

Characterizing Luminal A breast cancer heterogeneity and in vitro response to hormone therapy



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SIMONE GALLANT

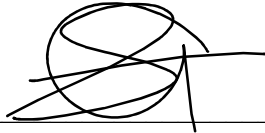
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A Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand,
Johannesburg, in fulfilment of the requirements for the degree of Master of Science in
Medicine (MSc.Med)

2024

Declaration

I, Simone Gallant, declare that this Dissertation entitled “Characterizing Luminal A breast cancer heterogeneity and in vitro response to hormone therapy” is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



Simone Gallant

____ 13 ____ day of ____ 06 ____ 2024 ____ in ____ Parktown ____

Abstract

Breast cancer is the most prevalent form of cancer diagnosed amongst women worldwide, responsible for a mortality rate of 6.9% and responsible for 684,996 deaths. Breast cancer is the most heterogeneous disease characterised by variations in genomic, epigenomic transcriptomic and proteomic profiles. The limited research on intratumoural heterogeneity in breast cancer and hormone therapy is the motivation for our study to further aid in understanding stemness markers influencing luminal A breast cancer and the effects hormone therapy has on biomarkers associated with breast cancer. In our study, we optimised modified essential 8 media to culture sorted cell populations in optimal conditions without differentiation ensuring stemness markers are maintained. Magnetic cell sorting was used to separate cells based on stemness markers CD133 and CD44. To verify these sorted markers flow cytometry was performed. The evaluation of the effects hormone therapy had on biomarkers was performed via immunocytochemistry and analysed using cell profiler. Our study revealed significant differences between subpopulations in MCF7 and T47D cell lines. It emphasizes the importance of CD44 and CD133's role in tumour progression and its possible influence in hormone therapy. Our findings show that in populations with both stemness markers present in T47D cell line there is a reduction in progesterone receptor expression when treated with Tamoxifen. We also noticed the difference between population and hormone therapy impact on these changes. Thus, stemness markers are vital in tumour progression and the interaction of biomarkers and hormone therapy. However future research in the biological process and pathway activation is needed to further understand the intricacies of CD44 and CD133 mechanism of action as well as its association to biomarkers common pathways.

Publications and Presentations Arising from This Dissertation

Health Sciences Faculty Research Day, University of the Witwatersrand. Title of the presentation “Changes in the expression of diagnostic markers in hormone-dependent breast cancer subpopulations following Anastrozole and Tamoxifen treatment.” Supervisors: Tanya N. Augustine, Dr Kutlwano Xulu Presenter: Ms Simone Gallant

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Lastly to my ever-present help, I'd like to give Glory to God. Every moment of this journey is a testament to His faithfulness. All my strength was wrapped up in knowing and being known by Him. Let my life be ever for His Glory.

Dedication

I would like to dedicate my work to all my lab partners whose determination and passion for science inspired me. Though they were unable to complete their degrees, their contributions to our research were invaluable.

List of Abbreviations

AF	Alexa fluor
AF-1	Activation function
Afs	Activation functions
AI	Aromatase inhibitors
uPA	urokinase-type plasminogen activator
BC	Breast Cancer
Bcl-xl	B-cell lymphoma-extra-large
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CDKs	Cyclin-dependent kinases
CSC	Cancer stem cells
CST	Cytometer setting and tracking
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagles' Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2	17 β - estradiol
E8	Essential 8
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-mesenchymal transition
ER	Oestrogen Receptor
ERE	Oestrogen responsive element
ERK	Extracellular related kinase
ERM	ezrin/radixin/moesin
FBS	Fetal bovine serum
Fe (NO ₃)	Ferric nitrate
FeSO ₄	Ferrous sulphate
FGF2	Fibroblast growth factor
FSC	Forward side scatter
GST	Gastric Stromal Tumour
HA	Hyaluronan
HER2	Human epidermal growth factor receptor 2
HREC	Human Research Ethics Committee
ICC	Immunocytochemistry
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
LBD	Ligand binding domain
MAPK	Mitogen-activated protein kinase
MFI	Median fluorescence intensity
MMP	Matrix metalloproteinases
mTOR	Mammalian target of rapamycin
NaHCO ₃	Sodium bicarbonate
NF κ B	nuclear factor-kappa B
NTD	N-terminal domain

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PR	Progesterone Receptor
PRE	Progesterone response element
PTEN	Phosphatase and tensin homolog
RTK	Receptor tyrosine kinases
RPMI	Rosewell Park Memorial Institute 1640 media
SERM	selective oestrogen receptor modulators
SRC-1	steroid receptor co-activator
SSC	Side scatter
TME	tumor microenvironment
TGFβ1	Transforming growth factor-β1
ZR	Zombie Red

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Chapter 1: Introduction

The breast is characterised by three major components: Lobules, ducts and connective tissue (**Fig 1**). It contains fatty tissue to dense tissue forming a network of lobes (Biswas et al., 2022). These lobes contain tube-like structures known as lobules containing milk glands (Biswas et al., 2022). Ducts connect the glands, lobules and lobes. It is common for breast cancer to begin in the ducts or lobules then metastasizing through blood vessels and lymph vessels, making 80% of breast cancer invasive (Biswas et al., 2022).

