin embedding programme as follows: T1: -25°C 24h; S1: 5°C increment per hour up to 25°C; T2: 25°C 24-100h (***see*** **Note 15**). The short colourless tube was changed for the short red temperature range tube. UV lamp was installed and the polymerisation programme started.

Notes

1. The Leica EM AFS unit is best programmed on Friday before 1 pm to ensures the freeze substitution runs over the weekend and allows sufficient time for acetone washes and Lowicryl infiltration the following Monday afternoon. This timing allows the 1 hour period of chilling to -90°C, 48 hours of incubation of the sample in fixative at -90°C, a warming up period up to -25°C taking 13 hours and another incubation in fixative at -25°C for 12 hours. In total, the sample is fixated for 73 hours. So the acetone washes ideally follow 3 days and 1 hour after the program started and require the temperature of -25°C. The time for acetone washes corresponds with the change from the program T2 to program T3. Because T3 is set at -25°C for another 50 hours (T3), the Leica EM AFS unit would keep the sample chilled longer if required (the exact timing is not crucial here, freeze substitution can run for instance for 24 hours longer at -90°C or for 24 hours at -25°C if needed) before proceeding with acetone washes.
2. Fixative tends to evaporate from the FT-chamber over the 73 hour period. To prevent the evaporation cryovials were used for the fixation step. Scratching the label into the side of the cryovial is suggested to make sure it is not washed away during freeze substitution.
3. Oxygen strongly inhibits the polymerization of methacrylate resins. Harsh stirring was avoided when mixing Lowicryl HM20 resin.
4. The longer the 100% Lowicryl infiltration the better. The optimal infiltration period is at least 24 hours.
5. The resin hardness improves when UV light is applied for at least 24 -72 hours at 25°C. The longer the polymerisation, the better the resin quality.

2.4.1.2.4: Sectioning and Staining of Semithin sections

When polymerisation is finished, the spider cover was removed with attached G-capsules from the Leica EM AFS substitution chamber. As the polymerised resin blocks were still in the planchettes, a

razor blade was used to remove the excess of the resin on the back of the planchettes; then the latter were removed by exposing or dipping to liquid nitrogen. Then resin blocks with sample were trimmed from G/FT-capsule-covering using razor blade, gloves were used for safety. Semithin sections, 0.5µm, of cells in Lowicryl HM20 MonoStep resin blocks were cut using a glass knife on a Leica Reichert Ultracut S Ultramicrotome. Semithin sections were stained for 1min over heat with filtered 1% Toludine Blue (Sigma), before rinsing with distilled water. Slides were blotted with filter paper and imaged on the Nikon Labophot.

2.4.1.2.5: Sectioning and Staining of Ultrathin sections

Ultrathin sections, 50-70 nm for TEM were cut using a glass knife on a Leica Reichert Ultracut S Ultramicrotome. The sections were floated on distilled water, and picked up using formvar coated 200 mesh copper grids. Once on grids, the sections were stained for 10 min with 1% Uranyl acetate in 70% alcohol, washed in water twice, then stained for 10min with Reynolds Lead Citrate, and taken for imaging on the TEM.

2.4.1.3: Grid Immuno-labelling

Ultrathin sections were picked up on Formvar coated nickel grids; rinsed with 0.1% glycine in PBS 3 times for 1 minute; blocked in 1% BSA in PBS 4 times for 1 minute; incubated with primary antibody, EGFR (1/20; 1/50), Importinβ (1/30) or Sec61β (1/50), in a wet chamber using 5-10 µl droplets per grid for 1 hour at room temperature or overnight at 4°C. Samples on grids were rinsed in PBS 4 times for 2 minutes. Then, incubated for 1 hour at room temperature with 1/20 or 1/30 colloidal gold conjugated secondary antibody 5 or 10nm in a wet chamber. The grids were rinsed in PBS 3 times for 5 seconds each; washed in PBS 4 times for 2 minutes each; washed in distilled water 10 times for 1 minute.

For Post-staining: the grids were floated for 10 minutes on a 20-50 µL droplet of 1% uranyl acetate; rinsed by dipping in distilled water ~ 20 times; then floated again for 10 minutes on 20-50 µL droplet of Reynolds lead citrate; rinsed by dipping in water ~ 20 times; then air dried on a filter paper and observed with TEM.

2.4.1.4: Image Acquisition

Light microscopy of Semithins was taken using Nikon Labophot microscope. Images of Ultrathins sections were obtained on a Hitachi H7600 Transmission electron microscope operating at 80 kV.

2.4.1.5: Cryosectioning & Immuno-labelling for Electron Microscopy

2.4.1.5.1: Cell Culture Specimens

Cells were grown in T25 culture dishes and the culture medium was replaced with a known volume of fresh culture medium the day before. Samples were fixed at ambient temperature by adding the same volume of double strength fixative to the culture medium covering the cells, mixed gently and after 2 min replaced with single strength fix for 2hrs or overnight. Cells were washed with PBS + 0.1% glycine, covered with 1-1.5ml of 1% gelatin in PBS at 37^{o}C for 10mins, and scraped from dish with cell scraper and transferred to an Eppendorf tube; centrifuged for 2-3mins at 200g and re-suspend in 10% gelatin at 37^{o}C for 10mins; and repelled by centrifugation, and then tubes placed on ice until gelatin set. The tip of the Eppendorf tube containing the cells was cut off with a razor blade. Using 2.3M sucrose as a lubricant, the tip was cut in half and the pellet separated from the Eppendorf tip, this was done on ice to keep the gelatin cool. Small blocks/pyramids (~1x1x1mm) were made from the pellet and store in 2.3M sucrose at 4^{o}C until ready for cryosectioning.

2.4.1.5.2: Mounting and Freezing

Once ready to section, the specimens were mounted on pins and frozen to provide a stable block for sectioning. The top of the pin were scratched to improve the grip of the specimen and washed in acetone to remove any dirt. This process was done as quickly as possible over ice to prevent water evaporating from the specimen surface and changing the concentration of the sucrose. After mounting specimens on pins, they were transferred to the cryomicrotome chamber. Frozen specimens (on specimen holders, pins) were stored in LN_2.

2.4.1.5.3: Cryosectioning, Semi-thin Sections and Ultrathin cryosectioning

Semi-thin sections of 100nm were cut for light microscope inspection at ~2.5-3 mm/s and picked up using a loop in a drop of 50:50 methyl cellulose/sucrose and transferred to a slide and stained with toluidine blue to check for light microscopy.

Ultrathin sections were cut at speed between 0.4 and 1.00 mm/s and feed at 70- 80nm and transferred onto nickel grids for immuno-labelling. An ioniser was used to stop sections curling up or flying away.

2.4.1.5.4: Imaging grids in the TEM

To check the grids and make sure the sections are satisfactory before immuno-labelling, a number of grids were placed onto cold 2% gelatin and melted in 37ºC oven for at least 20min to remove gelatine and sucrose/methyl cellulose mixture. The grids were rinsed in distilled water 5x2min, passed quickly over 2 drops of Uranyl Acetate/ Methyl Cellulose (UA/MC) on ice, and then left in 3rd drop for 5-10min. A wire loop was used to pick up grid. Pushed loop under grid and lifted from the droplet. The loop was tilted to an angle of 45-60^{o} (to dry the excess UA/MC underneath) and dragged along the filter paper until the excess was removed and a thin film remained. The grids were allowed to dry before carefully removing and imaging in the TEM.

2.4.1.5.5: Immuno-labelling of thin sections for EM

The grids, stored on the glass slides with Suc/MC at 4°C were transferred to cold 2% gelatin plates before melting at 37°C for at least 20 min, in order to remove the dried mixture of sucrose and methyl cellulose. Specimen grids were floated, section side down on a series of drops (100 µl) of PBS + 0.1% glycine(to quench free aldehyde groups) placed on the clean parafilm surface and left for 5 x 1min. Care was always taken to keep the section side of the grid wet and the back surface dry. The grids were then transferred, and floated on a drop of PBS containing 1% bovine serum albumin (blocking solution) and 1% Normal Goat Serum (NGS) for 3 min. Then transferred to a 5-10 µl drop of primary antibody (as stated in the figure legends) diluted in blocking solution (see table and notes for details on antibody dilution) and centrifuged prior to use. A plastic dish was used to cover the grids and left for 60 min at RT. After incubation, grids were floated on 4 drops of 0.1% BSA/PBS for 2 min each. Incubated on secondary gold conjugate (see table and notes for details on antibody dilution) diluted in PBS + 1% BSA for 30mins, again the grids were covered with a plastic dish. Grids were quickly rinsed through 3 drops of PBS 5 sec in each, washed by transferring to 4 drops of PBS, 2 minutes each drop, then stabilized with drops of 1% glutaraldehyde in PBS for 5 min finally washed in fresh distilled water 10 changes, 1min each.

2.4.1.5.6: Contrasting & drying of cryosections

Before drying, the sections were stained for contrast and supported by polymers in order to prevent drying artefacts. 3 drops of cold 2% methyl cellulose (MC) containing 4% uranyl acetate pH4 (UA) (standard mixture 1: 9) were placed onto a clean parafilm surface, on ice; the labelled grids were then transferred from the final water wash above and touched each onto the first two drops to wash away excess water and left on the final larger drop for 5- 10 min. Each grid was individually looped

off from the MC/UA solution using a 3.5 - 4mm diameter wire loop. Excess liquid was removed from the loop by placing the loop at a 45°- 60° angle onto a clean hardened filter paper, with the section side facing down toward the filter paper and the grid in the loop left to dry. Once dried, grids were carefully removed from the wire loop using pointed forceps and stored section-side up or immediately examined in the TEM.

2.4.2: Scanning Electron Microscopy

The method used in this project is a slight modification from (Goldberg and Fiserova 2010)

2.4.2.1: Sample preparation

Cells were grown on Silicon mounts (Agar Scientific Ltd, Stansted, UK) numbered with a diamond scribe (Agar Scientific Ltd, Stansted, UK) and cleaned with acetone, dipped in 70% ethanol and sterilised in a flame. Mounts were placed in cell culture dishes and cells allowed to grow to about 70% confluence for 1 or 2 days. Then, cells were serum starved overnight at 37^{o}C in an incubator. The next day, cells were briefly with PBS, treated with or without 100 ng/ml EGF under serum-starved conditions for 1h at 4^{o}C; then brought back to 37^{o}C for 30 min to initiate endocytosis before the mounts could be processed for Immuno-labelling.

2.4.2.2: Immuno-labelling

Immuno-labelling of cells was similar to immunofluorescence. Mounts were washed in PBS two times 5 min, fixed in 4% paraformaldehyde for 15 min, washed three times for 5 min, incubated in blocking buffer PBG (0.1% cold water fish gelatin (Sigma G-7765) and 1% BSA in PBS) for 1 h at room temperature. Whilst the block solution was being incubated, the extracellular domain primary antibody, Goat anti-Mouse IgG (InVitrogen), was prepared at optimised dilutions in PBS (As indicated in the Figure legends). After incubation for one hour, unattached antibodies were removed by washing with PBS three times 10min, and then further incubated with secondary antibody, EM BSA 5 and 10 nm Gold, for 1h at room temperature. After antibody labelling, unattached secondary antibody was removed by washing five times 10min. Then mounts were place in modified Karnovskys SEM fix (2% PFA in water, 2.5% glutaraldehyde in 0.1M phosphate buffer (35.61g $NA_2HPO_4.2H_2O$ and 27.6g $NA_2HPO_4.2H_2O$), pH7.4)) or sodium cacodylate for 10 min at room temperature or overnight at 4^{o}C. Two Petri dishes were filled with 0.1M sodium cacodylate and one with 1% osmium tetroxide in 0.1M sodium cacodylate. The mounts were transferred from SEM fix into the first dish containing sodium cacodylate for 1 min; mounts were transferred again into the second dish containing sodium cacodylate for 1 min, then into the dish with osmium tetroxide for

10 min. Cells were dehydrated in ethanol as follow, six Petri dishes were filled with distilled water and the remainder with 50, 70, 95, 100, 100% ethanol, respectively. Mounts were transferred into each of the dishes for 2 min with tweezers.

2.4.2.3: Critical Point Drying

After dehydration, mounts were transferred to a Critical point dryer (CPD) carrier under 100% dry ethanol. The CPD chamber was filled with 100% ethanol, the CPD carrier placed in chamber and the lid closed. The Bal-tec CPD 030 was used. Cooling was started and ethanol exchanged for liquid CO_2 with at least 10 changes until all the ethanol was replaced and it was left to stand for 30 min and 10 additional exchanges were performed. Chamber warmed to 40^{o}C, CO2 gas was slowly released over about 10 min.

2.4.2.4: Chromium Coating

The Cressington 328 coating system was used to coat the samples. Silicon mounts with the sample were placed on a clean glass slide in the vacuum chamber on the coating unit and pumped to $<10^{-6}$ mBar. The Cryo-pump was cooled with liquid nitrogen and by opening the isolation valve on the top plate of the cryo-pump the vacuum was allowed to improve until a vacuum of at least 5×10−7 mBar was reached. An Argon atmosphere was introduced to a pressure of around 10^{-3}m Bar and then chromium was sputtered for 30 sec with a shutter in place over the samples, until the plasma became skyblue. The shutter was then opened until 1–1.5 nm chromium was deposited on the sample and then the current was turned off. The valve on the cryo-pump was closed and the vacuum chamber vented, and then the glass slide with the specimens was removed and placed on a sheet of white paper; the colour of the metal coat on the glass was grey. Then, samples were imaged in the SEM.

2.4.2.5: Image Acquisition

For imaging the silicon mounts, the specimens were inserted into the Hitachi S5200 feSEM (Cotter, Allen et al. 2007) after coating. The microscope was set as follow, 10 kV accelerating voltage, and high emission current (20µa), large spot size. The secondary electron detector acquires a high resolution surface image, the secondary image; by detecting low energy electrons ejected from the sample surface (known as secondary electrons) to give an image of the sample surface. The backscatter detector was used to acquire the backscatter image.

2.5: Biochemical Fractionation

Cell Lysate

For the biochemical fractionation of cells, MCF-7 and MDA-MB-231 cells were grown on sterile culture dishes in the presence of DMEM with 10% serum for 1 or 2 days until they reached 70-80% confluence. The next day, the media was aspired off, cells washed with new media and grown further more in fresh DMEM with or without 10% serum overnight. Then the next day, cells were incubated at 4^{o}C with or without 100 ng/ml EGF for 1 h. While on ice, cells were then washed twice with ice-cold PBS and aspired; ice-cold lysis buffer (0.5% Triton X100, pH 7.4, 20 mM Tris, 100 mM NaCl, 1 mM EDTA) and Protease inhibitor was added and incubated on ice for 15min; vortexed at each 5 min. Cells were scraped with a plastic cell scraper and further disrupted and homogenised by passing through a 21-gauge needle. The cell lysis buffers containing the cell extract were immediately removed from the dishes and placed in ice microcentrifuge tubes; and centrifuge at 1000rpm for 10 min at 4^{o}C. Supernatant lysates were transferred to new tubes and diluted 1:10 with SDS-loading buffer, and boiled in boiling water bath for 5 minutes and then sonicated with 3-4 bursts of 5-10 seconds each; then frozen at -20^{o}C or processed for western blotting; the cell lysates were diluted at least 1:10 before determining the protein concentration because of the interference of the detergents in the lysis buffer with the Coomassie-based reagent and then frozen at this point for long-term storage at -80^{o}C.

2.6: Gel Electrophoresis and Immunoblotting

For proteins detection, the gel electrophoresis was incubated with chemiluminescent detection substrate Coomassie-based reagent. To process for Western Blot, proteins lysates were run on gel electrophoresis; and for immunoblotting, electrophoresed proteins were transferred the to a PVDF membrane. Membrane Blocking and Antibody Incubations: the PVDF were incubated for 1 hour at room temperature in Blocking Solution (25 mM Tris, pH 7.4, 0.15 M NaCl, 0.1% Tween® 20). The membrane was incubated overnight at 4°C in appropriate Antibody Solution containing primary antibody at indicated concentration shown in table 1. The next day, the membrane was washed at room temperature for 30-60 minutes with 5 or more changes of Blotting Buffer (2-5% non-fat dry milk in Blotting Buffer, pH to 7.4). Then the membrane was incubated for 1 hour at room temperature in Antibody Solution containing appropriate dilution of HRP-conjugated secondary antibody as indicated in table 2. The membrane was washed again for 30-60 minutes with 5 or more changes of Blotting Buffer. And finally exposed to film and developed images.

Chapter 3: Results

Many cancer research studies have focused on EGFR, its nature and mechanism of import to the nucleus. EGFR which is found to be over-expressed in cancer cells is thought to interact with Importinβ and Sec61β while translocating to the nucleus and into the NP. Much has been discovered about the transport of EGFR from the cell surface into the nucleus. Despite all the evidence, such as that the over-expressed EGFR that travels to the nucleus interacts with Importinβ and Sec61β and that it resides in the INM, little is understood. This project is carried out to confirm some of these claims. Firstly, the expression of EGFR was tested by western blots (WB) and by IF and EM studies in different cancer cell lines. Then immuno-fluorescence (IF) and Immuno-Electron Microscopy (immuno-EM) were also carried out to observe and confirm the translocation of EGFR from the cell surface in the cytoplasm to the nucleus and into the NP. MCF-7 and MDA-MB-231 human breast carcinoma and HeLa human cervical cell lines were used in this study and in all the experiments, although, it is by choice that some results only show one of the cell line; other results are not shown as similar to the one presented. The MCF-7 and MDA-MB-231 cells were used because they are found to particularly over-express EGFR. HeLa cells (human cervical adenocarcinoma) were used as control as they express endogenous EGFRs at close to physiological concentrations-approximately 50,000 EGFRs/cell; (Berkerset al. 1991, Dinneen and Ceresa 2004, Yu et al. 2009). Importantly, HeLa cells have been sufficiently characterized in their EGFR endocytic trafficking and signal transduction such that they are considered to function in a manner analogous to endogenous EGFRs; (Yu et al. 2009)

Detection of EGFR expression and localisation

Antibody specificity and effectiveness for EGFR, Importinβ or Sec61βI were first checked by WB, IF and by EM (SEM and TEM) in order to set the working concentration. As expected, WB experiment indicated that MCF-7 and MDA-MB-231 human breast carcinoma cells and HeLa human cervical cells expressed EGFR as a band of 170-180 kDa, Importinβ as a 97 kDa, and Sec61β a 14 kDa (Figure 7); and IF experiment image analysis confirmed the presence of EGFR (Figure 8.1), Importinβ (Figure 8.2) and Sec61β (Figure 8.3). EM images (Figure 9) of the same cell lines also confirmed expression of EGFR at the cell surface.

For WB studies, MCF-7 and MDA-MB-231 human breast carcinoma cells were grown with or without Fetal bovine serum (FBS) to study the effect of starvation on the culture, whether it affected the expression of EGFR, Importinβ or Sec61β. The samples were then treated with or without EGF

to observe the effect of EGF stimulation, whether EGF stimulated EGFR endocytosis. Starvation did not seem to have an effect on the expression of Importinβ or Sec61β when compared to the non-serum starved cells; (Figure 7, lanes 3, 4, 7, 8, 11, 12 versus lanes 1, 2, 5, 6, 9, 10); but there was a change in the expression of EGFR. Starved cell seem to have a higher expression of EGFR compared to the non-starved cells; (Figure 7, lanes 3, 4, 7, 8, 11, 12 versus lanes 1, 2, 5, 6, 9, 10). A slight increase in the expression of these proteins (EGFR, Importinβ and Sec61β) is observed in the cells when treated with EGF (Figure 7, lane 1 versus lane 2, lane 2 versus lane 4, 5 versus 6, 7 versus 8, 9 versus 10, 11 versus 12). These results are interpreted on the base that actin levels are the same in all cells.

For IF experiments, MDA-MB-231 and HeLa cells were treated accordingly; in short, cells were serum-starved in medium, then treated with EGF, finally fixed then labelled with appropriate antibody and analyzed using confocal microscopy. Image results clearly demonstrate the expression of EGFR localised on the cell-surface, as seen in Figure 8.1.

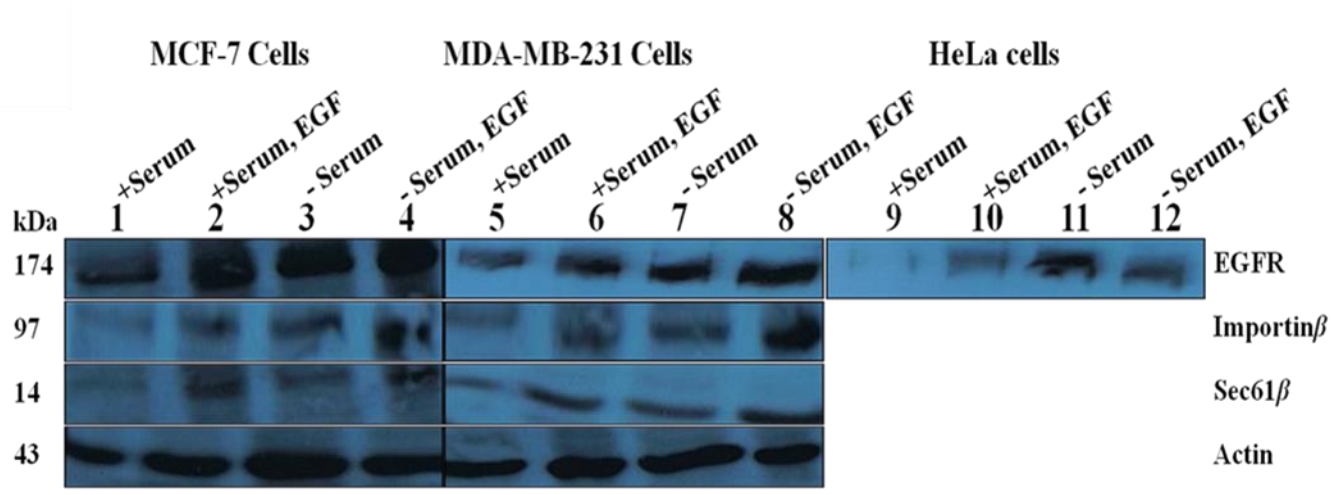


Figure 7. Expression and localisation of EGFR, Importinβ or Sec61β. Cells used for this experiment were maintained in serum-starved medium overnight (unless indicated otherwise), then treated with EGF for 30 min on ice, incubated for 5 min at 37^{o}C, finally fixed accordingly. **A**, Western blots; MCF-7, MDA-MB-231 and HeLa whole cell lysates were subjected to SDS–PAGE and WB analysis using EGFR, Importinβ, Sec61β and β-actin Abs. Actin was used as a control, as well as HeLa cells. Endogenous levels of EGFR, Importinβ, and Sec61β were simultaneously determined by WB analyses in which β-actin was also detected to serve as loading controls (As indicated).

For Immuno-EM experiments, TEM experiments were done to localize EGFR. MCF-7 and MDA-MB-231 cells were serum starved overnight, then treated with or without EGF (100 ng/ml) and incubated at 4^{o}C for 30 min and fixed; cells were fixed at 0 time (0 min) after bringing at room temperature. Results show that EGF stimulated samples have EGFR localised at the cell surface; yet some EGFR labelling was observed in the cytoplasm although samples were fixed at 0min (Figure

9, top panel); this is consistent with previous reports that indicate a basal level of EGFR in the cytoplasmic space; (Wang, Yamaguchi et al. 2010). Samples processed without EGF treatment also show a presence of EGFR on the cell surface but not in large number; almost no EGFR was found in the cytoplasm (Figure 9, top panel). SEM experiments were also carried out to localize EGFR. MCF-7 cells were treated with EGF (100 ng/ml) and incubated at 4^{o}C for 30 min and fixed as described in the method section at 0min after bringing at room temperature. Results clearly show the presence of EGFR on the cell surface (Figure 9, Lower panels). These observations are consistent with previous studies as EGFR is a cell-surface receptor for members of the EGF-family of extracellular protein ligands. These observations confirm the expression of EGFR in these cells.

Three-dimensionally constructed z-stack images using confocal microscopy experiment was performed to confirm the expression of EGFR (Figure 10). The figure shows HeLa (A) cells and MDA-MB-231 (B) cells grown without serum overnight and incubated with EGF for 30min at 4^{o}C, and treated with Qdot® Strepavidin Conjugate, then brought at room temperature and samples viewed live directly under the microscope as explained in the method section. The figure represents the expression of EGFR in cells. The bright white colour on images indicates Qdot®. Image A1 (Hela), B1 (MDA-MB-231) represent the top of the cell, and A12 and B8 the bottom of the cell as the cell is sequentially sliced down every 0.5 μM.

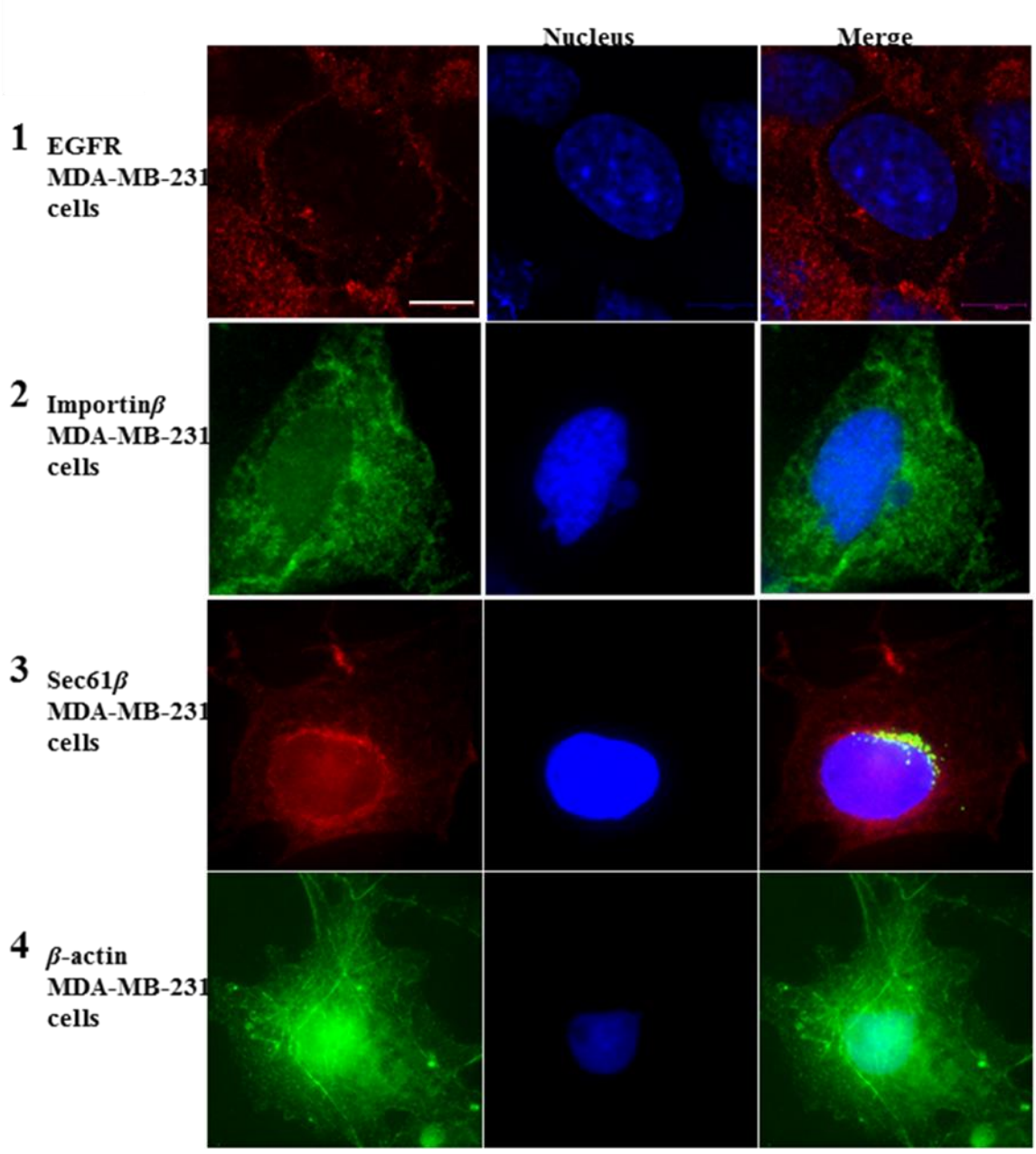


Figure 8. EGFR expression by IF/confocal analyses. Localisation of EGFR (1), Importin*β* (2), Sec61*β* (3) and *β*-actin (4) after EGF treatment. MDA-MB-231 and HeLa cells were immunostained with EGFR, Importin*β*, Sec61*β* and *β*-actin; analysed using confocal microscopy. First column represents the proteins, the Second column (DAPI staining) represents Nucleus; Third column is the Merge. Fourth column in first row is EGFR staining in HeLa cell. Bar, 5 µm

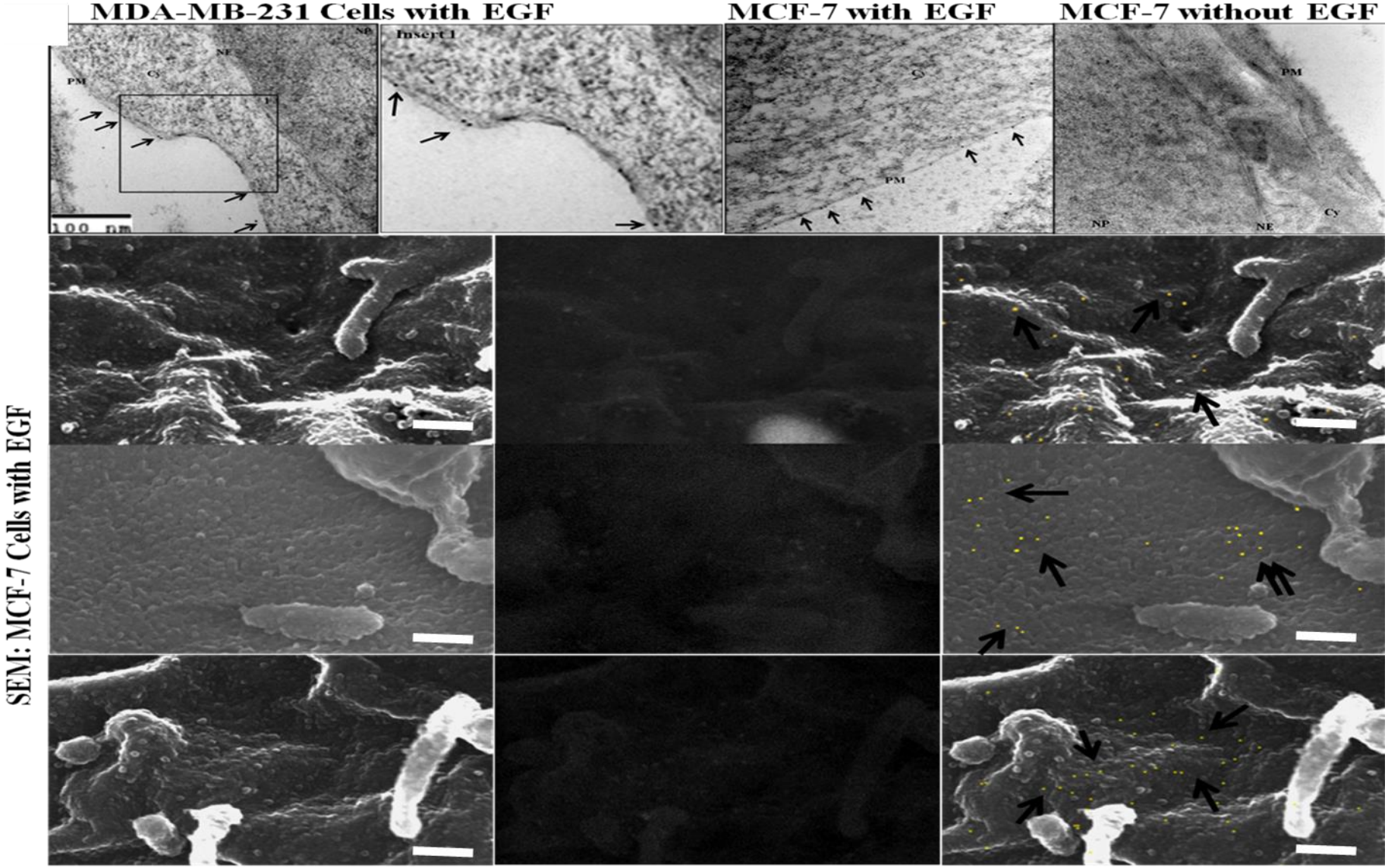


Figure 9. EGFR expression by EM analyses. EGF-induced nuclear translocation of EGFR was analysed using immuno-EM. MCF-7 or MDA-MB-231 cells were treated with or without EGF (100 ng/ml) and incubated at 4ºC for 30 min and fixed at 0 time (0min) after bringing at room temperature. **Top panel**: TEM High Pressure Freezing and Freeze Substitution; showing the expression and localisation EGFR at the cell surface in MCF-7 or MDA-MB-231 cells. **Lower panel**: Three examples of SEM images for the surface of MCF-7 cells. Secondary antibodies labelled with 10-nm gold particles were used to indicate EGFR. The first column represents raw data, the middle shows back-scatter images, and third column shows gold labelling of EGFR represented by yellow dots (shown by arrows). Antibody dilutions are shown in the table 1 and 2. PM, plasma membrane; Cy, cytoplasm; NP, nucleoplasm; NE, nuclear envelop. Bar, 10µm. All insets demonstrate enlarged high-resolution images

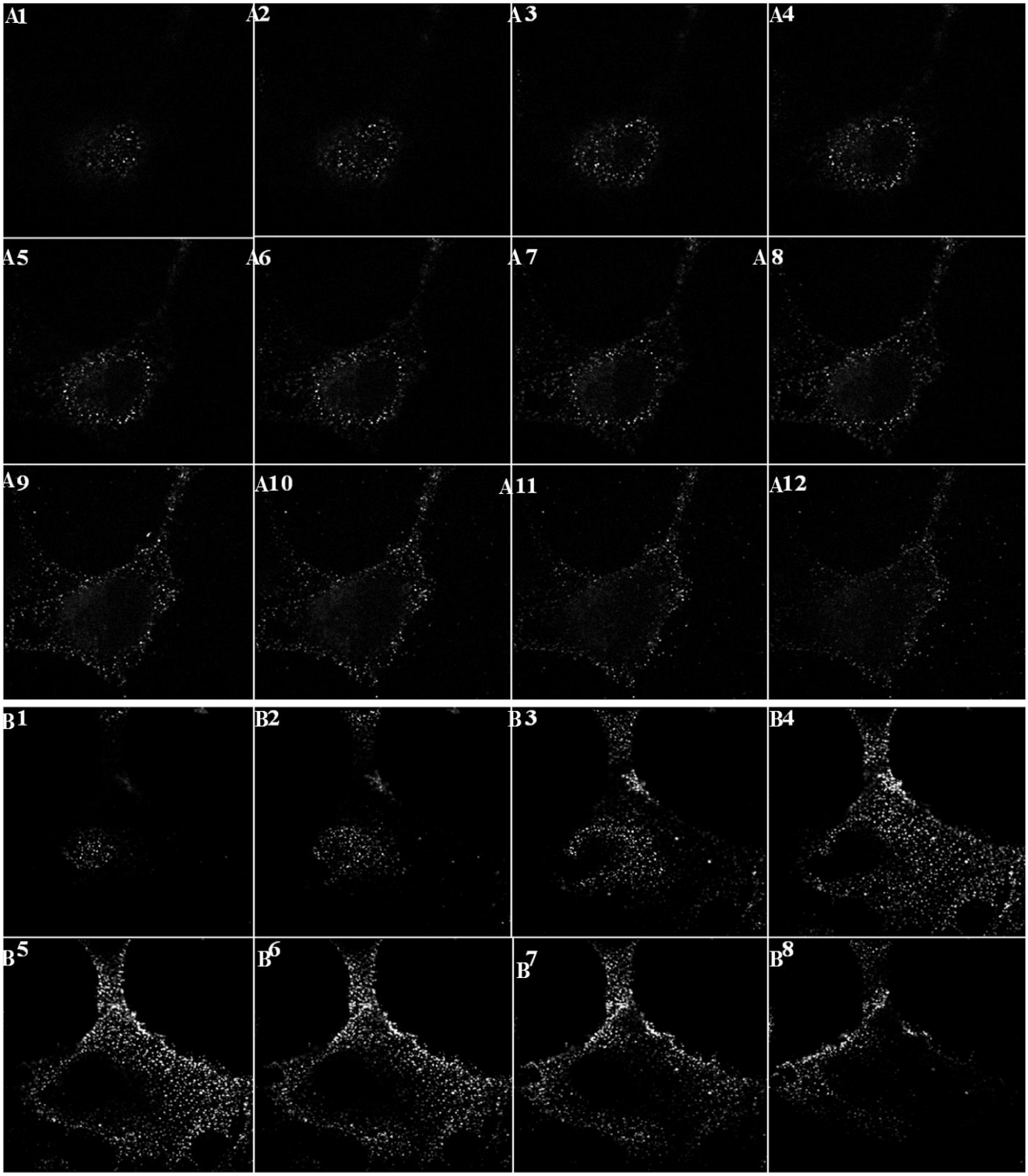


Figure 10. Z-stack images of EGFR expression by Spinning Disk microscopy. The bright white colour indicates EGFs conjugated to Qdot® Strepavidin 525, 625 bound to EGFRs shown by confocal Spinning Disk. Image stack of HeLa cells (**A**) and MDA-MB-231 cells (**B**) taken from above every 0.5 µM, A1 and B1 represent the top of the cell, and the cell is sequentially sliced down through horizontally, A12 and B8 represent the bottom, where the signal is spreading out as the cell stand flat in contact with the bottom of the petri dish. Cells were grown +/- serum, processed by treatment with EGF and Qdot® Strepavidin Conjugate and then viewed live on the spinning disk microscope.

The effect of serum starvation on cells was also checked by IF to observe the expression and location of EGFR. There was not a clear difference observed between MCF-7 and MDA-MB-231 cells that were serum starved compared to the non-serum starved cells. But a slight change was seen the serum starved cells where EGFR was mainly seen localised at the cell-surface, while the non-starved cells EGFR seem to be localised not only at the cell-surface but also in the surroundings of the cell surface, inside the cell. This could be due to the fact that in serum starved cells, endocytosis and other pathways are decreased by the reduced availability of growth factors; while the non-serum starved cells, EGFR endocytosis process is continual; (Figure 11 & 12). Similar observations were made in cells with and without EGF treatment; there was not a clear difference in cultures grown with or without EGF. Yet, a slight change was observed in cells without EGF, EGFR was seen localised mainly on the surface while in cells treated with EGF, EGFR localisation was seen to be spreading from the cell surface; (Figure 11 & 12). Our results are not conclusive; therefore more experiments are needed to clearly observe and confirm the differences between serum and non-serum starved cultures, and between cultures grown with and without EGF.