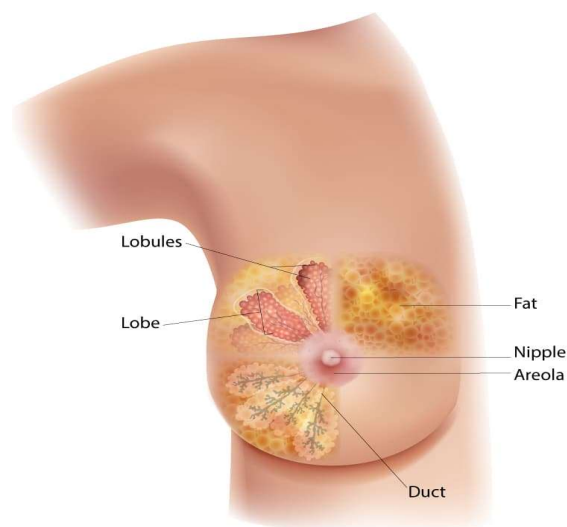


Figure 1: Major components of the breast (Centers for Disease Control and Prevention.,2023)

1.1 The incidence of breast cancer

Breast cancer is the primary form of cancer diagnosed amongst women globally, contributing to a mortality rate of 6.9% and responsible for 684,996 deaths in 2020 (Łukasiewicz et al., 2021). According to GLOBOCAN 2020 data, 2.3 million recent breast cancer cases (11.7%) were reported from 185 countries with a distribution of breast cancer cases and deaths across world regions (Kashyap et al., 2022) (Łukasiewicz et al., 2021). It is predicted that by 2030 new cases of breast cancer diagnosis will reach 2.7 million annually world-wide (Łukasiewicz et al., 2021). Breast cancer is more prevalent in developing countries (Harbeck and Gnant., 2017). In South Africa, breast cancer accounted for 22.6% of cancer cases in females and 16% cancer mortalities in the year 2016 (Ayeni et al., 2023). A rapid increase in breast cancer diagnosis across Africa was observed, South Africa experienced a 3% to 4% increase in breast cancer between the mid-1990s and mid-2010s per year (Sung et al., 2021). This could partly be due to late diagnosis due to limited resources, poor screening, and

availability to health care, which lowers survival rates (Lambert et al., 2020). Favourable prognosis was observed in the United States of America (USA) showing a 5% survival for 91% of non-Hispanic white women with breast cancer and 80% of African American women (Ayeni et al., 2023). As opposed to high income countries, a 3-year overall survival rate amongst black South African women was estimated to be 59% (Ayeni et al., 2023).

Risk factors such as age, gender, genetic mutations, and obesity are all involved in advancing Breast cancer (Ayeni et al., 2023). In addition, factors such as reproductive history (pregnancy, breast feeding, menstruation and menopause) and female sex are linked to increased risk as a result of hormones oestrogen receptor (ER) and progesterone receptor (PR) levels (Ayeni et al., 2023). Thus, emphasizing the importance of establishing the intricacies of the disease (Ayeni et al., 2023).

1.2 Tumour heterogeneity in Breast cancer

Breast cancer is a heterogenous disease based on its differences in genomic, epigenomic, transcriptomic and proteomic profiles attributed to different disease manifestations (Guo et al., 2023). It is known to be the most heterogenous disease in women worldwide (Guo et al., 2023). Tumour heterogeneity is characterised as intertumoural heterogeneity and intratumoural heterogeneity (Seoane and De MattosArruda, 2014) (Grzywa et al., 2017). Intertumoural heterogeneity defines differences amongst tumours in different patients or within the same patients with diverse tumour deposits (Seoane and De Mattos-Arruda, 2014) (Grzywa et al., 2017). It is estimated that 50% to 80% of recent breast cancer diagnosis is characterised as invasive ductal carcinoma (IDC) with other cases classified as invasive lobular carcinoma (ILC) (Henry., 2019). Invasive ductal carcinoma can show no distinct morphological characteristics with no histological type and thus be characterised as “no specific type”. However, if they present with distinct characteristics including cellular and molecular features (morphology, architecture, and growth patterns) they can be classified as “special type” (Henry., 2019). Examples of specialised types of breast cancer include medullary carcinoma, neuroendocrine carcinoma, pleomorphic lobular carcinoma, classic lobular carcinoma, cribriform carcinoma, tubular carcinoma, metaplastic carcinoma, mucinous carcinoma, and apocrine carcinoma.

Mucinous tumour accounts for 1% to 2% of breast cancer, commonly characterised as hormone receptor-positive and human epidermal growth factor receptor 2 (HER2)- negative (Henry., 2019). It is classified by their distinct histology as well as their variation genomically such as lower genomic instability and decreased amounts of mutations in genes in the PI3K

pathway (Henry., 2019). Inflammatory breast cancer is an aggressive type of Breast cancer and is identified by clinical changes such as edema of the breast and the progression of diffuse erythema (Henry., 2019). It accounts for 2.5% of breast cancer diagnosis (Henry., 2019).

Inherited breast cancer accounts for 5% to 10% (Henry., 2019). Mutations in genes such as *BRCA1*, *BRCA2*, *TP53*, and *PTEN* contribute to increasing probability of breast cancer (Henry., 2019). Histological subtypes linked to mutations in gene *CDH1* include lobular carcinoma, encoding e-cadherin, and medullary carcinoma with *BRCA1* (Henry., 2019).