EGFR nuclear translocation involves endocytosis and endosomal sorting machinery

Observations were made during this study confirming that EGFR translocation into the cell cytoplasm involves endosomal sorting machinery; (Figure 13); this is also in accordance with other studies that suggest that for cell surface EGFR to enter the nucleus, it might require EGF mediated internalisation; endocytic vesicles serving as carriers. After cells were incubated with EGF on ice and briefly incubated at 37°C for up to 5 min, EGFR was found in invaginated endocytic vesicles (Figure 13). EM images also showed transported EGFR apparently not enclosed by membrane; (Figure 13, indicated by solid arrows, top panel). However this could be because fixation was not completely successful, making it hard to see some membranes. With these observations we confirm that, upon EGF treatment, the ligand binds to the extracellular domain of EGFR and the latter is endocytosed, embedded in endocytic vesicles, then transported to the nucleus.

Immuno-SEM experiments were also carried out to show the endocytosis of EGFR; (Figure 14). The experiment was performed to see what happens on the cell surface; as demonstrated by the images in these figures, EGFR presence detected by labelling with gold particles (represented by the Yellow dot in the figure) are seen in large number on the cell surface at 0 time. There is a visible decrease in the gold particles at time 5 min and more at time 15 min. Statistical analysis

also shows the decrease of EGFR expression on cell surface with time; (Figure 15; Histogram). The decrease in the number of gold particles on the cells surface of the SEM images indicates that EGFRs are being endocytosed into the cell; yet not all EGFRs labelled by gold particles disappeared at time 60 min (Results not shown). These observations in Figure 13 and 14 confirm the endocytosis and transport of EGFR from the cell surface and are consistent with previous studies.

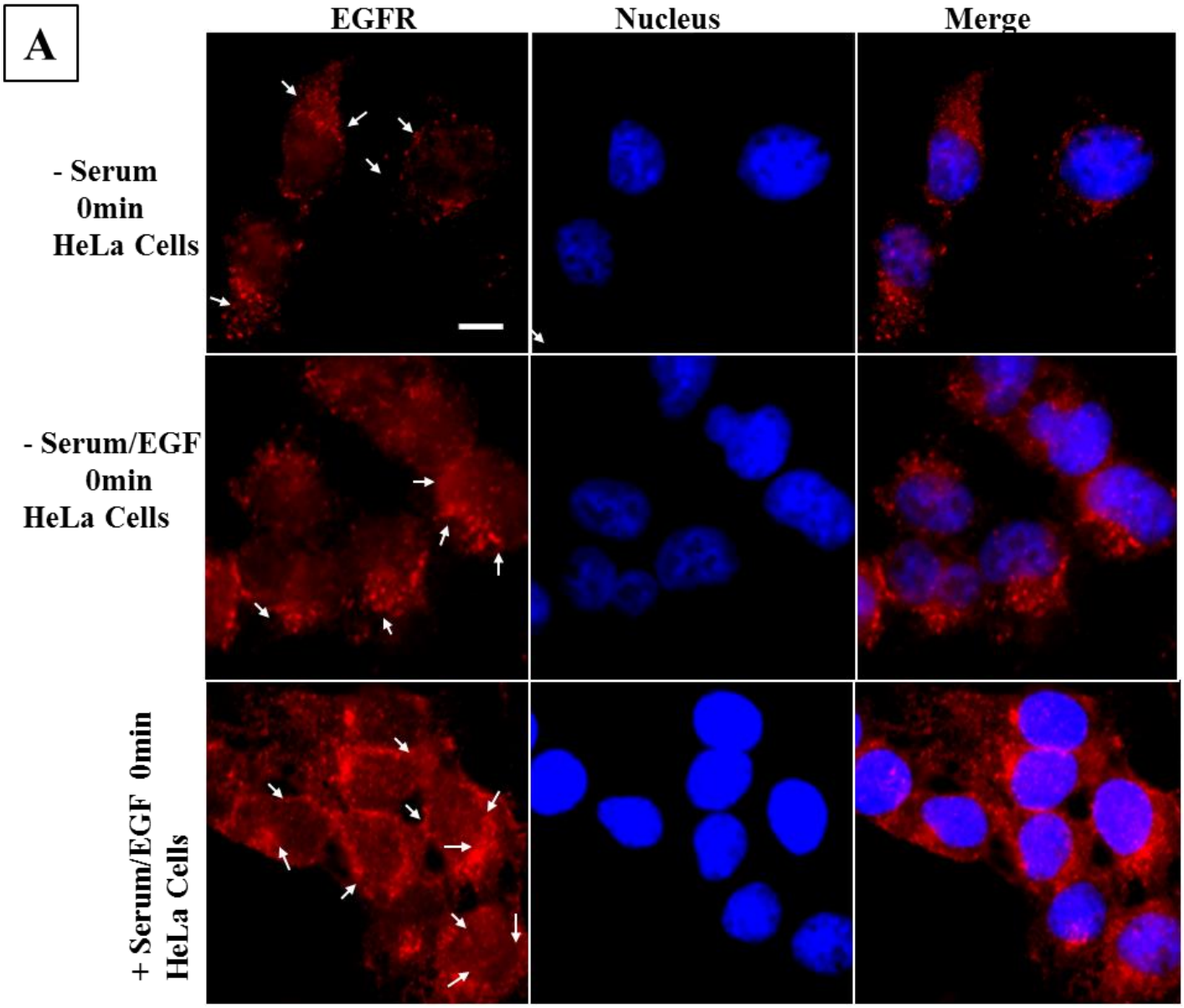


Figure 11. IF microscopy analysis of HeLa cells treated without/without Serum and EGF. Cells were incubated with or without FBS overnight and then treated with or without EGF for 30 min on ice and then fixed at 0 time. Antibody dilutions are shown in the table 1 and 2. EGFR is indicated by the red signals and the nucleus stained with DAPI is represented by the blue signal. Arrows indicate the location of EGFR in the cell. Arrows in –Serum cells shows EGFR mostly on the PM; in –Serum arrows show EGFR spread inside the cell. Bar, 10µm.

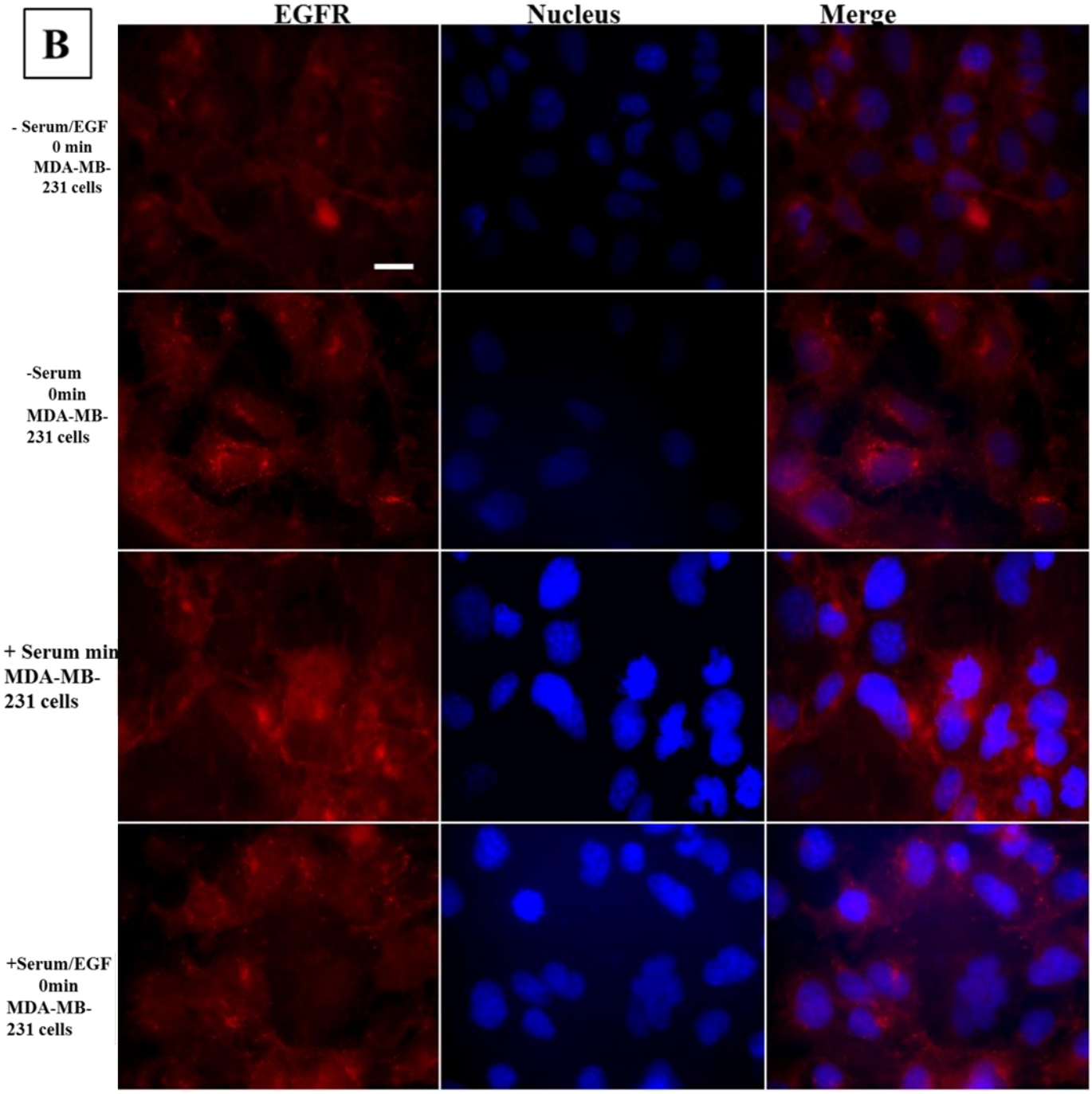


Figure 12. IF microscopy analysis of MDA-MD-231 cells treatment with/without Serum and EGF. Cells were treated as described in the figure legend **(A)** far above. Antibody dilutions are shown in the table 1 and 2. EGFR is indicated by the red signals and the nucleus stained with DAPI is represented by the blue signal. Bar, 10µm.

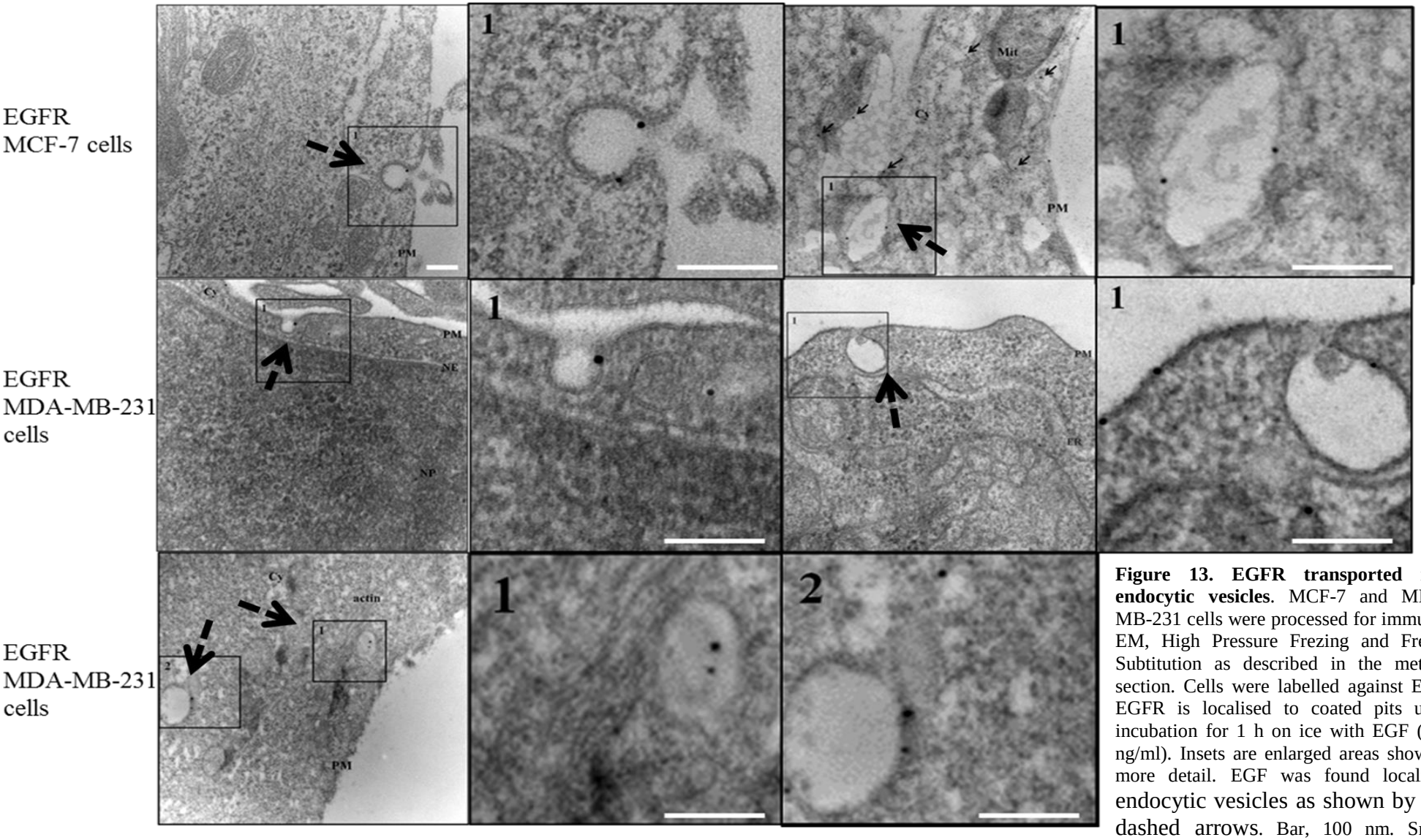


Figure 13. EGFR transported into endocytic vesicles. MCF-7 and MDA-MB-231 cells were processed for immuno-EM, High Pressure Frezing and Freeze Subtitution as described in the method section. Cells were labelled against EGF. EGFR is localised to coated pits upon incubation for 1 h on ice with EGF (100 ng/ml). Insets are enlarged areas showing more detail. EGF was found localized endocytic vesicles as shown by big dashed arrows. Bar, 100 nm. Small arrows shows

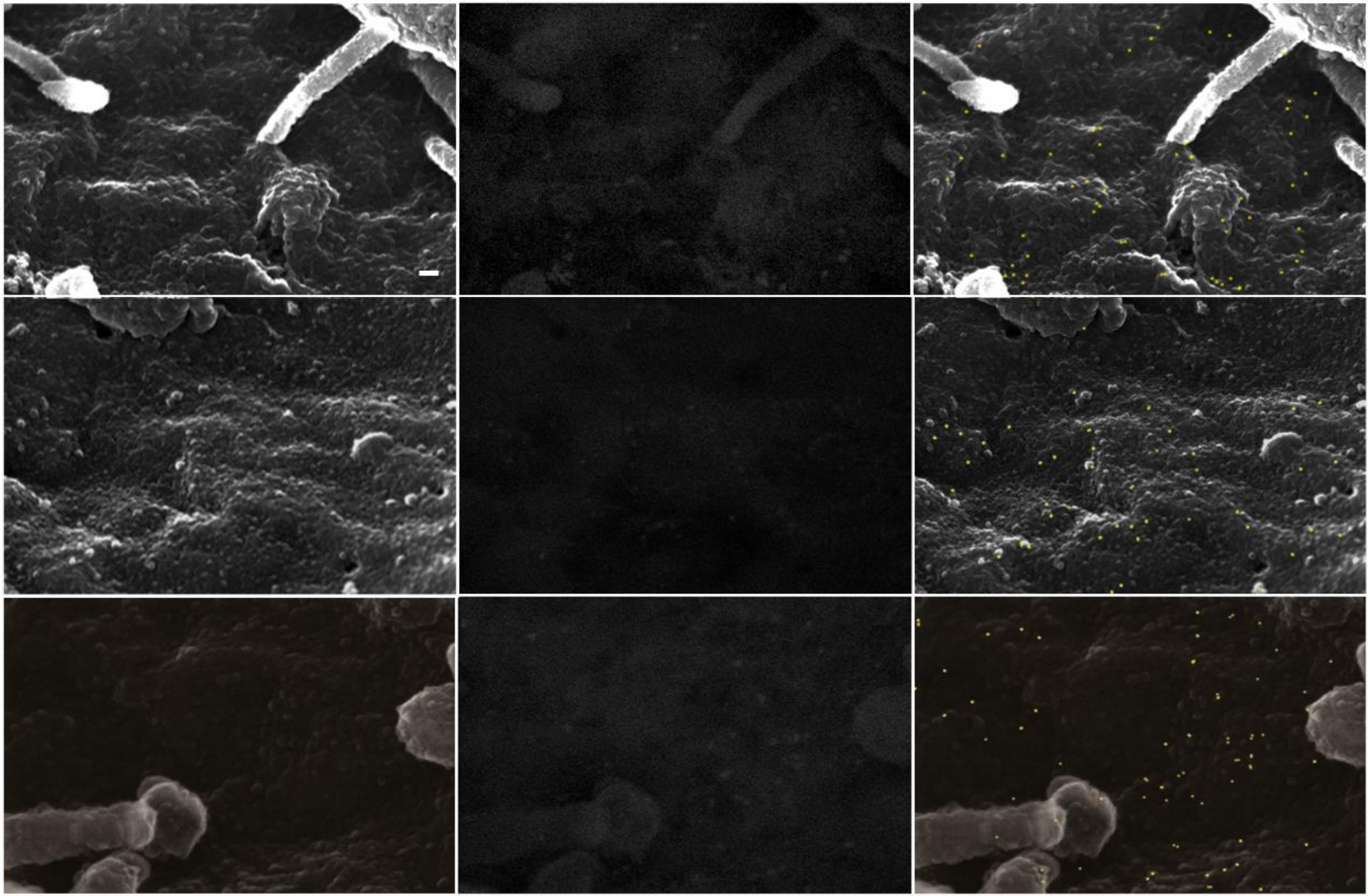


Figure 14.A. EGFR endocytosed from the cell surface processed by Immuno-SEM time 0 min. MCF cells were treated with a secondary goat anti-mouse IgG, 10-nm gold particles represented by Yellow dots. Middle column is the backscatter image, left column are enhanced images by Photoshop CS6 (64 Bit) software program. Bar, 100nm.

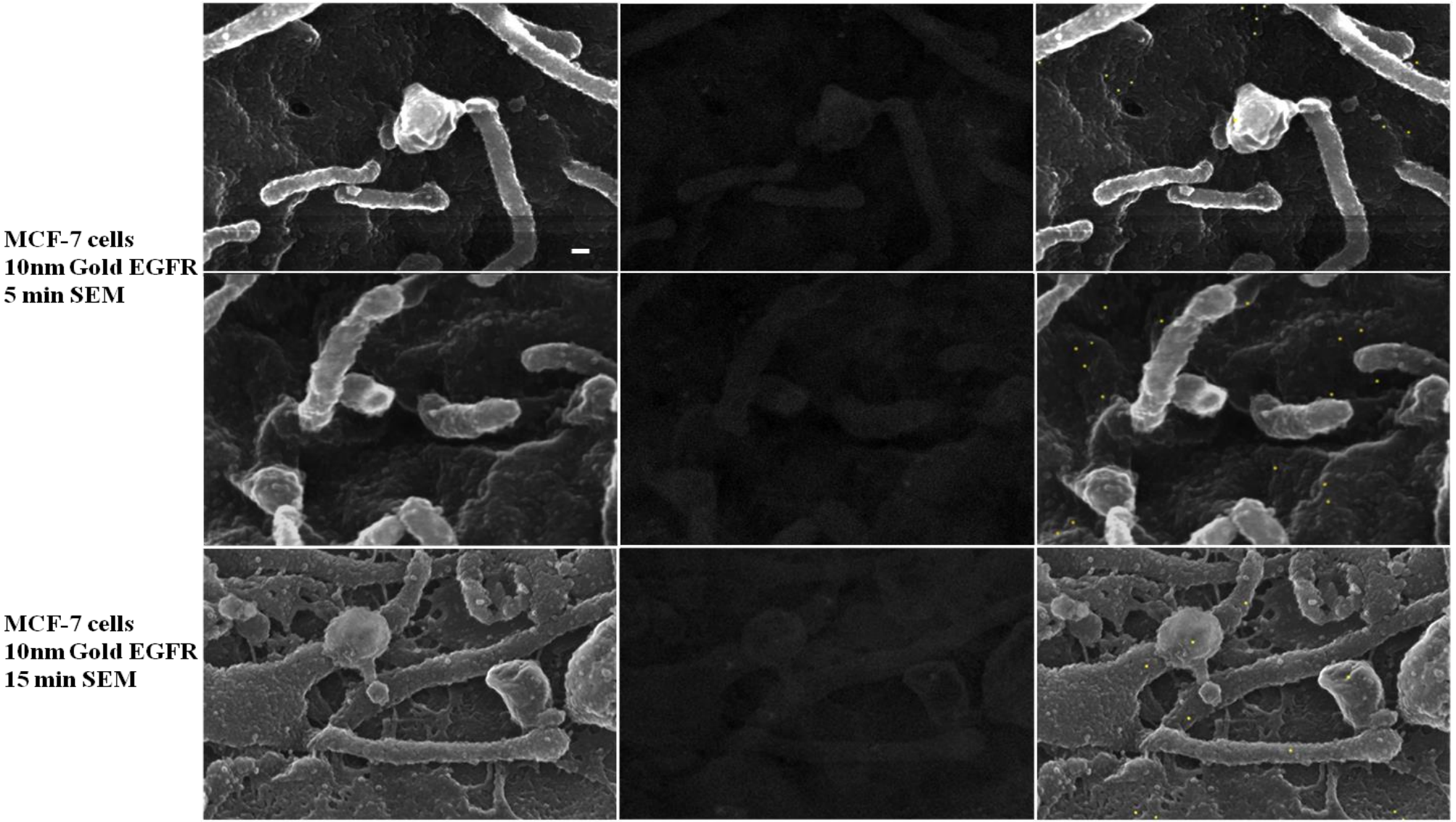


Figure 14.B. EGFR endocytosed from the cell surface processed by Immuno-SEM time 5 and 15 min. MCF cells were treated with a secondary goat anti-mouse IgG, 10-nm gold particles represented by Yellow dots. Middle column is the backscatter image, left column are enhanced images by Photoshop CS6 (64 Bit) software program. Bar, 100nm.

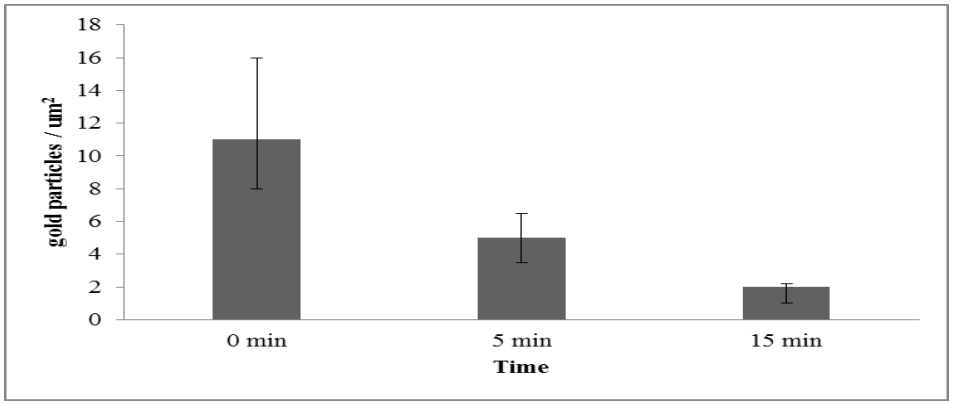


Figure 15. Histograms showing the distribution of EGFR gold nanoparticles labelling over different cell SEM fixation times. Bars represent average densities (±S.E.M.) of gold particles over 2 um^2. The values are pooled data from three single-labelling experiments.

Endocytotic vesicles co-localise with actin

It was tested to see whether vesicles containing EGFR were transported in an actin-dependent way as some observations were made in some reports. IF images seem to show EGFR embedded in vesicles moving along actin; (Figure 16), yet it is uncertain whether the vesicles are traveling on the actin as the images of the EGFR and Actin signal show the nucleus signal. Similar observations were also seen by immuno-TEM analysis; (Figure20). MCF-7 cells immuno-stained with EGFR antibody with and secondary antibody conjugated to gold nanoparticles showed many filaments in the cytoplasm, on which many gold particles were seen in close proximity or possibly attached to. The vesicles enclosing EGFR are small in size and cannot be seen on the images (Figure 20); this could be due to the difficulties of immune-EM fixation. These observations, of vesicles travelling on actin, are consistent with the function of the latter. Actin is a highly conserved protein that participates in many important cellular processes, including vesicle and organelle movement, protein-protein interactions, and the establishment and maintenance of cell junctions and cell shape. Despite this, these observations are inconclusive as we believe more is need to study in details to confirm these observations and understand how the vesicles interact with actin; moreover, it is necessary to clearly show by Z-stack confocal microscope and by protein interaction studies and also by live cell imaging that the vesicles are really moving on the actin and investigate the interaction mechanism of the vesicle membrane with actin filaments. We suggest for IF live and fixed (three-dimensional structures and interactions determined by field emission in-lens scanning electron microscopy) experiments staining the vesicles and the actin with different colour, this will allow a close observation of interaction.

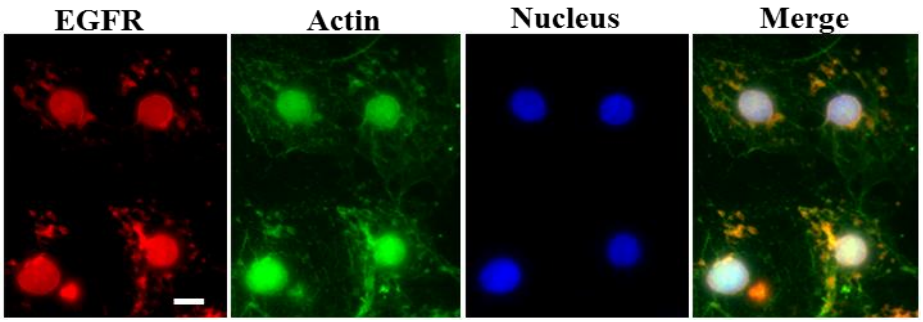


Figure 16; EGFR endocytic vesicles transported on Actin to the nucleus. IF images of MDA-MB-231 cells analysed using immunostains of EGFR and *β*-actin, the nucleus was stained with DAPI. Bar 10 µm.

EGFR is imported to the nucleus

Upon EGF binding EGFR on the cell surface, EGFR is endocytosed in vesicles; it is then thought to be imported to the nucleus. Few studies have shown in detail EGFR import by EM, therefore this work was set to see in detail by EM the import of EGFR from the plasma membrane to the nucleus. In order to investigate this, different techniques were performed to characterise the system in different cell lines (MCF-7 and MDA-MB-231), Qdots, confocal, and EM analysis.

A timed IF microscopy study was carried to study the localisation of EGFR in +/- serum cells. This experiment was designed to observe at different time points the effect of serum starvation on the import of EGFR. For this, HeLa and MDA-MB-231 cells treated as described in the method section; in short, samples were incubated overnight with or without FBS (serum starvation) and then stimulated with EGF for 30 min at 4^{o}C before bringing at 37^{o}C to be fixed with PFA at time points, 0, 5 and 10 min; (Figure 17 & 18). Results of this experiment seem to support previous reports. It was observed that EGFRs were located and concentrated at cell surface at time 0 min (fixation time) in serum starved cells, and were thought to be moving from the cell surface towards the nucleus at time 5 and 10 min. However, in non-starved cells, EGFRs were seen both on the plasma membrane and inside the cell (the cytoplasm) at time 0 min; and there appear to be minimal difference in the location of EGFRs in time 0 min and time 5 and 10 min; (Figure 18). Despite these observations, there is little evidence to clearly prove the difference in the effect of serum starvation on EGFR location. We suggest more IF analysis with better antibodies in order to confirm previous studies.

For more studies of the import of EGFR to the nucleus; an experiment was also performed with Qdot® Strepavidin Conjugate. As this experiment was never done in the study of EGFR in breast cancer cells, it was performed to observe the import mechanism of EGFR by IF and EM. Qdot®

Strepavidin Conjugate which are single crystals of semiconductor materials (CdSe) were used because of their unique properties: correlative light and electron microscopy; and for its real-time receptor ligand tracking. These nanometer-size materials are very sensitive, multicolor, stable and highly fluorescence; for more information details, please find appendix on Qdot® Strepavidin Conjugate.

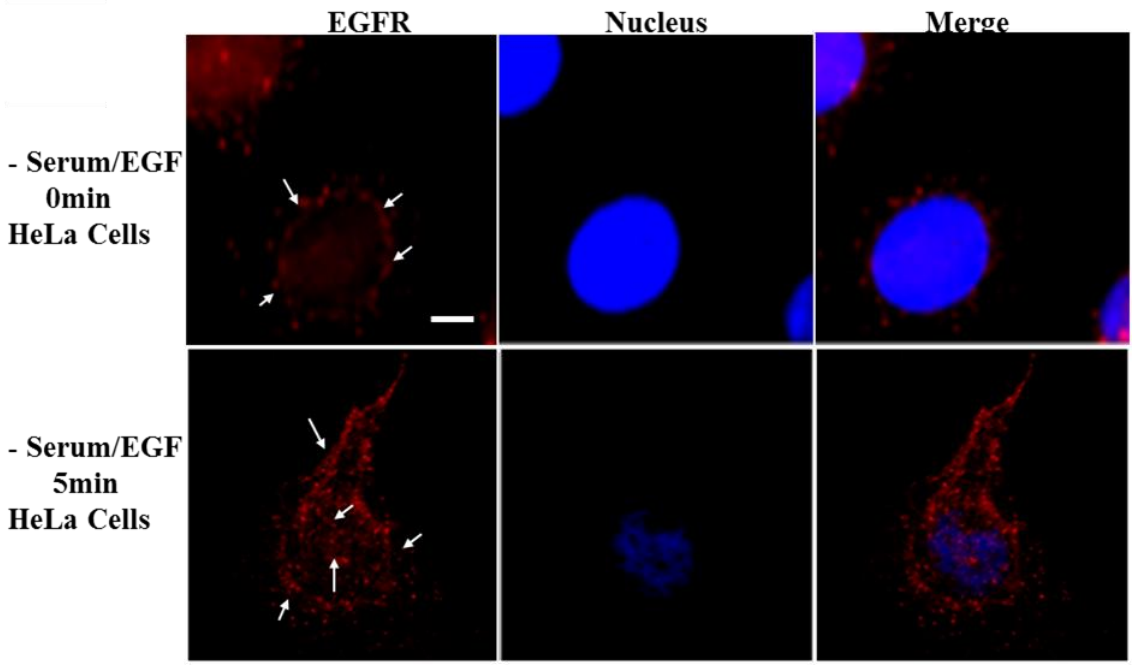


Figure 17. EGFR is imported to the nucleus. Internalization of Alexa 488-EGF shown by IF/confocal. HeLa cells were grown +/- serum, processed by treatment with EGF, fix with PFA at different time points and labelled with Alexa 488, EGFR antibody, Bleu represent DAPI. Bar 10 µm.

For this experiment, cells were treated as for IF and confocal microscopy studies seen in the method section. In short, MCF-7 cells were grown with serum for 1 or 2 days until confluent; then cells were serum starved for one more day. The next day, cells were incubated at 4oC with Biotin EGF and then incubated with Qdot® Strepavidin Conjugate 525, 625 at 100 ng/ml, and moved to 37oC to initiate endocytosis; then cells were fixed with PFA and imaged (Figure 19. A, Top panel); or cells were viewed live on the confocal spinning disk (Figure 19. B, Bottom panel). Qdot® Strepavidin Conjugate experiments by immuno-EM was not successful in our study, (see technical steps further below).

The fixed samples with PFA at time 0 min showed the extracellular localisation of EGFR confirming previous studies. The brightness and the colour of these images proved to be better than images of samples treated with antibodies.

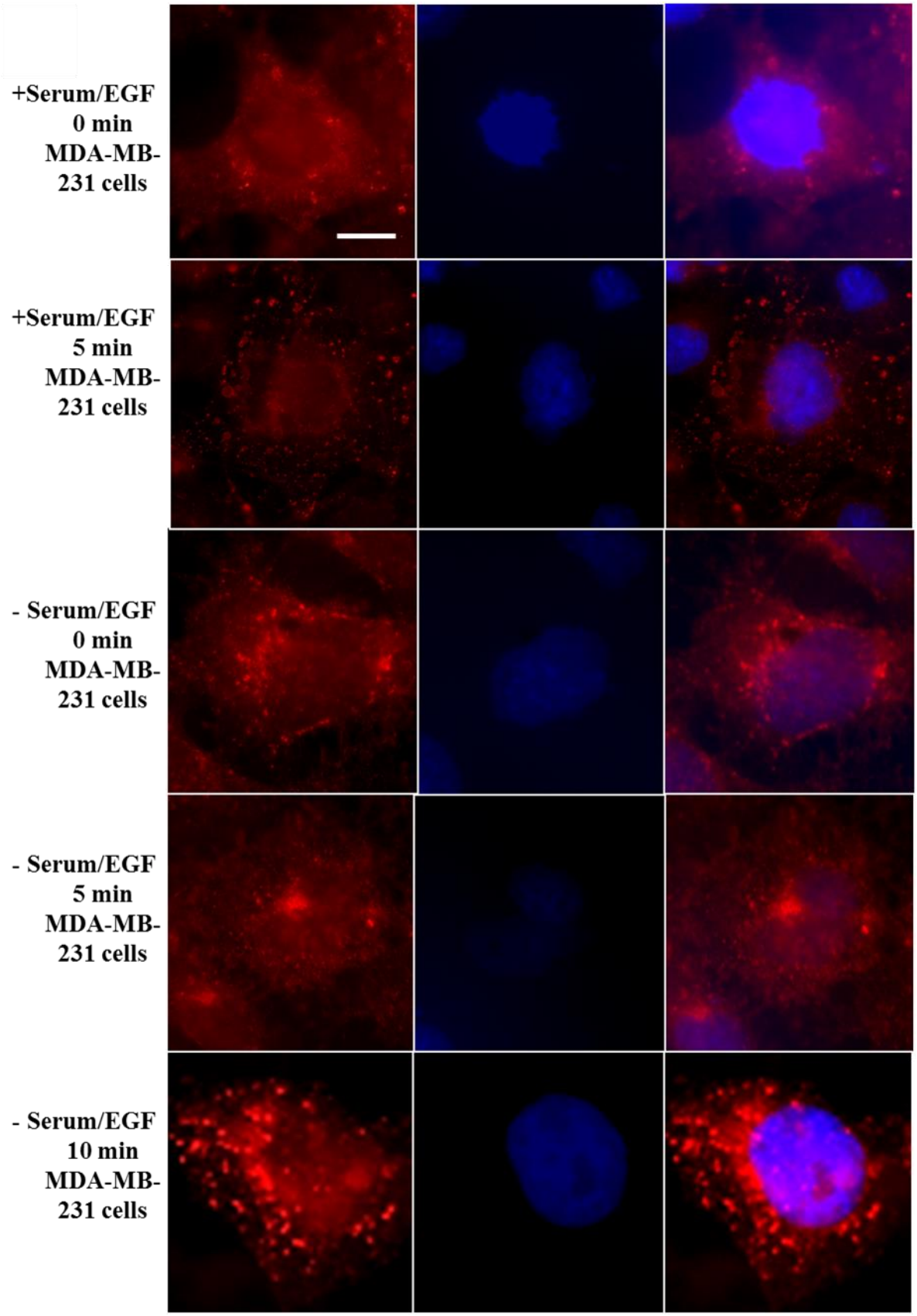


Figure 18. EGFR is imported to the nucleus. Internalization of Alexa 488-EGF shown by IF/confocal. MDA-MB-241 cells were grown +/- serum, processed by treatment with EGF, fix with PFA at different time points and labelled with Alexa 488, EGFR antibody, Bleu represent DAPI. Bar 10 µm.

The live samples were imaged using a confocal Spinning Disk at different time points to observe the import of EGFR, time 0 and 3 min. The images showed at time 0 min the localisation of EGFR at cell surface, this is in line with the fixed cells. At time 3 min, EGFR can easily be seen not concentrated on the cell surface compared to time 0 min but the red stains are seen away from the cell surface towards the nucleus (Figure 19. B, Bottom panel); after time 3 min, nothing was seen happening in the cells, this could be due to photo-bleaching by the laser. These results support the notion that cell surface EGFR is endocytosed into the cell after EGF stimulation and translocates towards the nucleus.

To further confirm these findings, immuno-gold EM studies were carried out in MCF-7 cells using the anti-EGFR antibody. Conventional TEM Processing and High Pressure Freezing, Freeze Substitution (HPF/FS) Processing methods were used to observe the process of translocation of EGFR. Conventional methods are quicker and more straightforward, but HPF/FS may be preferable to catch rapid dynamic processes such as endocytosis and nuclear transport and is less prone to artefacts, particularly of membranes. Similarly to IF observations above, EM images clearly demonstrated that EGF induced EGFR endocytosed and translocated to the nucleus; (Figure 20). The immuno-EM studies in human breast carcinoma MCF-7 cells showed that EGFR was mainly localized on the cell surface plasma membrane (PM) at time 0 min after EGF treatment and that after EGF stimulation, EGFR could be seen in the cytoplasm (Cy) and at time 5 min. EGFR are seen in the cytoplasm in vesicles and Golgi compartments at time 5 min, and it is suggested that it is moving towards the nucleus; (Figure 20. Middle panel). With further time, EGFR was detected in the NE at time 10 min; (Figure 20. Bottom panel). As translocation of molecules from the surface membrane to the nucleus is a continuous process, EGFRs are seen in the nucleus and at the same time in the cytoplasm at time 10 min. These observations are consistent with other observation made in this study and with previous reported analysis.