The molecular heterogeneity of breast cancer is used to classify breast cancer into intrinsic molecular subtypes including luminal A (Estrogen Receptor (ER)+/(Progesterone Receptor (PR)+ and Ki67-low, absence of HER2, luminal B (ER+/PR+ and HER2+ or HER2-, and Ki67-high) (Lüönd et al., 2021), HER2 enriched (ER+, PR+, HER2+, KI67: 15-30%) with an increased expression of *ERBB2* gene and (HER2+ ER-,PR-, KI67 >30%), and triple-negative breast cancer (ER-, PR-, HER2-)(Xulu et al., 2023). This classification of subtypes aid in providing appropriate treatment plans (Orrantia et al., 2022).

Intratumoural heterogeneity is the presence of special cellular populations with distinct genetic, epigenetic, and phenotypic features within the same tumours (JamalHanjani et al., 2015). As such tumours can flourish in varying conditions (Tuasha and Petros, 2020). Intratumoural heterogeneity can lead to poor prognosis of the disease as the prescribed treatment may not effectively target all cell populations within the tumour thus, may lead to disease relapse (Tuasha and Petros, 2020).

The mechanisms involved in breast cancer heterogeneity are not fully understood, there are however, two common theories which may explain this phenomenon including clonal evolution and cancer stem cells (CSC) theory (Tuasha and Petros, 2020). Clonal theory is founded on Darwin's theory of evolution, whereby a mutation in a cell is passed on to the daughter cells as it divides (Iseghohi, 2016). Thus, inducing more mutations as it divides expanding clonally overtime (Iseghohi, 2016). The clonal evolution theory suggests that cancer cell populations evolve and develop because of heritable epigenetic and genetic changes in a multistep tumorigenesis process (Tuasha and Petros, 2020). The cancer stem cell theory occurs when cells undergo symmetric division and form two identical daughter cells that are copies of them (self-renewal) or two identical daughter cells that are progenitors (non-CSC) (Iseghohi, 2016). The asymmetric division is when the stem cell divides to form two non-identical daughter cells, one replicating the stem cell and the other non-identical (non-CSC) (Iseghohi, 2016). Malignant cells exhibit characteristics of stemness defined as

the cells ability to expand its lineage, formulating differentiated cells, and engage with its environment to sustain a balance between quiescence, proliferation, and regeneration (Aponte and Caicedo, 2017). The common features of stemness between stem cells and cancer cells including self-renewal, differentiation potential and organization of stemness supporting microenvironments promote cancer development and survival (Aponte and Caicedo, 2017).

Tumour heterogeneity can also be divided into temporal and spatial heterogeneity (Fumagalli & Barberis, 2021). Temporal heterogeneity refers to the differences in molecular makeup of cancer cells during progression, while spatial heterogeneity is characterised by the interactions between different cell populations (Fumagalli & Barberis, 2021). A clear indication of temporal heterogeneity is the variation of ER, PR HER2 and Ki67 expression found between primary tumours and metastatic lesions (Fumagalli & Barberis, 2021). The extent of temporal intratumoral heterogeneity can be influenced by the time of diagnosis and the time of treatment (Fumagalli & Barberis, 2021). As a result, increased delays can aid in further advancement of tumour heterogeneity making treatment difficult (Fumagalli & Barberis, 2021). An indication of spatial heterogeneity could include interaction with stem cell markers of different cells and interaction with the microenvironment (Guo et al., 2023).

1.3 Luminal A breast cancer cell lines: MCF7 and T47D

Breast cancer cell lines in humans are important for breast cancer research in understanding tumour progression and treatment (Adjo Aka and Lin, 2012). MCF7 and T47D cell lines are human hormone dependent cell lines commonly used in *in vitro* and *in vivo* studies to study different processes including analysing gene expression, protein function and drug efficacy assessment (Adjo Aka and Lin, 2012; Lee, Oesterreich and Davidson, 2015). T47D cell lines originated from the pleural effusion of a 54-year-old breast cancer patient. These cells exhibit epithelial morphology and form monolayers in culture. MCF7 cells were isolated from a pleural effusion of 69-year-old women with metastatic disease (Seban et al., 2015). The patient from which MCF7 cells were derived had a history of undergoing a mastectomy for a benign tumour 7 years prior to primary cell culture (Seban et al., 2015). After 4 years the patient underwent a mastectomy of her breast for a malignant adenocarcinoma and 3 years later underwent radiotherapy and hormone therapy (Seban et al., 2015). After 2 months of nodular recurrence, samples were taken from a pleural effusion for laboratory studies (Seban et al., 2015). These cell lines were derived from a metastatic site by pleural effusion and are generally classified as luminal A (Adjo Aka and Lin, 2012). Both MCF7 and T47D cell lines possess several key characteristics specific to the mammary epithelium (Yu, Kim, Yoo and

Kang, 2017). Although their phenotypic and molecular characteristics are known to be similar, one study indicated the differences between these two cell lines revealing the protein variations expressed between them (Yu, Kim, Yoo and Kang, 2017). A study was carried out by Adjo et al., (2012) and showed several proteins related in cell growth, apoptosis regulation and carcinogenesis were greatly expressed in T47D cells than in MCF7, emphasising heterogeneity in Luminal A breast cancer (Adjo Aka and Lin, 2012). Studies in our lab have also shown that T47D cells were more likely to respond to combined antiplatelet therapy with Anastrozole and differed significantly from MCF7 (Xulu, Duarte and Augustine, 2020). After treatment with Anastrozole and antiplatelet therapies, T47D cells displayed rounded morphology and cell clusters, and membrane ruffles which indicated that cells were obtaining a more aggressive phenotype than MCF7 cells (Xulu, Duarte and Augustine, 2020). T47D cells also revealed lower levels of ESR1 (a gene encoded for ER α) in comparison to MCF7. In addition, T47D revealed higher expression of ESR2 (a gene encodes for ER β) when compared to MCF7 cells (Pather K., 2019).