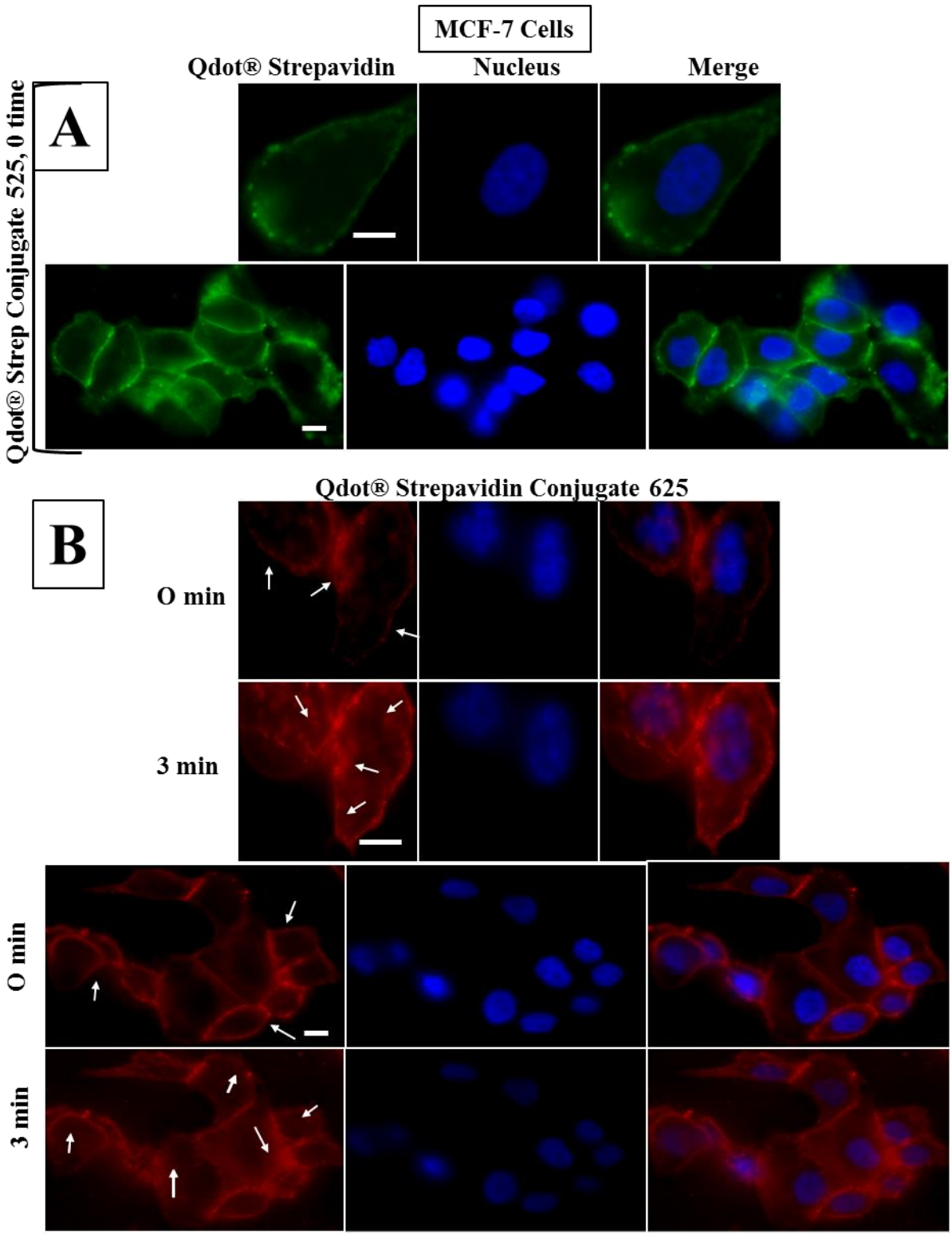


Figure 19. A and B. Translocation of EGFR to the nucleus. MCF-7 cells were analysed by live confocal Spinning Disk with Qdot® Strepavidin Conjugate. The green **(A, Fixed samples)** and red **(B, Live samples)** colours indicate EGFR Qdot® Strepavidin Conjugate and the blue colour the nucleus. Cells were serum starved, and treated with EGF then incubated with Qdot® Strepavidin Conjugate and then viewed under the confocal microscope. Images were taken at the time series indicates. Arrows indicate the localisation of EGFR. Bar 5 µm.

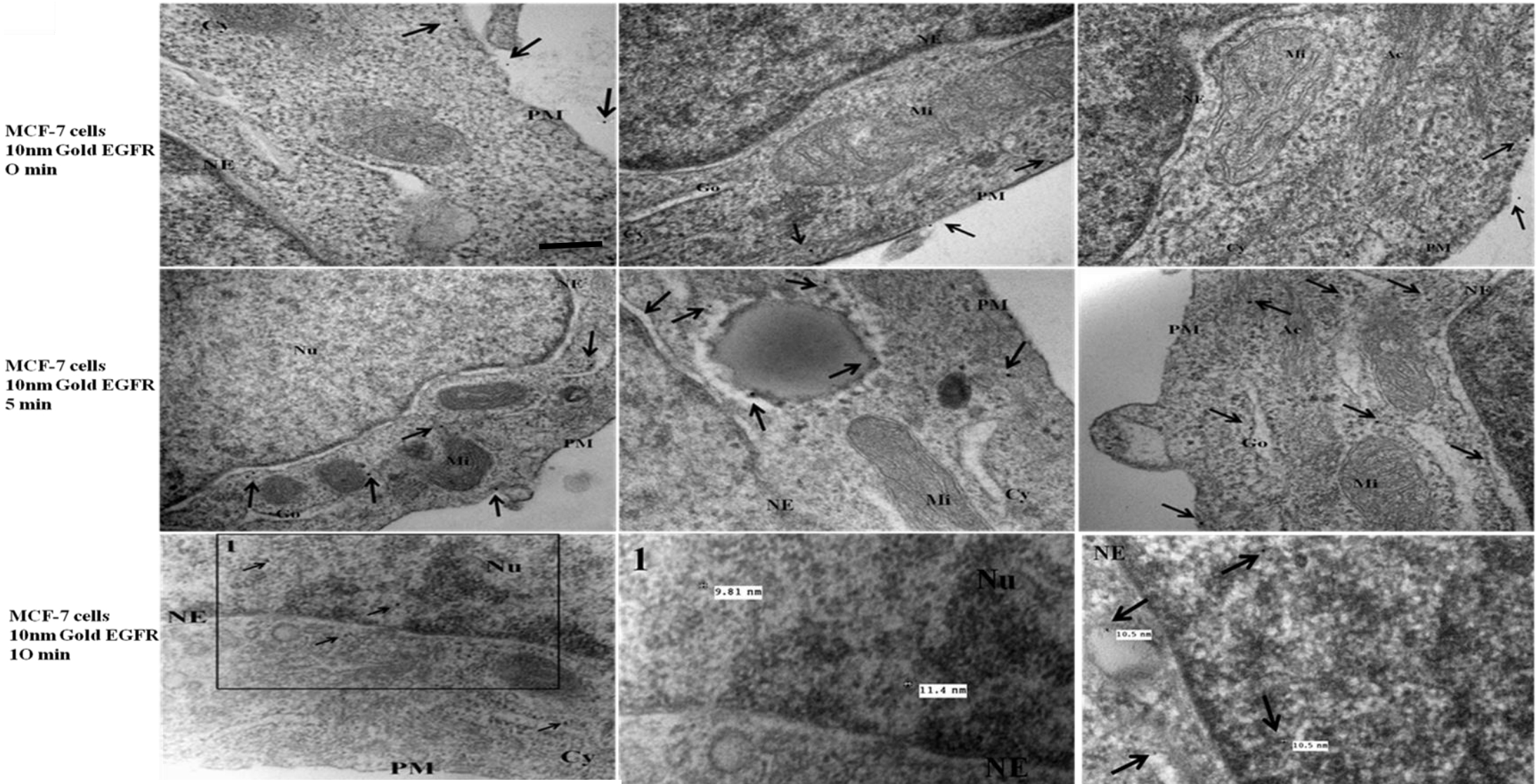


Figure 20. Translocation of EGFR to the nucleus: Immuno-EM studies, Resin embedding of human breast carcinoma MCF-7 cells at time points also showed that EGFRs labelled with 10 nm gold particles were mainly localized on the cell surface plasma membrane and that upon EGF stimulation, EGFR moved across the cytoplasm to the nucleus. Cy, cytosol; Nu, nucleus; NM, nuclear membrane; Mi, mitochondria; PM, plasma membrane; Go, golgi; Ac, actin. Arrows shows 10 nm gold particles labelling EGFR. Bar, 100 nm.

EGFR is imported by Importinβ1 to the nucleus

Importinβ1 is involved in the nuclear import of EGFR, (Wang, Yamaguchi et al. 2010). Many studies have reported the involvement of Importinβ1 in the regulation of EGFR transport to the nucleus. It is responsible for the EGFR trafficking to the INM and the nucleus. Importinβ1 is suggested to be initially inserted into the ER membrane and the Golgi compartment then targeted to the INM through the NPCs. In this study, it was tested to confirm these reports. The interaction of EGFR and Importinβ1 was tested by both by IF and Immuno-EM.

As Importinβ1 is known to import protein to the nucleus, Immuno-EM studies were also performed to confirm the interaction of Importinβ1 and EGFR. Conventional TEM Processing and High Pressure Freezing, Freeze Substitution Processing methods were used to compare the results. MCF-7 cells were treated with EGF for 30 min, fixed as per EM methods and then labelled with secondary Abs 5 and 10 nm EM Streptavidin gold particles were used to indicate importinβ1 and EGFR, respectively. The samples were fixed at different time points, 0 min, 5 min and 10min, to observe the interaction of Importinβ1 and EGFR while trafficking from the cell surface to the nucleus.

The images analysis results show that at time 0 min, EGFRs are seen at the cell surface (CS) and in the proximities of the CS and Importinβ1 was seen in the cytoplasm (Figure 21; Top panel. Figure 22; 1). These EM analyses confirm that EGFRs interact with Importinβ1 and the interaction enhanced by EGF ligands; as it is a well notion that once cells are stimulated with EGFs, EGFRs are endocytosed, and taken up into vesicles in the cytoplasm. Image results also show the unbound EGFR on the CS of EGF treated MCF-7 cells. Importinβ1 molecules labelled by 5 nm gold particles are also seen in the cytoplasm freely not interacting with EGFRs. At time 5 min (Figure 16; Middle panel. Figure 22; 2), some EGFRs tagged to gold particles are clearly seen interacting with Importinβ1 gold particles in the cytoplasm. And at time 10 min (Figure 21; Bottom panel. Figure 22 3), EGFRs particles are observed interacting with Importinβ1 in the cytoplasm but also in the vicinity of the nuclear envelop (NE) and inside the nucleus. EGFRs/ Importinβ1 interaction was even observed in the INM; (Figure 21. Bottom panel; far right Inset). These co-localization patterns strongly support previous studies that EGF-dependent EGFRs nuclear translocate into the nucleus in the INM while interacting with Importinβ1. It is noticed that the integrity of the nuclear and plasma membrane is disrupted; this could be due to the high pressure freezing processing when not highly successful. The Immuno-EM studies in human breast carcinoma MDAMB-231 cells also showed that EGFR was mainly localized on the cell surface plasma membrane,

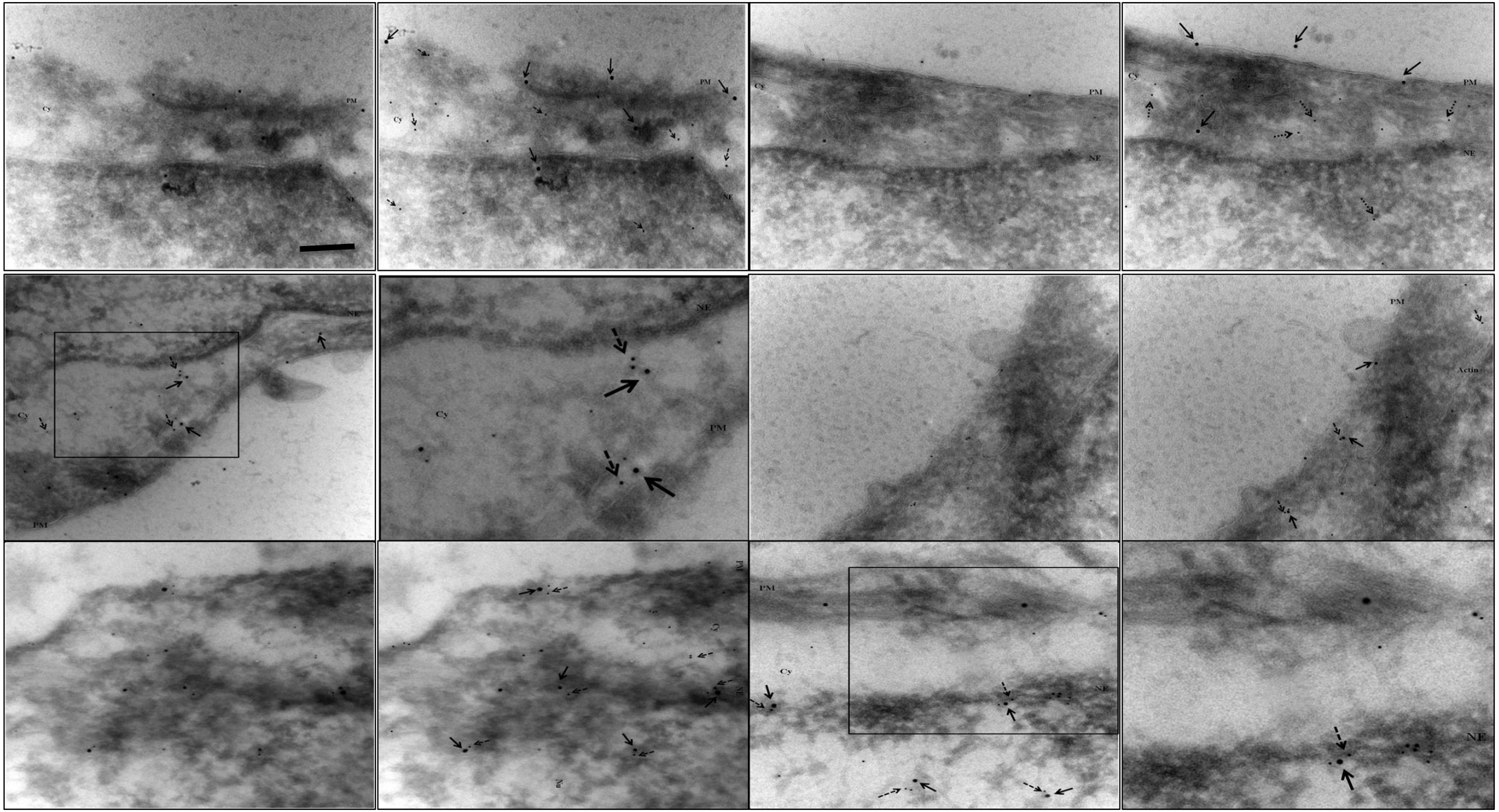


Figure 21. Co-localization and Interaction of EGFR and importinβ1 by High Pressure Freezing, Freeze Substitution Processing. Association of EGFR and importinβ1. MCF-7 cells were treated with EGF (100 ng/ml) for 30 min. Secondary Abs tagged with 10-nm and 5-nm colloidal gold conjugate particles were used to indicate EGFR and importinβ1, respectively. Top row set indicates time 0 min, middle row: 5 min, bottom row: 10 min. Solid arrows mark EGFR and dashed arrows indicate importinβ1. Note: the integrity of the plasma and nuclear membrane is disrupted; this could be due to the high pressure freezing processing. Cy, cytosol; Nu, nucleus; NM, nuclear membrane; PM, plasma membrane; NE, Nuclear Envelop; Bar, 100 nm. For the EM information studies, the 10-nm gold particles are capable of absorbing 5.8 IgG molecules per particle. With this regard, each gold particle represents 1–5 molecules of primary/secondary antibody and 1–10 EGFR molecules.

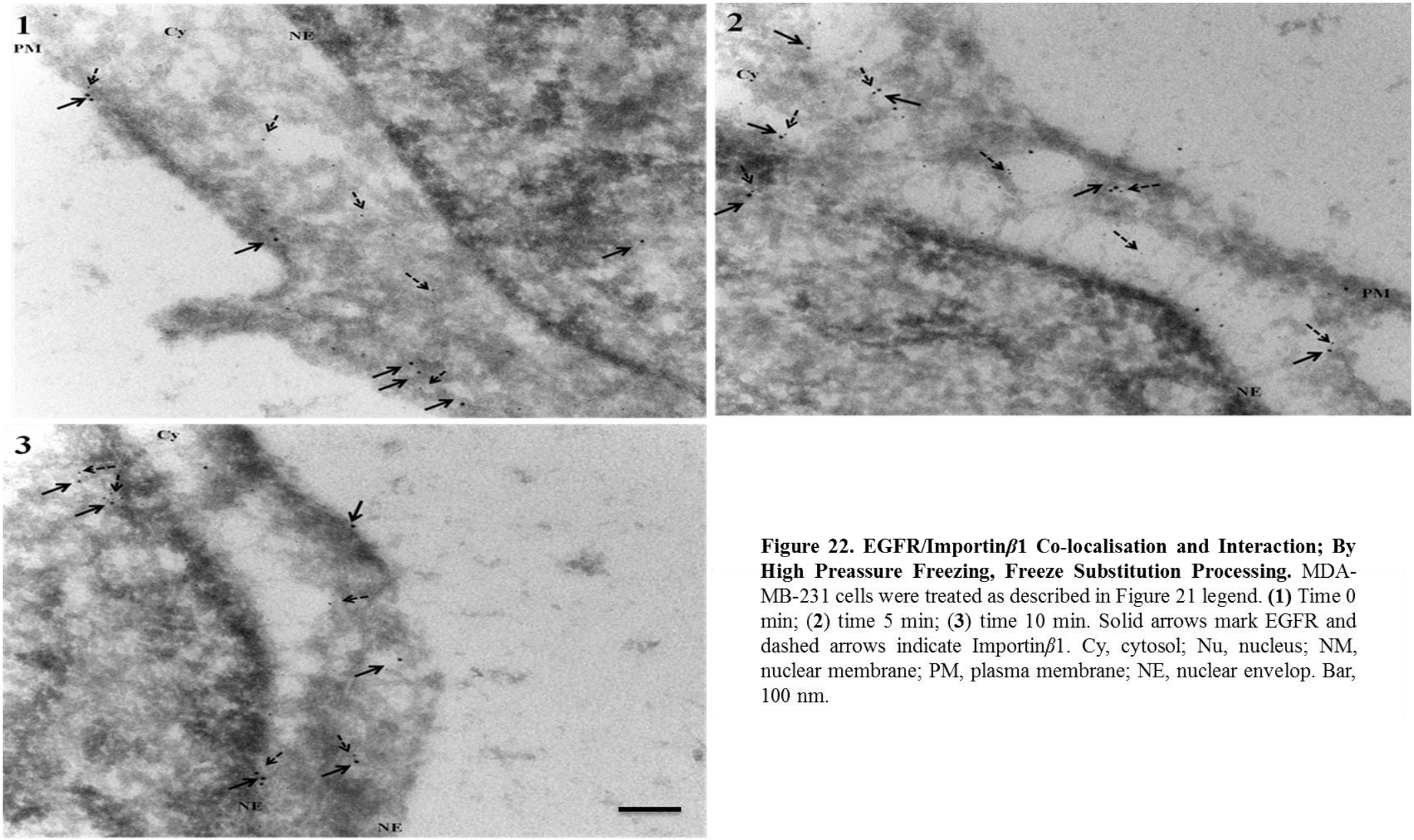


Figure 22. EGFR/Importinβ1 Co-localisation and Interaction; By High Preassure Freezing, Freeze Substitution Processing. MDA-MB-231 cells were treated as described in Figure 21 legend. **(1)** Time 0 min; (**2**) time 5 min; (**3**) time 10 min. Solid arrows mark EGFR and dashed arrows indicate Importinβ1. Cy, cytosol; Nu, nucleus; NM, nuclear membrane; PM, plasma membrane; NE, nuclear envelop. Bar, 100 nm.