T47D cell lines express lower ER but higher PR than MCF7 cells, suggesting a more aggressive phenotype (Horwitz et al., 1983; Pather et al., 2019). T47D cells express a mutated, nonfunctional form of p53, which is a tumour suppressor protein that is a transcription factor able to induce growth arrest or apoptosis (Alkhalaf and El-Mowafy, 2003; Welsh, 2013). p53 is commonly inactivated by mutation or other mechanisms in human breast cancer (Alkhalaf and El-Mowafy, 2003). In breast cancer cell lines, ER α is known to cause the proliferative response of 17 β - estradiol (E2) (Zhou and Liu, 2020). In T47D cells, using real-time PCR a study revealed the primary ER mRNA is ER α (Zhou and Liu, 2020). Another study revealed that the expression of ER β in the breast cancer cell line T47D inhibits proliferation in response to E2 treatment (Strom et al., 2004). MCF7 cell line reveal a vast variation in the expression of ER, PR and HER2 and growth conditions may influence the proportion of the dominant receptor in MCF7 cells thus influencing the phenotype (Şerban, 2015). The predominance of ER-positive phenotype can be sustained when exposed to small amounts of oestrogen in the fetal bovine serum (Şerban, 2015). However, with the reduction of oestrogen further growth would select variants that are dependent on Epidermal Growth Factor Receptor (EGFR), HER2 and other stimulators of the signalling pathways AKT (PKB; protein kinase B), extracellular related kinase (ERK) and mammalian target of rapamycin mTOR (Şerban, 2015).

In MCF7 cells expressing ER α , E2 resulted in proliferation and tumour formation. However, when ER β was expressed with ER α , MCF7 cells underwent antiproliferation and antitumour

pathways in the presence of oestrogen (Paruthiyil et al., 2004; Omoto and Iwase, 2015). This finding agreed with another study which showed that the over expression of ER β inhibited cellular growth and prevented tumour growth in immunodeficient mice (Omoto and Iwase, 2015). Thus, emphasizing the potential importance of ER β detection in clinical diagnosis.

1.4 Heterogeneity of diagnostic and prognostic biomarkers of luminal A breast cancer

1.4.1 Oestrogen Receptor

Oestrogen can be regulated via direct genomic signalling or indirect genomic signalling (Fuentes & Silveyra, 2019). Indirect genomic signalling oestrogen binds to the ERs, the complex translocates to the nucleus activating transcriptional changes in the oestrogen-responsive genes with or without EREs (Fuentes & Silveyra, 2019). The indirect genomic signalling pathway is characterised by membrane-bound ER that activates cytoplasmic events including the regulation of the membrane-based ion channels, second-messenger cascade and transcription factors (Fuentes & Silveyra, 2019).

The ER-independent pathway is activated by oestrogen inducing antioxidant effects (Fuentes & Silveyra, 2019). Non genomic signalling is common in steroid hormones and is characterised by the activation of different protein-kinase cascades resulting in changes in gene expression via phosphorylation of transcription factors. It is commonly linked to an increase of membrane bound ER namely, GPER1, ER α and ER β . Once oestrogen binds to ERs on the surface of the cell it facilitates intracellular calcium mobilization, adenylate cyclase induction and mitogen-activated protein kinase induction, production of cyclic adenosine monophosphate (cAMP), phosphoinositol 3-kinase signalling pathway activation and inducing the membrane tyrosine kinase receptors. GPER1 is linked to the activation of both adenylate cyclase and EGFR (Vrtačnik P et al., 2014).

ER is a transcription factor responsible for regulating the transcription of many genes resulting in cell division, mammary gland development and cell proliferation (Paterni et al., 2014). ER are characterised by functional domains including N-terminal transcriptional activation function (AF-1) domain, DNA-binding domain (DBD) found within the AF-1 domain, zinc- finger domain important for identifying the oestrogen responsive element (ERE) and a second zinc finger important for stabilization of protein-DNA interactions (Paterni et al., 2014). Between the AF1 and ligand binding domain (LBD) is the hinge region that makes up the second activation domain (AF-2) (Paterni et al., 2014).

1.4.1.1 ER variants: alpha and beta

Sequence patterns of amino acids for ER α and ER β show a 59% sequence identity in their ligand binding domains (LBD) (Paterni et al., 2014). These variations are based on differences in the ligand binding cavities of two amino acid positions namely Leu384 and Met421 in ER α and Met336 and Ile 373 in ER β . In addition, there are also differences in ER β binding pocket that is smaller and narrower in comparison to ER α (Paterni et al., 2014). This could be due to the bigger sequence assortment beyond the ligand binding pocket as opposed to the small variations at the two pocket residue positions (Paterni et al., 2014). ER α has been found to support growth and differentiation in breast cancer (Paruthiyil et al., 2004). Other studies have indicated that ER α has also been known to contribute to tumour promotion (Paruthiyil et al., 2004). According to some studies, ER β is expressed in many tumours (Paruthiyil et al., 2004). About 70% of breast tumours express ER β with many studies indicating that ER β is associated with poorer prognosis than tumours expressing ER α (Paruthiyil et al., 2004). ER β is linked to increased cell proliferation markers, Ki67 and cyclin A in human breast cancer (Paruthiyil et al., 2004). Increased ER β mRNA is shown to be increased in Tamoxifen-resistant tumours in comparison to Tamoxifen-sensitive tumours thus suggesting its ability to promote tumour progression and cell proliferation (Paruthiyil et al., 2004).