Where and when does EGFR recruit Importin*β*

In this study, we asked to find out when and where EGFRs coo-localise with Importin*β*1. EGFR/Importin*β*1 co-localisation and interaction were observed is in the cytoplasm and in the nucleus, (Figure 21; 22). Interactions were also seen at the NE, at the ONM, and even in the INM as reported by previous studies (Figure 16 bottom panel RIGHT; Figure 22, 3). During the EM analysis, we observed an unprecedented localization of EGFR/Importin*β*1 co-localisation. EGFRs particles were observed co-localising and interacting with Importin*β*1 in the proximity of the PM (at the cell surface of MCF-7 cells) (Figure 22, 1), and in the intracellular membrane (ICM), (Figure 21 bottom panel; LEFT; Figure 22, 2). These observations of co-localization at the PM and ICM by Immuno-EM analyses have not been reported to date to our knowledge. We suggest more investigations to be done in order to also confirm these claims.

It was observed that over 45 % of EGFR gold particles were seen co-localising with Importin*β*1 in 75 sample cells (Figure 24). The rest of EGFR gold particles were at distances that we dimed not to be interacting, that is over 15 nm. More statistical analyses are found further below.

EGFR/Importin*β*1 co-localisation was observed following EGF stimulation only at time 5 and 10 min. There was no apparent co-localisation at time 0 min as EGF-dependent EGFRs were not yet endocytosed. At time 5 min, the co-localisation and interaction of EGFR/Importin*β*1 is seen in the ICM of the cell surface and in the cytoplasm; and at time 10 min, EGFR/Importin*β*1 co-localisation and interaction was observed in the cytoplasm and in the NE, the INM and in the nucleus. EGF-induced EGFR/ Importin*β*1 interaction and translocation to the nucleus were further confirmed by immunofluorescence (IF)/confocal analyses; results not shown.

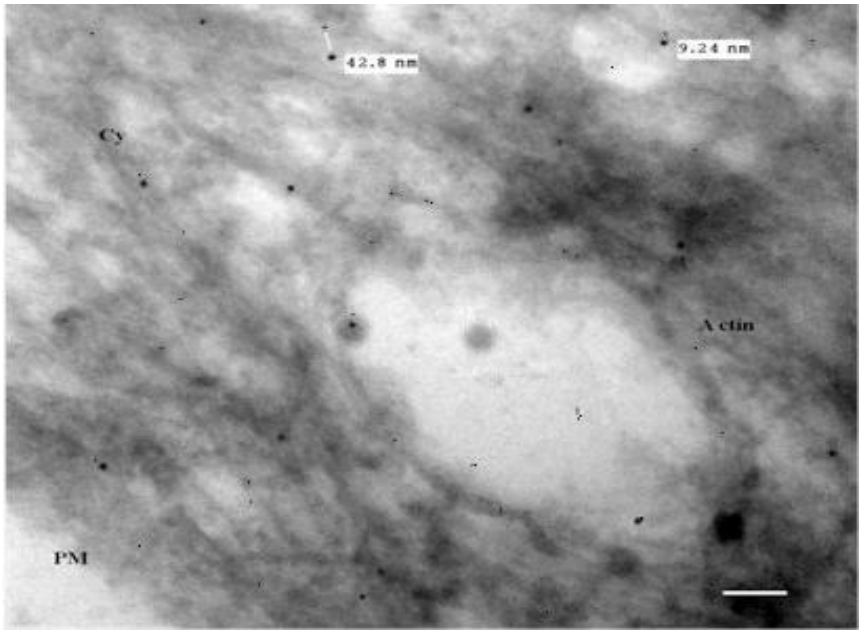


Figure 23. EGFR/Importin*β*1 interaction distance. MCF-7 cells were treated with EGF (100 ng/ml) for 30 min. Secondary Abs tagged with 10-nm and 5-nm colloidal gold conjugate particles were used to indicate EGFR and importin*β*1, respectively. PM, plasma membrane; Cy, cytoplasm; Nu, nucleus. Bar, 2 µm.

To further confirm whether EGFRs interacted with importinβ1, ImageJ software program (version 1.38x; National Institutes of Health) and Adobe Photoshop CS6 (64 Bit) software programs were used to measure the distances between the EGFRs 10-nm and importinβ1 5-nm colloidal gold conjugate particles (Figure 23). Analyses of the interaction distance were carried out in order to confirm whether the particles were co-localizing or not. We suggest that acceptable interaction distances could be up to 12nm. The diagrams show a MCF-7 cells which were treated as described in the method section with the interacting EGFRs/ importinβ1 and the non-interacting particles of EGFRs and importinβ1.

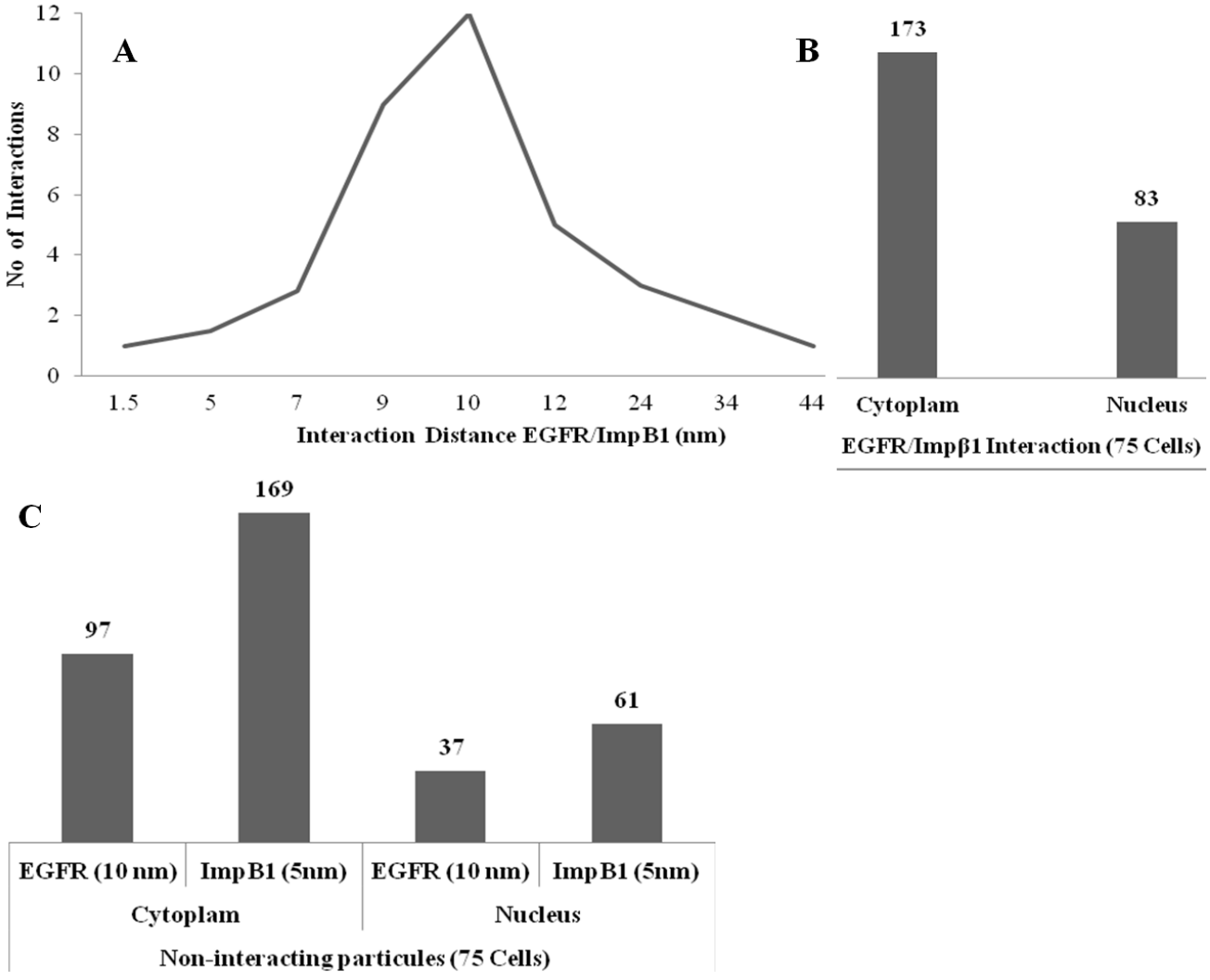


Figure 24. EGFR/Importinβ1 interaction. (**A**) The chart diagram indicates the distance between interacting EGFR/Importinβ1. (**B**) The bar diagram indicates the number of EGFR/Importinβ1 co-localization for MDA-MB-231 different cells. It shows the interaction between EGFR and Importinβ1 after EGF treatment at time 10 min. (**C**) The bar diagram shows non-interacting particles of EGFR and Importinβ1 in the same cells as in diagram (B). Figure measurements are made were made using the ImageJ software program (version 1.38x); nm, nanometres; Imp1β, Importinβ1

Statistic studies of MDA-MB-231 cells show that over 50% of EGFR/Importinβ1 interactions observed are ranging between 8.5 and 11.5 nm; the shortest being 1.3 nm and the longest possible being over 43 nm (Figure 24; A); data set derived from two different cell lines (MDA-MB-231 and MCF-7 cells) and three independent labelling experiments. More EGFR/Importinβ1 interactions were observed in the cytoplasm and at the nuclear membrane then in the nucleus (Figure 24; B). It was observed that for every single interaction in the nucleus there were two interactions in the cytoplasm. Consistent with previous reports, after EGF treatment, EGFRs are internalized from the plasma membrane by endocytosis; endocytic vesicles serving as carriers of EGFRs then fuse with Importinβ1 and then traffic towards the nucleus (Figure 6); there are two other routes undertaken by EGFR, degradation and recycling, this could be the reason why there more Importinβ1 particles than EGFRs. Furthermore, two different sized gold particle-labeled secondary antibodies, including those labeling anti-EGFR (goat anti-mouse IgG, 10-nm gold particles, arrows) and anti- Imp1β (goat anti-rabbit IgG, 5-nm gold particles, arrowheads) were used to specify the identity of the targeted proteins. Analysis of the data collected from the microscopy images shows the localization of each protein. There non co-localizing particles of EGFR and Importinβ1 are localised mostly in the cytoplasm then in the nucleus (Figure 24; C); plotted diagrammatically from data of 75 cells.
Consistent with the previous studies, these results show that, EGF-dependent EGFR nuclear translocation associates with Importinβ1. It strongly support the notion that cell surface EGFR translocates to the INM and the NP, which is regulated by Importinβ1 (being responsible for the EGFR trafficking to the INM and the nucleus), through the NPCs in response to EGF.

Imported EGFR is translocated from the ER-INM to the Nucleoplasm by Sec61β

The translocon Sec61β is thought to capture newly synthesized INM proteins in the ER. It is also known that it associates with EGFR in the ER and in the INM. To further support these reports, an experiment for the co-localization of EGFR with Sec61β was performed using immuno immuno-EM with the specific primary antibodies followed by incubating with two different sized gold particle-labeled secondary antibodies, including those labeling anti-EGFR (goat anti-mouse IgG, 10-nm gold particles, arrows) and anti- Sec61β (goat anti-rabbit IgG, 5-nm gold particles, arrowheads) (Figure 25). The results of our study clearly show the interaction of EGFR with Sec61β in the cytoplasm (Figure 25) and also in the NP, nucleus; (Figure 25, bottom right). The results clearly showed that EGFR/Sec61β co-localization inside the nucleus (Figure 25, bottom right) when specific primary antibodies against EGFR and Sec61β were treated. Reports suggest that the co-localization of EGFR with Sec61β in the cytoplasm indicates that EGFR associates with the translocon in the ER. Consistent with previous reports, we observed a high number of Sec61β in the

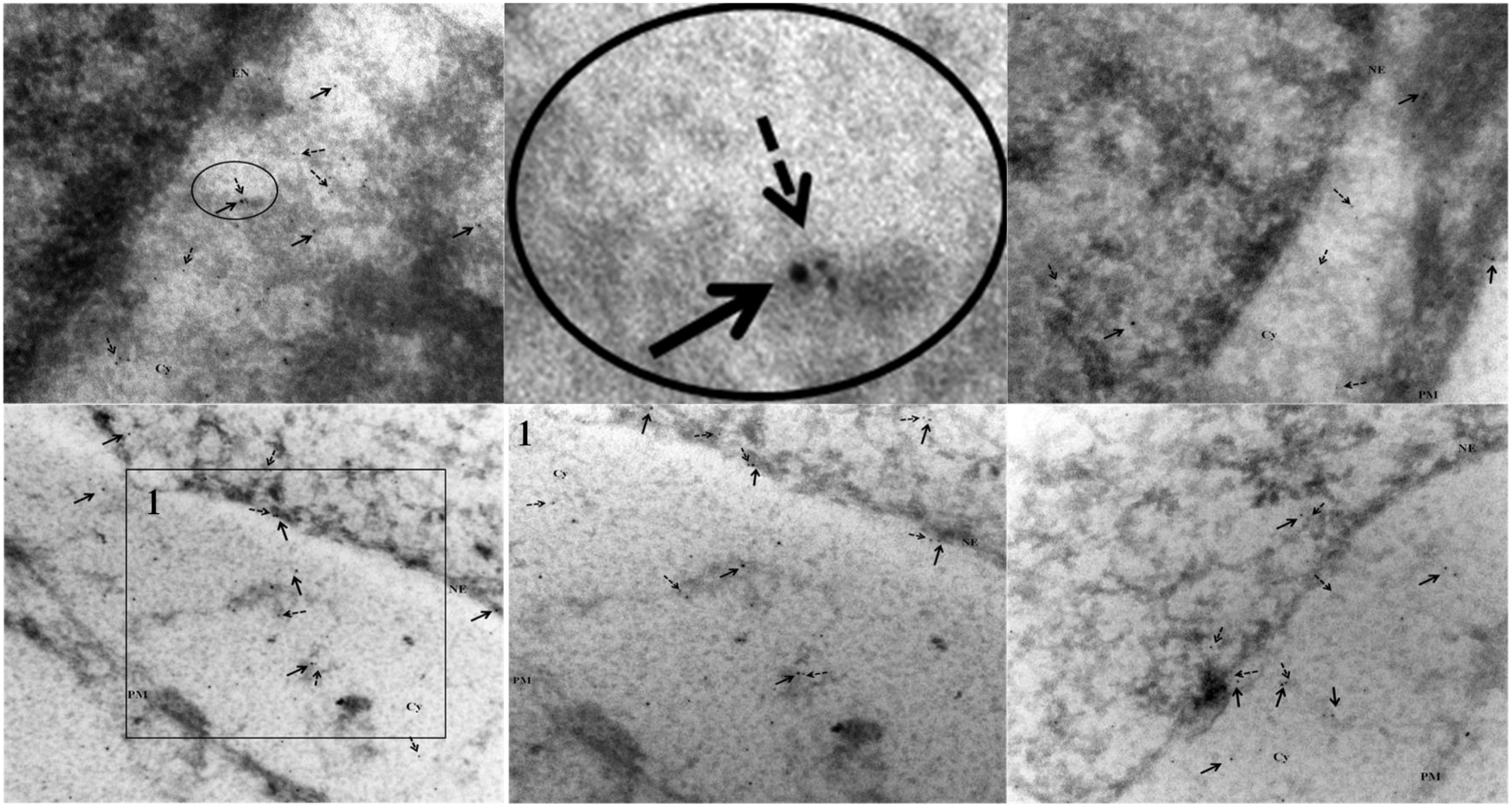
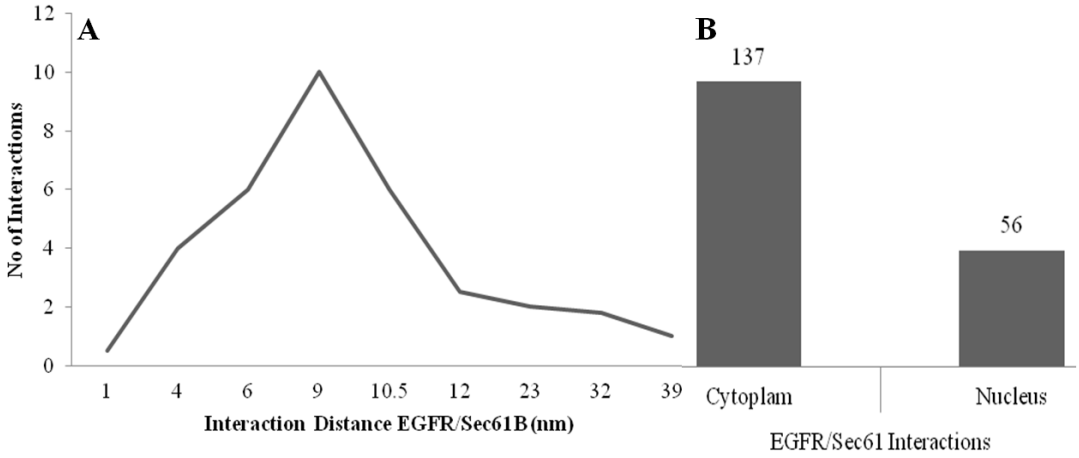


Figure 25. Co-localization and Interaction of EGFR and Sec61β in MCF-7 cells. Association of EGFR and Sec61β. Cells were treated as described in the method section. Secondary Abs tagged with 10-nm and 5-nm colloidal gold conjugate particles were used to indicate EGFR and Sec61β respectively. Solid arrows mark EGFR and dashed arrows indicate Sec61β. Cy, cytosol; Nu, nucleus; NM, nuclear membrane; PM, plasma membrane; NE, Nuclear Envelop; Bar, 100 nm.

cytoplasm compared of that in the NP. According to some studies, both Sec61α and Sec61β are found in the ER or the cytoplasm where they serve as translocon, but only Sec61β is found in the NP; this supports our observation of Sec61β detected in low number in the NP compared to the cytoplasm. Together with previous reported studies, these observations suggest that EGF-dependent EGFR transport to the NP involves membrane-bound trafficking and that the translocon Sec61β associates with EGFR in the ER.

Statistical analysis of our results shows that most interaction distances in MCF-7 and MDA-MD-231 cells are between 7 and 10.5 nm (Figure 26, A). Over 43% of cells have EGFR/Sec61β interaction ranging from 7 and 10 nm as quantified using the ImageJ software program (version 1.38x; National Institutes of Health), and calculated from two different cell lines (MDA-MB-231 and MCF-7 cells) and three independent labelling experiments The bar chart (Figure 26, B) show the number of interactions in the cells. It compares the number of interacting EGFRs/ Sec61β particles in the cytoplasm compared to the nucleus. There is almost 3/1 ratio of interaction in the cytoplasm than in the nucleus (Figure 26, B); made from a pool of 50 cells, which were positive for nuclear localization of EGFR under EGF stimulation.

It is known to our knowledge that upon EGF stimulation, EGFR is endocytosed. Once inside the cytoplasm, the EGFR passes through the Golgi and then the ER, fusing with the ONM where it is inserted in the INM by Sec61. We observed that there was a little EGFR/Sec61 interaction in the NP compared to the cytoplasm. This is consistent with previous reports, which show that Sec61β only assists translocate EGFR into the NP but does not stay in the nucleus.



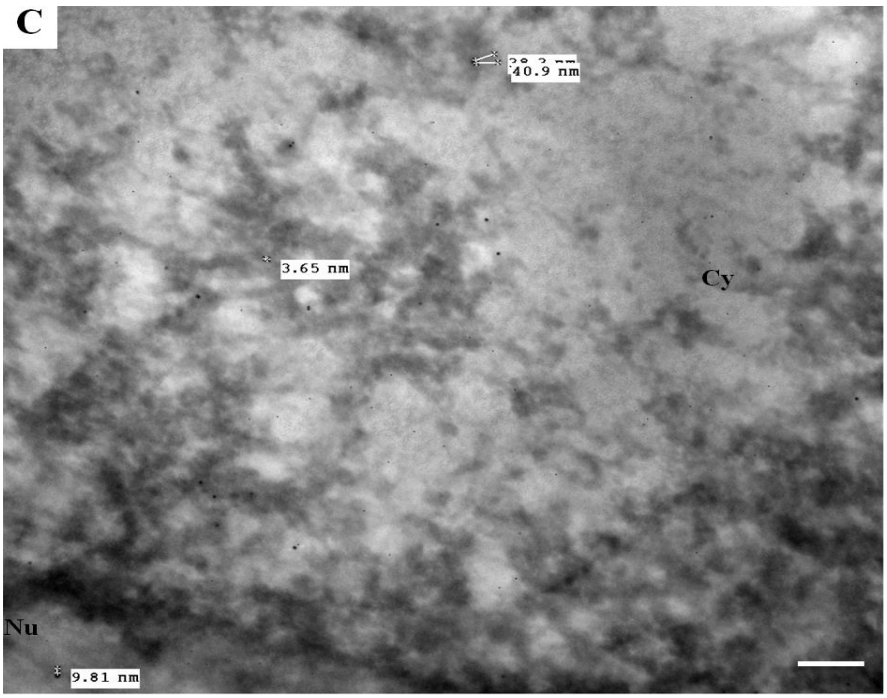


Figure 26. EGFR/Sec61*β* interaction. (**A**) The line chart diagram shows the distance between interacting EGFR/Sec61*β*. It indicates EGFR and Sec61*β* interaction range in MCF-7 cells. (**B**) The bar diagram shows the number of EGFR/Sec61*β* co-localization for 75 different cells. It shows the number of interactions between EGFR and Sec61*β* after EGF treatment at time 10 min. Figure measurements are made were made using the ImageJ software program (version 1.38x); nm, nanometres; Imp1*β*, Importin*β*1. (**C**) EGFR/Sec61*β* co-localizing distance. MCF-7 cells were treated with EGF (100 ng/ml) for 30 min. Secondary Abs tagged with 10-nm and 5-nm colloidal gold conjugate particles were used to indicate EGFR and Sec61*β*, respectively. Cy, cytoplasm; Nu, nucleus. Bar, 2 µm.

Chapter 4: General Discussion

Accumulating reports present various observations of full-length membrane receptors signalling pathway. For instance, they recently presented an EGFR signalling pathway that shuttles directly an activated EGFR into the cell nucleus, instead of the well known the traditional transduction cascades pathway. The new recently discovered pathway allows trafficking of in a membrane-embedded form from the cell surface to the nucleus.

In this report, we proposed to confirm the EGFR full-length membrane receptors signalling pathway by studying the interaction of EGFR/ Importinβ and EGFR/Sec61β by IF and EM microscopy studies, as it is known that EGFR is a key agent that is invaluable in the signalling pathway of some cancer types. We believe that understanding of its particular different pathways might be of therapeutically advantages.

Observations made in this work support previously reported mechanisms of the EGFR signalling pathway. EGFR is expressed at high level mostly in cancer cells. Here we confirm its expression in human breast carcinoma cells: MCF-7 and MDA-MB-231, and in human cervical cancer cell HeLa, by different methods, Biochemical methods, Immunoblotting analysis of cell fractions, confocal-immunofluorescence, real time confocal imaging, and Immuno-Electron Microscopy studies. All these techniques indicates that EGFR moves from the cell surface to the nucleus, and also localises in the nucleus; (Figure 4; 7, 8, 9) (Wang, Wang et al. 2010). Being a cell surface membrane receptor, EGFR is observed to internalize into the cell upon ligands stimulation.

Our observations during experiments showed the internalization of EGFR by endosomal sorting machinery; this is in agreement with previous reports that have shown that internalized EGFR are carried by endocytic vesicles (Lo and Ali-Seyed et al. 2006). Internalized EGFR can also be observed not in endosomes; these have also been reported; it is suggested that they are eventually degraded by lysosomes (Sorkin and Von Zastrow 2002). We confirm that upon EGF treatment, EGFs bind primarily to the EGFR on the cell surface; then, as time elapses, EGFR is endocytosed by invagination of the plasma membrane into the cell, forming vesicles that are then able to fuse with other endosomes and enter the endolysosomal membrane system (Sorkin and Von Zastrow 2002).

It is known to our knowledge that EGFR is imported from the cell surface into the nucleus. EGFR is known to be regulated by certain Karyopherins in order to traffic from the PM. Karyopherins are important to mediate the translocation event of RTKs to the nucleus. For instance, Dynamins are found to have an essential role in the endocytic progress in mammalian cells; (Henley, Krueger et al. 1998, Zuleger, Kelly et al. 2011). It is also found to involve the *trans*-Golgi network, endosomes and podosomes (Damke, Baba et al. 1994, Smaczynska-de, Allwood et al. 2010, Zuleger, Kelly et al. 2011).

Among the Karyopherins involved in the translocation of EGFR, importins are the most important and are essential for EGF-dependent EGFR import to the nucleus and into the INM. Importinβ1 is crucial in EGFR nuclear import; its knock down has a major impact on the translocation of EGFR into the INM then the NP. Although we did not carry out experiment to confirm these claims, we believe that without Importins, EGFR would still be internalzed but stay in the cytoplasmic space and eventually be degraded. Our immuno-EM analyses clearly demonstrate clearly the co-localisation and interaction of EGFR with Importinβ1. Interestingly, Importinβ1 was observed to interact with EGFR in the proximities of the PM of cells. We believe this has not been previously reported or so not been presented by EM studies. Nevertheless, we strongly recommend further studies in order to confirm these observations and get an insight of the role and mechanism of co-localization at this location.

EGFR is reported by various researches to accumulate in the NP of cells after trafficking from the cell surface. Importinβ1 regulates EGFR translocation to the nucleus. Yet, in order to get to the INM, another protein is identified to have a major role. The well-known ER associated translocon Sec61β which is found to reside in the INM (Liao and Carpenter 2007) regulates EGFR import in the nucleus and was recently reported to be essential in the release of membrane-embedded EGFR from the INM into the NP; (Lu, Ladinsky et al. 2009, Wang, Yamaguchi et al. 2010). In our experiments, we localised Sec61β in both the ER and the ER-Golgi intermediate compartment (Figure 7, 3 and Figure 25). This suggests that the Sec61β seen in the EM images in the cytoplasm are located in ER-Golgi intermediate compartment; (Figure 8; Figure 25); this is in line with previous reports; (Osborne, Rapoport et al. 2005). Regarding the distribution of the core components of the Sec61 translocon, they are reported not to reside permanently in the ER as none of the Sec61 subunits contain any known ER retention or retrieval signals normally associated with ER resident proteins. Thus, there are doubt that Sec61 translocon is localized in the ER and

ER-Golgi intermediate compartment. It is however thought to exist and remains in the INM for the release of EGFR in the NP; (Wang et al., 2010).

We also confirm that there was little or no Sec61β found in the NP as analysed by immuno-EM; this is consistent with other reported data that used various methods to locate Sec61β; (Wang, Yamaguchi et al. 2010). The ER-associated translocon Sec61β has a major in the translocation of membrane-embedded proteins into from the INM to the NP; it serves for nuclear translocation in addition to the well-known NPC; yet it is not found in the NP, only in the INM. It releases EGFR from the INM to the NP, or if knocked down, EGFR accumulates in the INM as previously reported by Wang's group; (Wang, Yamaguchi et al. 2010). We confirm that EGFR translocation activities from the cell surface to the NP increases with time, this is strongly supporting previous reports. During this study, we also frequently observed a basal level of activated EGFR and nuclear EGFR without EGF ligand stimulation; (Figure 9) (Wang, Yamaguchi et al. 2010).

Chapter 5: Conclusion

Together, our study of the EGF-dependent kinetics of EGFR translocation from the ER to the INM then NP further confirm previous reports and provide strong back up to hard and long studies that have been carried out for more than a decade; yet, prompt for an urgent need to further unravel unknown properties of this signaling pathway. In conclusion this report helps see clearly by IF and Immuno-EM analyses the route for the nuclear translocation of EGFR from the cell surface in response to EGF and may be a general mechanism for nuclear transport of full length RTKs or other cell surface receptors. We also conclude that importinβ stays bound to EGFR all throughtout in the import until in the NP, and that importinβ was found in the NP, even interacting with EGFR, and no Sec61β was found in the NP.

The EGFR is a complex signalling system important in normal physiology and in the maintenance of the tumorigenic state. Studies of its biochemistry and biology have already made deep contributions to cell signalling and there are bound to be many more surprises in the near future. However, there are many basic questions that must be answered about the mechanism of the EGFR import, i.e., the proteins that interact with EGFR and their nature, what is/are the exact route(s), does nuclear EGFR plays a crucial role in the genesis, progression, metastatic growth and/or therapeutic responses of human cancers? What is the cellular mechanism by which cell-surface EGFR gains nuclear entry (the main focus of Martin W. Goldberg research, the supervisor

of this study)? Particularly, this will be crucial in advancing our knowledge of the nature of the nuclear RTKs and cytokine receptor pathways, as a mechanism has yet been described to account for their nuclear translocalization. Further experiments need to be done to explore the full mechanism of EGFR import from the cell surface into the nucleus, to the NP. It is critical that these experiments are performed under conditions carefully designed with intelligent methods and by experienced researchers in order to get strong evidences of the pathway.

Chapter 6: Technical steps

We carried out many experiments during this study; some of which were not successful. In this section, we present some of the difficulties accounted.

Qdot® (Quantum Dot) Strepavidin Conjugates were chosen to be used in this project as there were no reports of their use in similar studies. We desired to observe the EGFR pathway for the first time by IF and Immuno-EM using Qdot® (Quantum Dot) Strepavidin Conjugates.
IF experiments with Qdot® (Quantum Dot) Strepavidin Conjugates show clear signal and the results are comparable to results of experiments using conventional antibodies (Figure 13; 19, A and B)

The same approach was used for EM studies as. Nevertheless, the results were not conclusive. The main purpose of using Qdot® Conjugate was for their unique properties: correlative light and electron microscopy. Although experiments with light microscopy were successful, the TEM experiments were not of a success. Although Qdot® Conjugate are claimed to have better EM labels than colloidal gold because of superior penetration in samples, the labelling with Qdot® Conjugate was hard to distinguish in the TEM images. Normal backgrounds of TEM images without any labelling were comparable to images of samples treated with Qdot® (Quantum Dot) Strepavidin Conjugates. The image background of the TEM labelled with the Qdot® Conjugate (Figure 27, A) did not allow the distinction with a non-labelled image (Figure 27, C). Control experiments were performed to observe Qdot® Conjugate on their pure state (Figure 27, B. or Figure 31, A), these confirmed the difficulties accounted when samples were labelled with Qdot® Conjugate for TEM analysis. Therefore, we suggest if Qdot® Strepavidin Conjugates could be made in different shape so they are distinctly recognisable compared to ribosome and other more contrast structure in the cell; that will help for imaging QD in biological structures.

SEM experiments with Qdot® Conjugate (Figure 28) were successful compared to the TEM experiments, as discussed above. SEM samples labelled with Qdot® Conjugate (Figure 28, A-C) was clear and successful and not to confuse with pure Qdot® Conjugate in water (Figure 28, D).

During the studies, multiple staining with other dyes were tried but without success compared to single Qdot® Conjugate staining, although the latter is designed to allow the detection of more parameters in a single experiment. Qdot® Conjugates were used staining EGFR, but when other dyes where used for the Golgi, the ER and the cell membrane, the high brightness of Qdot® Conjugate was lost. This might be due to the fixation protocol: long incubation period and the multiple washes. But yet this should have an effect of the brightness of Qdot® Conjugate as the staining is biological. This loss of brightness might also be due to the photo-brightening when imaging. The Qdot® Conjugates with multiplexed colour cell labelling allows the detection of more parameters in a single experiment but probably not when used with other antibody dyes. More experiments were performed to get good brightness yet without success. More experiments are needed to be done in order to optimise the protocol.

While doing TEM experiments, difficulties were accounted with the fixing. This made it difficult to distinguish labelling with background contrast of images. It could have been due the fixing agents used; the time, washings and other factors may have contributed to this.

Experiments were carried out to test the difference in gold labelling before and after embedding in resin for TEM analysis. It was found that labelling before embedding in resin was more efficient than labelling after embedding. This could be to the fact that before embedding in resin, gold labelling could efficiently penetrate the sample. Furthermore, it was found that labelling after embedding was more unspecific compared to labelling before embedding which was more specific and reliable. The efficiency and specificity of labelling before embedding could be to the multiple washings done. All image figures used in this report are from labelling before embedding.

We also tested to see the efficiency and specificity of TEM and SEM double labelling with two different gold particles sizes; 5 and 10 nm gold particles were used for both TEM and SEM targeting two different proteins, EGFR and Impβ or EGFR and Sec61β. To do this, the two different gold particles were mixed together for the double labelling or labelling was done individually in two stages, the first protein (5nm) then for the second protein (10nm). Results of experiments with the mixed gold particles (5 and 10nm together) labelling proved to be less specific but more efficient than individually labelled gold particles in two stages.

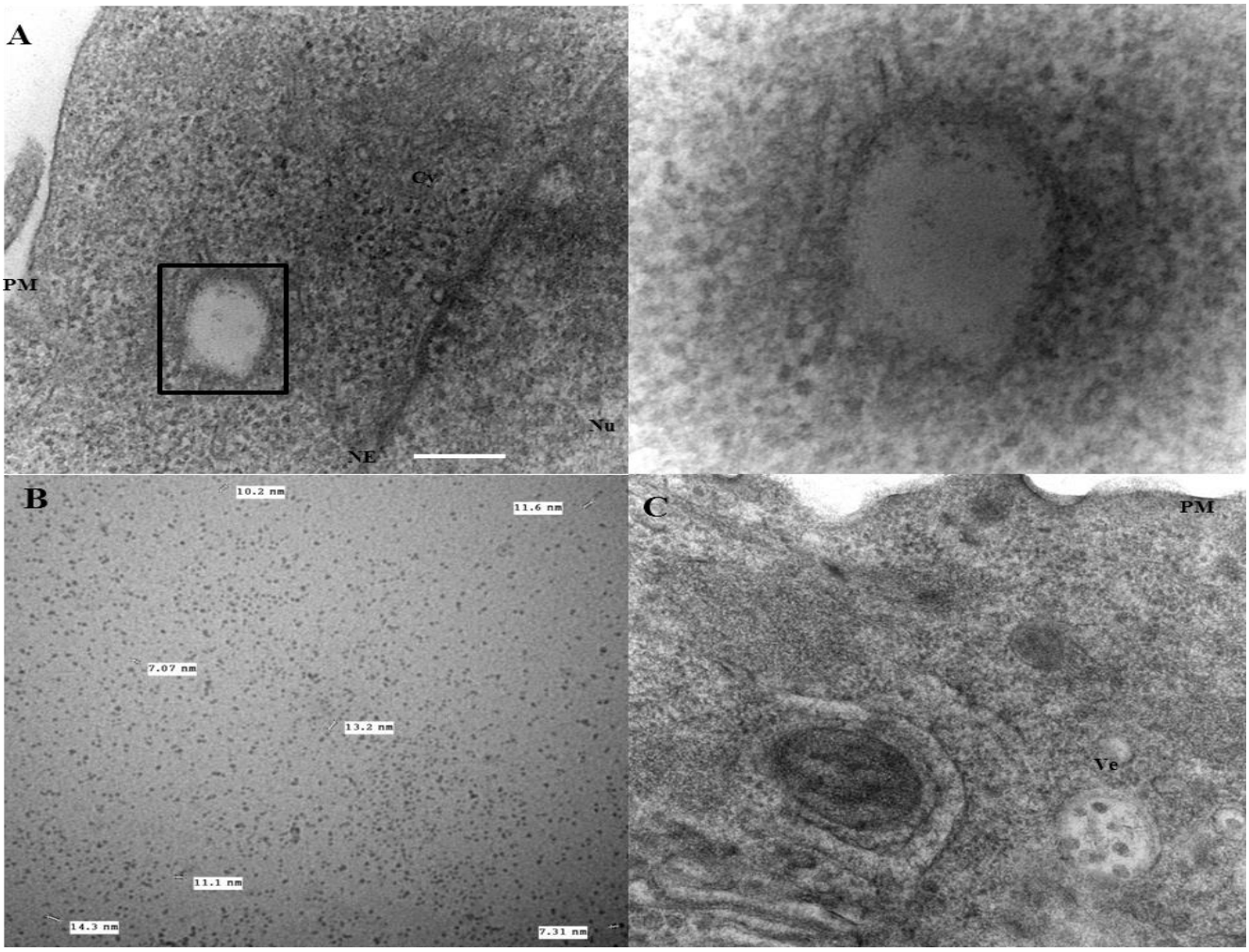


Figure 27. Qdot® Conjugate in TEM imaging. A. MDA-MB-231 human breast carcinoma cells processed for immuno-EM, embedded in resin and labelled with Qdot® Conjugate. B. TEM of pure Qdot® Conjugate in water. C. MDA-MD 231 cells processed for immuno-EM, embedded in resin with no labelling. PM, plasma membrane; Cy, cytoplasm; NE, nuclear envelop; Ve, Vesicle. Bar, 10 nm. Inset shows enlarged high-resolution image.

The mixed gold particles labelled more but were not specific; it showed more 5nm particles labelling in favour of the 10nm; this could be a binding competition due to the size of the gold particles. The small size gold particles are expected to bind in favour of the bigger particles. Nonetheless, the small gold particles should be specific to a targeted protein. Therefore, we found that it is preferable to use individual labelling stages instead of pre-mix of gold particles.

IF studies of EGFR/Impβ and EGFR/Sec61β proved to be challenging as suitable antibody to do double staining were challenging to find. There is a limited choice of antibody colours between the EGFR, Impβ and Sec61β, double labelling proved to be very challenge. Also the range of excitations of most of the available antibodies we got to hand were too close making it difficult to distinguish the signal.