Several clinical data showed a relationship between ER and kinase signalling pathways (Brown et al., 2018). RTKs, such as EGFR and HER2, and downstream signalling through phosphoinositide 3-kinase (PI3K) and Mitogen-activated protein kinase (MAPK) can induce different pathways of cell proliferation and survival bypassing ER blockade (Brown et al., 2018). In addition, these signalling pathways can induce ER by posttranslational alterations, including ER phosphorylation, in a ligand independent manner. Furthermore, ER can activate transcription of growth factor receptors and ligands (Brown et al., 2018). Thus, this crosstalk suggests that in ER⁺ breast cancer, it is vital to target both the ER pathway and RTK signalling pathways (Brown et al., 2018). The ER α signalling pathway is the essential pathway in hormone dependent breast cancer. It is involved in cellular growth, survival, architecture, and gene expression regulatory mechanism (Bouris et al., 2015). According to other studies, down-regulating ER α in MCF7 cells promotes cellular phenotypic changes, gene, and protein expression (EGFR and HER2 receptor tyrosine kinases) related to epithelial-to-mesenchymal transition (EMT) (Bouris et al., 2015).

1.4.2 Ki67 in breast cancer

Ki67 is a proliferative marker, a human nuclear antigen involved in cell division in normal and malignant tissue (Mannell, 2016). It plays an essential role in the cell cycle and mitosis making it vital in its association with cancer progression (Mannell, 2016). Luminal A is characterised by a low Ki67 (<14% positive cells) in comparison to Luminal B with a high Ki67(>14% positive cells) making it important when considering its role in tumor heterogeneity (Atoui A et al., 2022). It has therefore become a marker of interest in many studies (Mannell, 2016). According to (Mannell, 2016) a low expression of Ki67 ($\leq 15\%$) was linked to five-year disease-free survival with 87% and 89% overall survival rate while patients with a high expression of Ki67 (>45%) had disease free survival with an overall survival of 76% and 83% (Mannell, 2016). In breast cancer Ki67 is used regularly in the selection of treatment (Mannell, 2016). Thus, Assessing the response that hormone therapy such as Tamoxifen and Anastrozole, has on Ki67 is therefore essential (Mannell, 2016). According to (Joosten et al., 2021) Tamoxifen treatment in breast cancer patients caused a decrease in IHC-based Ki67 levels, established through mRNA-based cell proliferation markers (Joosten et al., 2021). Therefore, Ki67 has become a biomarker for Tamoxifen response in premenopausal breast cancer patients (Joosten et al., 2021).

Ki-67 is characterised by nuclear localization patterns, featured in distinct parts of the cell-cycle (Uxa et al., 2021). Tandem repeats are featured on the central region of Ki-67 and consist of residues phosphorylated by CDK1 when undergoing mitosis (Uxa et al., 2021). Phosphorylation is known to influence Ki-67 mitotic localization and *in vitro* DNA binding (Uxa et al., 2021). During interphase, the phosphorylated Ki67 results in a fibre-like structure that surround the nucleoli and crosses the peri nucleolar heterochromatin (Uxa et al., 2021). This is vital as it indicates that the reduction in Ki67 decreases the interaction of heterochromatin around the nucleoli (Uxa et al., 2021). When Ki67 becomes hyperphosphorylated during mitosis, there is less binding to the DNA, making it more mobile on the chromosome periphery upon anaphase (Uxa et al., 2021).

Ki67 is induced by cell cycle regulator such as cyclin-dependent kinases (CDKs), the initiation of the subunit of the ubiquitin ligase APC/C-CDH1, that is responsible for the destruction of mitotic cyclins and cell cycle transcription factor B-Myb (Mrouj et al., 2021). Thus, Ki67 expression does involve major steps of tumorigenesis such as initiation, progression and metastasis, immune responses, and drug sensitivity (Mrouj et al., 2021). By maintaining transcriptional process involved in tumour cell adaptations, Ki67 plays an important role in tumourigenesis. This was shown in a study performed on mutant mice,

whereby Ki67 knock-out in cancer cells reduced the ability to induce angiogenesis (Mrouj et al., 2021). In addition, due to the knockout Ki67 expressing cells, epithelial and mesenchymal characteristics of the cells were altered and was therefore unable to induce metastasis (Mrouj et al., 2021). According to Mrouj et al., human colorectal cancer cells DLD-1 revealed that Ki67 knockout decreased the number of cells expressing the antigen CD133 (Mrouj et al., 2021).

1.4.3 Progesterone Receptor

PR is associated with the steroid nuclear receptor family of ligand-dependent transcription factors (Li et al., 2022). It is characterised by the central region of the DNA binding domain (DBD), C-terminal ligand binding domain (LBD) and amino-terminal domain (Li et al., 2022). PR consists of two transcriptional; activation functions (Afs) that assists in coregulatory protein surface interactions (Li et al., 2022). AF1 is localised in the N-terminal domain (NTD) and AF2 is localised in the LBD (Li et al., 2022). Once PR are induced, they activate functional domains and enzymes to the promoter to induce transcriptional regulation via co-activators such as steroid receptor co-activator (SRC-1) (Li et al., 2022). SRC-2 and SRC-3, cyclin AMP response element binding protein (CBP/p300) (Li et al., 2022). Furthermore, they will attach the progesterone response element (PRE) of PR and induce transcription of target genes (Li et al., 2022). There are two common isoforms of PR: A and B, PR-B is the full-length receptor, with PR-A being an N-terminally shorter version (without the first 164 amino acids found in PR-B) (Daniel et al., 2011). They can both be responsible for the regulation of either the identical or different groups of target genes and are comprised of both ligand dependent and independent activities (Daniel et al., 2011). PR-B and PR-A are both substrates for MAP kinases such as CDK2, MAPKs, CK2 (Daniel et al., 2011). Once the receptors are phosphorylated with coregulators, they regulate promoter selection and sensitivity of PR target genes to progesterone, EGF, FGF and IGF (Daniel et al., 2011).