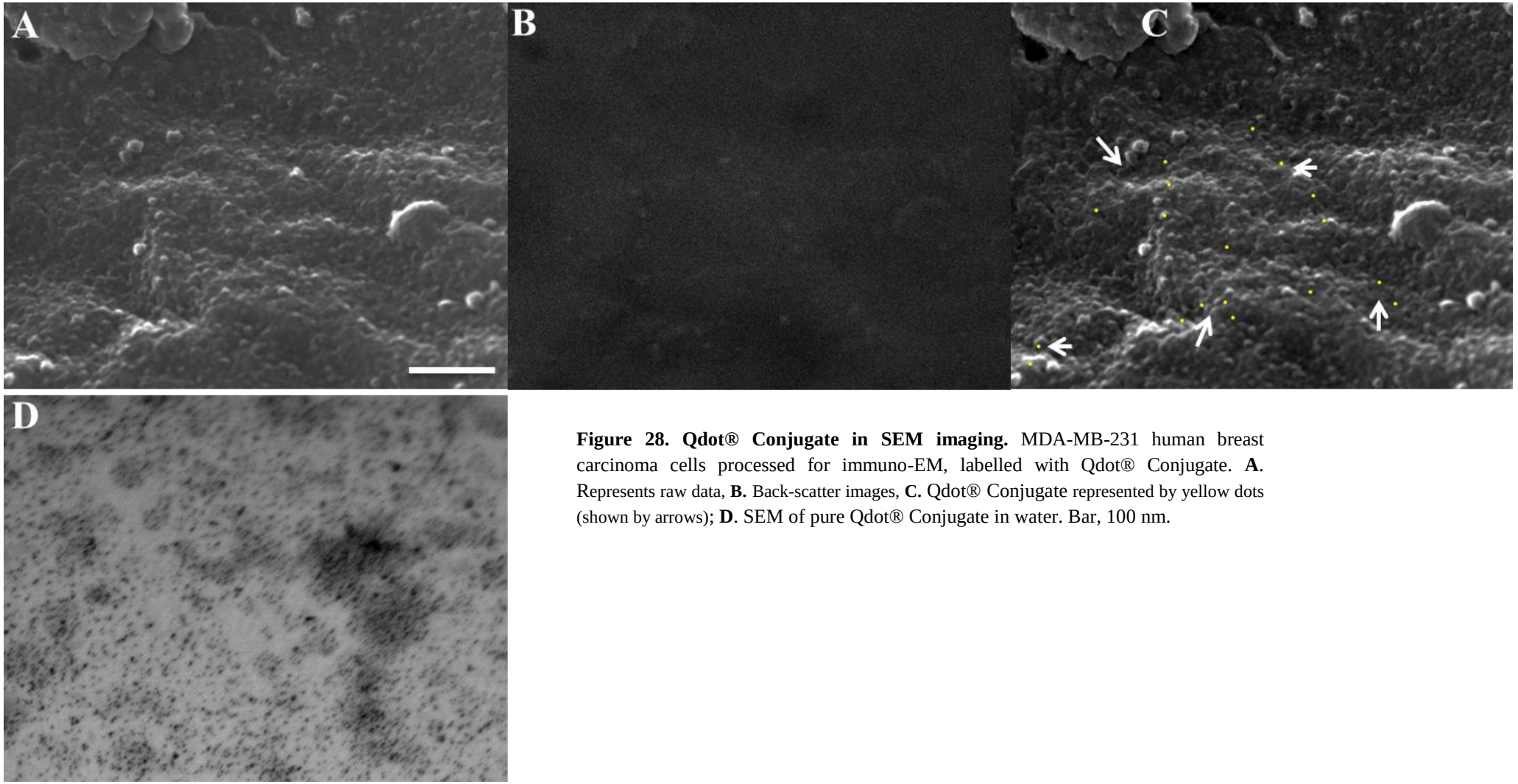


Figure 28. Qdot® Conjugate in SEM imaging. MDA-MB-231 human breast carcinoma cells processed for immuno-EM, labelled with Qdot® Conjugate. **A**. Represents raw data, **B.** Back-scatter images, **C.** Qdot® Conjugate represented by yellow dots (shown by arrows); **D**. SEM of pure Qdot® Conjugate in water. Bar, 100 nm.

Chapter 7: Further experiments

The main purpose of this part is to suggest further experiments that can be undertaken in order to determine the mechanism for membrane protein translocation through the nuclear pore complex (NPC): when and where do karyopherins and Ran start and stop interacting with the cargo during translocation? How do nucleoporins FG domains change conformation during translocation? How does NPC architecture change? How does this facilitate translocation? Why is Egfr transported through the Golgi/ER? Do all the EGFR transported go through the Glogi/ER? If not, what is the transport mechanism difference at the NPC? What is the percentage of Egfr transported by each pathway? (Because Egfr is glycocylated in Golgi/ER what could be the effect of not being glycocylated?).

- There is a need to get more detailed time course live cell imaging with confocal microscopy to observe if/how EGF affect interaction of EGFR/Importinβ or EGFR/Sec61β and also observe the route of passage of EGF tagged with fluorescent Quantum Dots bound to EGFR from the plasma membrane to the nucleus. Different compartments, endosome, ER, Golgi, actin, cell membrane and nucleus can be can be labelled either chemically (Life Tech ER tracker) or with GFP markers (Life Tech CellLight Reagents). These experiments will reveal in time and in space the pathway of EGFR. Proteins like Importinβ and Sec61β that are known to interact with EGFR can also be labelled to further more understand in 3D its interaction.
- To further more understand where and when importinβ binds to EGFR, a simple experiment can be tested. Transfection of cells with EGFR-GFP, cloning or acquisition of CFP-EGFR and YFP-importinβ for FRET analysis +/- transport factor mutants (Ran, karyopherins, etc…). Similarly the spatiotemporal relationship between EGFR and Sec61β can be studied, as well as Sec61β and importinβ. This will examine; the expression of GFP tagged importinβ with CFP-EGFR
- Drugs can be used to further study EGFR nuclear pathway. Inhibition of endocytosis, inhibition of different interactions EGF/EGFR, EGFR/ Importinβ, EGFR/Sec61β inhibition of Importinβ and Sec61β would reveal more to the pathway. For instance, expression of the Q69L mutant of Ran (a GTP-locked form) should inhibit this interaction and prevent nuclear import. Expression of the T24N mutant (which inhibits RanGEF and depletes the cell of RanGTP) should prevent the dissociation of EGFR from importin β, probably locking EGFR at the inner nuclear membrane or in the NPC. Inhibition of CRM1 export with leptomycin B (LMB) would allow clear observation of EGFR import without any perturbation. These effects can be further characterised by immuno-EM.

- Ligand can also be gold-tagged on the extra-cellular domain of EGFR and immuno-gold label the cytoplasmic domain, it is possible to examine the precise route the protein takes through the NPC. Such experiments will prove unequivocally whether EGFR remains as an integral membrane protein, or a soluble protein that is extracted from the membrane (by Sec61β) prior to NPC translocation. This will also test to determine if Sec61β is transported with EGFR or separately and determine the mechanism.
- EM tomography and serial "nano-sectioning" and 3D reconstruction can be used to reconstruct the route travelled in 3D space. The effects of Ran and karyopherin mutants will help dissect this. This trafficking can be recapitulates in Xenopus oocytes, by ectopic expression of tagged EGFR then isolation of the nuclear envelope and examination by field emission scanning electron microscopy. After detergent extraction or fracturing it is possible to determine how membrane-bound cargo interacts with NPC substructures, which is unknown for any integral membrane protein. The effects of mutants can be examined by ectopic expression.
- EM experiments,

Sonification of cells and SEM treatment to study the transport of EGFR through the NPC on the INM, and also observe EGFR/ Importinβ interaction to understand where Efgr recruits Importinβ: (1) Does it recruit it at cell membrane? (2) In the cytoplasm? If so, at what stage? And what is the timing after endocytosis?

Examine the localisation of EGFR and the mechanism of nuclear import by immuno-gold TEM in time course experiments to see how it progresses from one compartment to the next and how it travels through the NPC. Double labelling immuno-TEM, microinjection in Xenopus oocytes and feSEM. High pressure freezing/freeze substitution of cells. Live-FRET can be performed to examine the mechanism of nuclear import, the expressions of GFP tagged importinβ with CFP-EGFR and determine exactly when and where two proteins start and stop interacting.

- Test if EGFR is exported from the nucleus by the exportin, CRM1 and if it is exported as a membrane or soluble protein.

Appendix

More IF image results of MDA-MD-231 cells

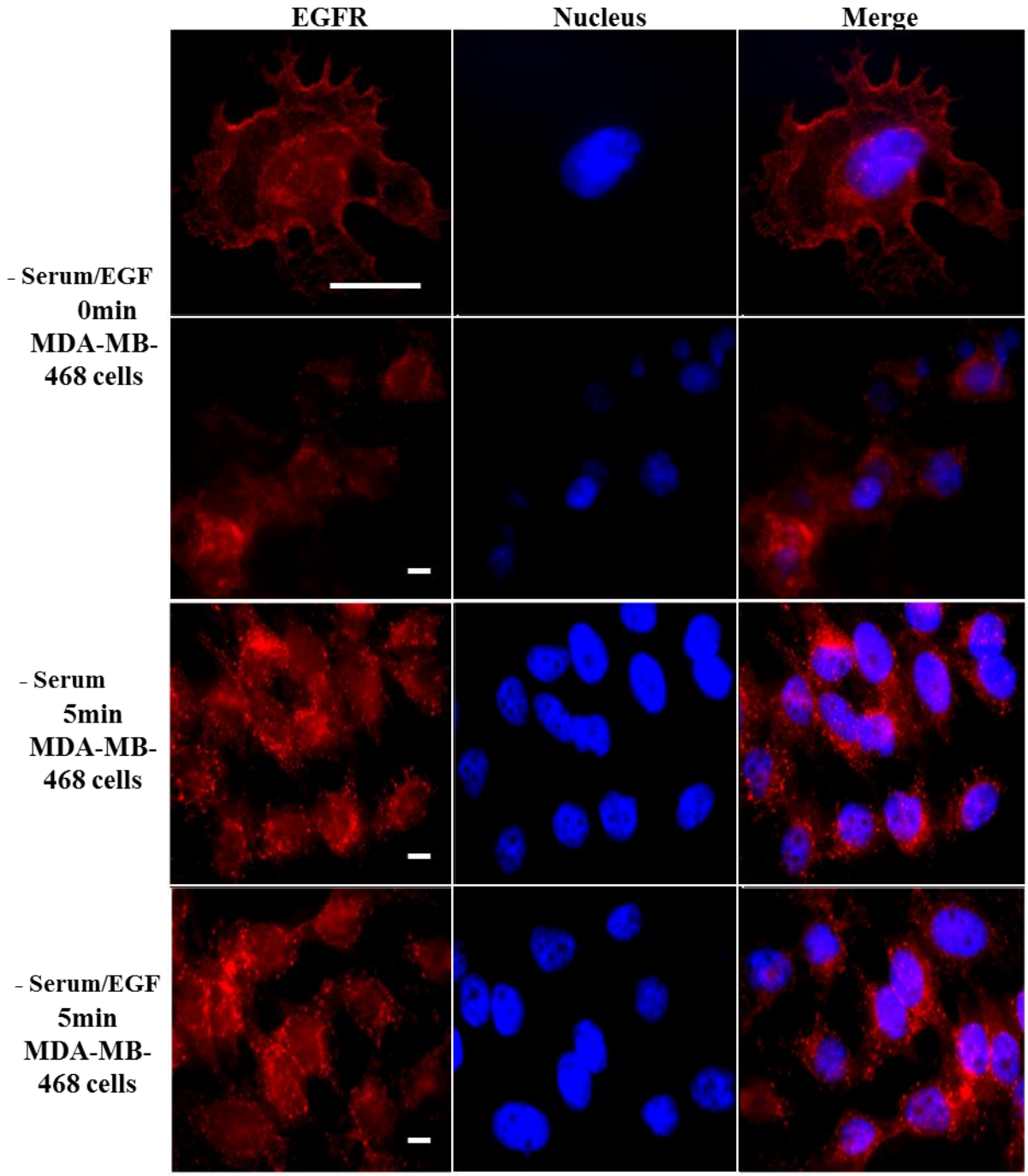


Figure 29; Serum starved +/- EGF stimulated MDA-MB-231 cells. MDA-MB-231 cells shown by IF/confocal analysis. Cells were grown serum starved then processed by treatment with/without EGF, fixed with PFA at different time points and labelled with Alexa 488, EGFR antibody. Bar 10 µm

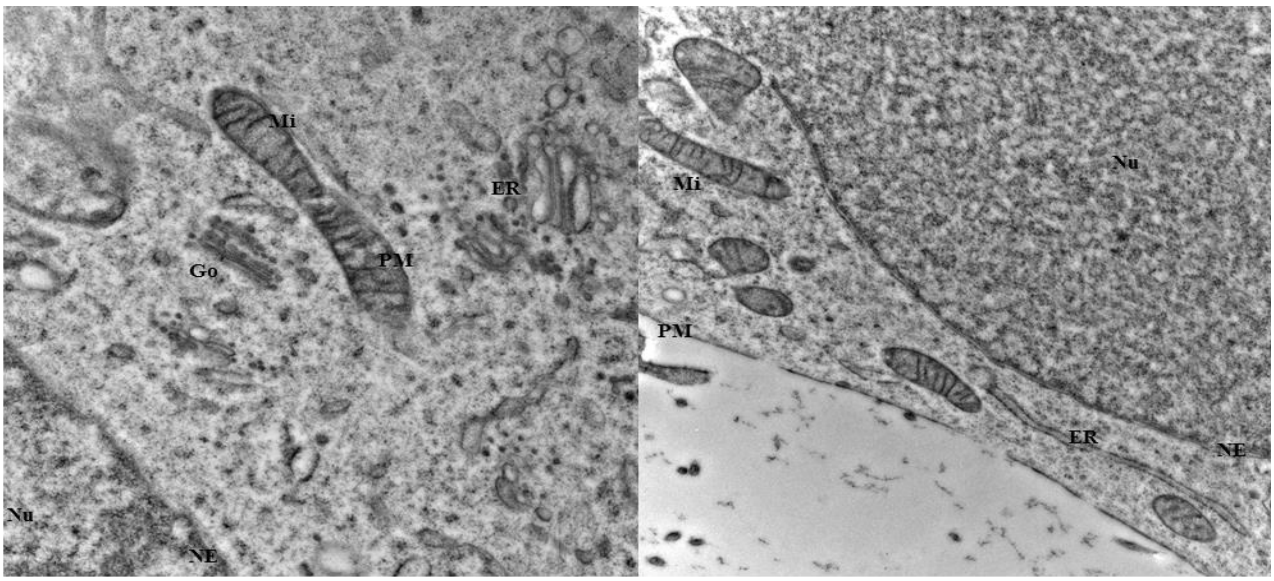


Figure 30. Well preserved TEM images. MDA-MB-231 human breast carcinoma cells processed for immuno-EM, embedded in resin with no labelling. PM, plasma membrane; Cy, cytoplasm; NE, nuclear envelop; Ve, Vesicle; Mi, Mitochondria; Go, Golgi; ER, Endoplasmic Reticulum. Bar, 10 nm. Inset shows enlarged high-resolution image.

The Qdot® streptavidin conjugate

The Qdot® streptavidin conjugate, made from a nanometer-scale crystal of a semiconductor material (CdSe), coated with an additional semiconductor shell (ZnS) in order to improve the optical properties of the material. These materials have a narrow, symmetric emission spectrum with the emission maximum near 525 nm (Q10141MP), 565 nm (Q10131MP), 585 nm (Q10111MP), 605 nm (Q10101MP), 625 nm (A10196), 655 nm (Q10121MP), 705 nm (Q10161MP), or 800 nm (Q10171MP). This core-shell material (Figure 31 B) is further coated with a polymer shell that allows the materials to be conjugated to biological molecules and to retain their optical properties. This polymer shell is directly coupled to streptavidin (Figure 31 B). The Qdot® streptavidin conjugate is the size of a large macromolecule or protein (~15–20 nm).

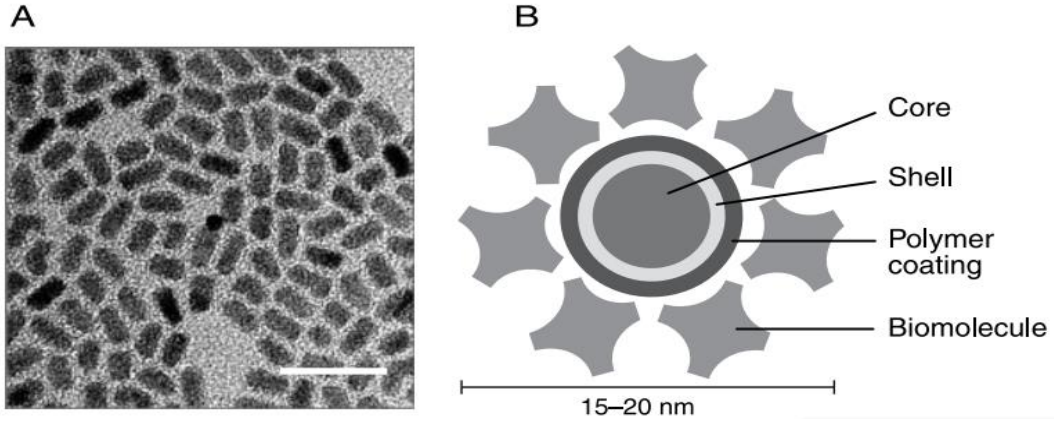


Figure 31. Qdot® (Quantum Dot) Strepavidin Conjugates. **A**. Transmission electron microscope image of core-shell Qdot® nanoparticles at 200,000x magnification. Scale bar = 20 nm. **B**. Schematic of the overall structure of a Qdot® streptavidin conjugate. The layers represent the distinct structural elements of the Qdot® nanocrystal conjugates, and are roughly to scale.
Core Nanocrystal (CdSe)- Determines color. Inorganic Shell (ZnS)– Improves brightness and stability
Polymer/Organic Coating - Provides water solubility and functional groups for conjugation
Biomolecule-Covalently attached to polymer shell (Immuoglobulins, Streptavidin, Protein A, Receptor ligands, Oligonucleotides). Note: Figure is a copyright of Molecular Probes, Invitrogen detection technologies.

Spectral properties of Qdot® Conjugate are higher than Organic dye (FITC) that the former has Large “Stokes shift”, Single-source excitation, Narrow emission, Excellent photostability. Qdot® Conjugate provides excellent brightness for high sensitivity. Five colour multiplexed cell labelling allows the detection of more parameters in a single experiment; and direct conjugates provide ultimate flexibility and high quality images.
Size of the nanocrystal determines the color; it is tunable from ~2-10 nm (±3%) and its distribution determines the spectral width.

The key issues denoted for Qdot® Conjugate are fixation protocol, filter selection, PAP Pen, quenching, photobrightening.

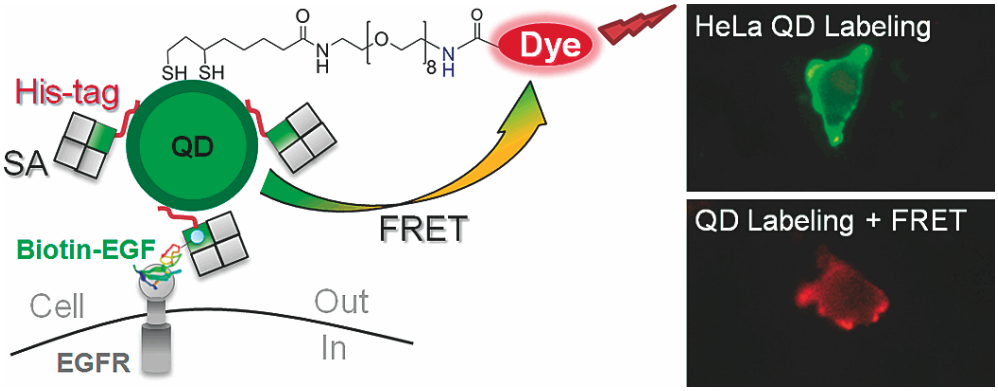


Figure 32. TEM of pure Qdot® (Quantum Dot) Strepavidin Conjugates (Image from manual). (Left) Schematic showing conjugation of His6-tagged streptavidin (hSA) to 20% aminoQDs also combined hSA conjugation with covalent conjugation of dye to 20% aminoQDs

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