PR also acts to initiate signal transduction pathways involved in pro-proliferative signalling in the breast (Daniel et al., 2011). Some studies indicate that PR extranuclear action results in the induction of protein kinases (MAPK, PI3K/Akt and c-Src) via the ligand induced association between PR and c- Src kinase (Daniel et al., 2011). In addition, some studies also revealed that progesterone induced c-Src and ERK2 (MAPK) in breast cancer cells (T47D), thus increasing T47D cell growth (Daniel et al., 2011).

Progesterone aids in biological functions primarily by binding to the nuclear PRs (Chen, B. *et al.*, 2020). Via regulatory pathways of CSC activity, progesterone binds to both nuclear and

cell membrane PRs (mPRs) (Chen, B. *et al.*,2020). As PR have two common isoforms PR-A and PR-B ratios of these isoforms can impact different functions (Chen, B. *et al.*,2020). PR-A was found to induce CSC expansion with PR-B promoting anchorage-independent proliferation in T47D cells (Chen, B. *et al.*, 2020). In addition, when comparing PR-B⁺ tumourspheres to PR-A⁺, PR-A tumourspheres revealed more CSC populations including ALDH1⁺ and CD44⁺CD24⁻ cell populations (Chen, B. *et al.*,2020).

1.5 Stem Cell markers and Intratumoural heterogeneity

Intratumor heterogeneity is associated with clonal variation and influences of the microenvironment (Prasetyanti & Medema, 2017). CSCs are thought to contain most of a cancer's tumour- promoting and metastatic attributes (Tuasha and Petros, 2020). It was observed that a small subpopulation of breast cancer cells, had the capacity to develop tumours in mice, showing plasticity reminiscent of stem cells (Dittmer, 2018). Breast Cancer Stem Cells (BCSCs), which constitute a small population of CSC, have been associated with chemoresistance and relapse in breast cancer (Palomeras, Ruiz-Martínez and Puig, 2018). BCSCs are identified by the expression of cluster of differentiation (CD) surface markers, CD44 and CD133, and low or no expression of surface marker CD24 and aldehyde dehydrogenase (ALDH) activity (Dittmer, 2018).

1.5.1 CD44

Cluster of differentiation 44 (CD44), a transmembrane adhesion glycoprotein expressed in embryonic stem cells, differentiated cells and cancer cells (Hassn Mesrati et al., 2021). CD44 is involved in physiological process including organ development, immune functions, and haematopoiesis (Hassn Mesrati et al., 2021). In addition, CD44 also mediates processes such as T-cell differentiation, branching morphogenesis, proliferation, adhesion, aggregation, and migration (Hassn Mesrati et al., 2021). In normal cells, CD44 is predominantly expressed in lymphocytes, smooth muscle, fibroblasts, and different types of epithelia (Hassn Mesrati et al., 2021). Various studies have revealed increased levels of CD44 mRNA in lymphoma, breast, colon, and endometrial cancer (Hassn Mesrati et al., 2021). Increased levels of CD44 are often linked to tumour progression due to its physiological functions (Hassn Mesrati et al., 2021). CD44 is characterized by two isoforms due to alternative splicing via transcription, namely the standard isoform (CD44s) and the variant isoform (CD44v) (Hassn Mesrati et al., 2021).

According to Hassn Mesrati et al., CD44 isoforms impact tumour initiation and tumour progression. CD44s promoted tumour initiation and CSCs gene via the PDGFR β /STAT3

cascade signalling pathway (Hassn Mesrati et al., 2021). In addition, CD44s was shown as the dominant isoform in breast CSCs and its downregulation diminished CSCs signatures (Hassn Mesrati et al., 2021). Similarly, a change from variant to standard CD44 isoform was associated with EMT via the AKT signalling pathway in breast cancer cells (Hassn Mesrati et al., 2021). CD44s is also known to interact with phosphorylated cort-actin activated invadopodia, which mediates metastasis of breast cancer cells to various organs (Hassn Mesrati et al., 2021). CD44s is also associated with TGF- β signalling, mediating the mesenchymal phenotype (Hassn Mesrati et al., 2021).

The interaction between HA and CD44 enables binding of adaptor molecules to cytoplasmic region in CD44 inducing signalling pathways Ras, ErbB2, mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinases (PI3K) (Härkönen et al., 2019). In addition, HA binding to CD44 was found to increase proliferation via the β -catenin signalling and nuclear factor-kappa B (NF κ B) transcription activity enhancing P-glycoprotein and B-cell lymphoma-extra-large (Bcl-xl) genes in MCF7 breast cancer cell lines (Härkönen et al., 2019). These pathways are also linked to stemness and malignant changes of cells (Härkönen et al., 2019).

1.5.2 CD133

CD133 in normal breast cancer cells is involved in morphogenesis, ductal branching, and the ratio of luminal to basal cells (Brugnoli et al., 2019). A study conducted with murine mammary tumours revealed that CD133⁺ cells had an increased colony forming ability, increased proliferation and elevated ability to form tumours (Brugnoli et al., 2019).

CD133 is vital for various cellular processes such as glucose and transferrin uptake, autophagy, membrane-membrane interaction, and matrix metalloproteinase functions (Li, 2013). CD133⁺ CSCs are linked to tumour metastasis, chemotherapy- or- radiotherapy resistance (Li, 2013). CD133 is an important biomarker for identifying and isolating cell subpopulations (cancer stem cells) in various forms of neoplasms such as breast cancer. Cells expressing CD133⁺ consist of stemness properties including drug-resistance, self-renewal, differentiation ability; high proliferation and are able to form tumours in xenografts (Tume, Paco, Ubidia-Incio and Moya, 2016).

In some studies, CD133 in both normal and cancer stem cells revealed that CD133 reduction was influenced by the cell cycle G1/G0 portions (Brugnoli et al., 2019). Other influences of CD133 include hypoxia and the microenvironment which increased CD133 expression via the hypoxia inducible factor-1 α (HIF-1 α) (Brugnoli et al., 2019). In addition, CD133 could

also be involved in the regulation of the PI3K/AKT signalling in CSCs (Brugnoli et al., 2019).

1.5.3 CD24

CD24 is a sialoprotein that promotes cellular adhesion, proliferation, and metastasis (Jaggupilli and Elkord, 2012; Crabtree and Miele, 2018). BCSC are characterised by low CD24 expression, and *in vitro* studies indicate that the upregulation of CD24 inhibits stemness in breast cancer cells (Crabtree and Miele, 2018). Breast cancer subtypes differ regarding the proportions of CD44⁺CD24^{-/low} CSC subpopulation, being higher in basal-like tumours (>10%) than in luminal A and B tumours (Dittmer, 2018) (Vikram et al., 2020). CD44⁺/CD24^{-/low} BC cells have been characterised by inducing increased invasive activity and tumour promoting qualities (Deng et al., 2017). In addition, tumours with increased CD44⁺/CD24⁻ revealed poor clinical outcome and increased metastasis (Deng et al., 2017).

1.6 Hormone Therapy in breast cancer

Breast cancer heterogeneity increases the difficulty in treatment and diagnosis. Treatment for breast cancer consists of local therapies: surgery, radiotherapy and systemic therapies such as hormonal therapy, chemotherapy, and targeted therapy (Drăgănescu and Carmocan, 2017). Systemic therapy is chosen based on the recurrence risk determined through diagnostic score of each patient (Drăgănescu and Carmocan, 2017). Of particular interest to this study is the luminal phenotype breast tumours (accounting for 68.30% pooled frequency of luminal phenotype in Southern Africa) which, as a result of positive status of hormone receptors, can be treated with hormone therapies including selective oestrogen receptor modulators (SERM) that act to limit the signalling capacity of the ER or with aromatase inhibitors (AI) which limit the availability of oestrogen (Abimbola F et al., 2023) (Szostakowska et al., 2018; Nascimento and Otoni, 2020). Oestrogen is a regulator of cellular growth and differentiation through the genomic pathway which regulates transcription of specific target genes by binding to associated deoxyribonucleic acid (DNA) regulatory sequences via ER α and ER β , or via the non-genomic pathway whereby it involves indirect regulation of gene expression via a variety of intracellular signalling events (Paterni et al., 2014; Lee, Kim and Choi, 2012; Fuentes and Silveyra, 2019).

1.6.1 Tamoxifen

Tamoxifen is a triphenylethylene derivative SERM which acts by binding to the ER and inhibiting the proliferative action of oestrogen on mammary epithelium (Sporn and Lippman,

2003). It is the most commonly used hormone therapy for ER-positive breast cancers in both pre- and postmenopausal women; however, a significant number of patients either present with Tamoxifen-resistant tumours or acquire resistance to the drug during the course of treatment (Chang, 2012). Most patients that relapse on Tamoxifen treatment respond to other therapies including ER down regulators/ER antagonists or AIs (Chang, 2012). Tamoxifen mechanism of action is competing with 17β -estradiol (E_2) at the receptor site and reducing the action of E_2 in breast cancer, or via binding to DNA once after metabolic activation thereby initiating carcinogenesis (Chang, 2012).

Tamoxifen is a treatment commonly used in ER α positive breast cancer (Sellitto et al., 2020). Tamoxifen's interaction with ER α is controversial. According to Sellitto et al., Tamoxifen has an agonist/antagonist effect on ER α (Sellitto et al., 2020). According to some studies ER β binds to Tamoxifen, however, the evidence of its interaction in ER α -negative breast cancer is contradictory. According to Sellitto et al., low levels of ER β expression was associated with Tamoxifen resistance, in addition ER β expression was also linked to the benefit of overall survival in Tamoxifen-treated tumours (Sellitto et al., 2020). Some studies finally concluded that Tamoxifen has an agonist/ antagonist function for ER α but an antagonist effect for ER β (Sellitto et al., 2020).

According to Gruvberger-Saal et al., 2007 ER β was revealed to bind Tamoxifen, with low levels of ER β linked with Tamoxifen resistance (Gruvberger-Saal et al., 2007). Although other studies indicated that ER β expression was linked to increased overall survival in patients treated with Tamoxifen; untreated patients were shown to have no association, indicating to ER β 's potential as a Tamoxifen sensitivity marker (Gruvberger-Saal et al., 2007).

1.6.2 Anastrozole

The primary source of oestrogen is via the conversion of androgens to oestrogens in muscle, the skin, adrenal glands, and adipose tissue (Peters & Tadi, 2022). Anastrozole is a common type of aromatase inhibitor and acts by inhibiting the aromatase enzyme through competitive inhibition allowing the decrease in oestrogen production (Milani, Jha and Potter, 2009). (Peters & Tadi, 2022). Breast cancer cells have high aromatase activity, which is a common source of oestrogen (Peters & Tadi, 2022). The inactivation of the aromatase enzyme suppresses serum oestrogen levels, thus decreasing cancer cell proliferation in hormone receptor positive breast cancer (Peters & Tadi, 2022). Aromatase inhibitors are vital in the therapy of ER α breast cancers (Ingle et al., 2020). Their mechanism of action includes

aromatase inhibition (Ingle et al., 2020). In addition, other studies have also revealed that Anastrozole can also bind to ER α creating an additional method to mediate the decrease of oestrogen levels (Ingle et al., 2020). Studies have shown that Anastrozole degrades ER α specifically in the presence of oestradiol (E2) and reveals that E2 sensitizes cells to Anastrozole (Cairns et al., 2020). The interaction of Anastrozole and ER α is clearly interesting and could explain the changes in levels of expression of ER α once treated with Anastrozole (Cairns et al., 2020). According to Cairns et al., 2020 Anastrozole initiates ER α -dependent transcription but only at specific concentrations as increased concentration of Anastrozole resulted in the activation of ER α degradation. The study also revealed that Anastrozole treatment reduced ER expression in breast cancer patients (Cairns et al., 2020). Anastrozole was tested to understand its ability to inhibit E2-stimulated ER α activity in CRISPR KO T47D cells (Ingle et al., 2020). It is known that Anastrozoles mechanism of action is to decrease oestrogen production from androgenic precursors by inhibiting aromatase (**Fig 2**) encoded by the gene CYP19A1 (Ingle et al., 2020). According to Ingle et al., 2020 an additional mechanism of action of Anastrozole includes binding to the ligand of ER α (Ingle et al., 2020) (Hu et al., 2015).

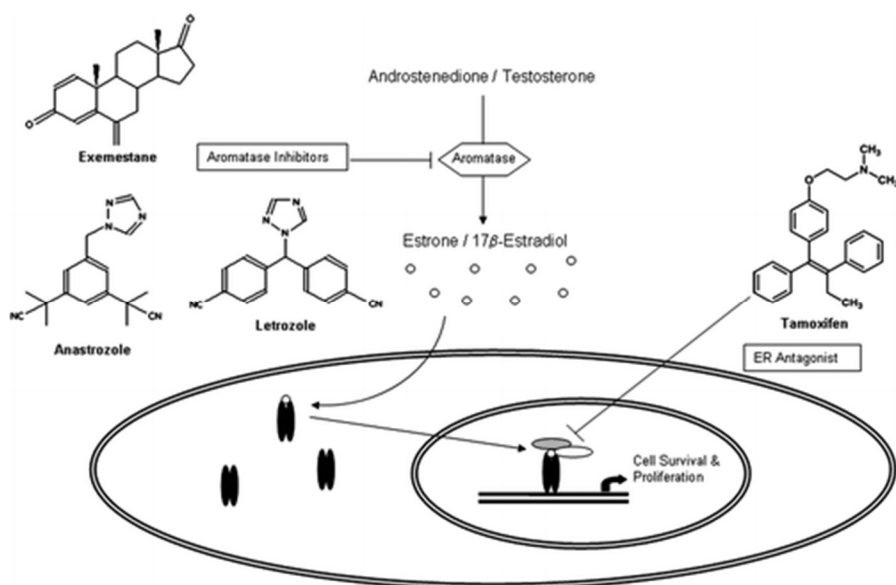


Figure 2: Anastrozole and Tamoxifen mechanism of action (Shiuan Chen., 2011)

1.7 Rationale

Breast cancer is characterised by both intertumoural and intratumoural genomic heterogeneity that expands further than localised disease and is driven by intricate mechanisms of tumour dissemination. Furthermore, the association between breast cancer subtypes and its genomic features during BC evolution is poorly understood (Lluch et al., 2019). The primary challenge of cancer is associated with the large tumour heterogeneity and

plasticity (Lluch et al., 2019). Evidently, optimal profiling methodologies, the timing of tumour sampling, and the effects of tumour heterogeneity affect disease management and long-term treatment efficacy (Lluch et al., 2019). There is a gap in research on the evaluation of intratumoural heterogeneity within breast cancer cell line populations and how they interact with tumour therapy based on their differences. Intratumor heterogeneity reduces the efficacy of cancer treatment and surviving tumour cells post-therapy may cause cancer relapse. Thus, there is an importance for understanding how cancer cells gradually transform and how treatment of different subpopulations in cancer will affect the cancer outcome (Januškevičienė and Petrikaitė, 2019). Thus, understanding the molecular and cellular characteristics of tumour heterogeneity is essential for optimising treatment strategies to minimise resistance to therapy (Januškevičienė and Petrikaitė, 2019). By establishing the variations between populations using suitable biomarkers and their response to hormone therapy (Tamoxifen and Anastrozole) we can potentially aid in the management of breast cancer using hormone therapy.

1.8 Aims

To investigate the effects of hormone therapy using Anastrozole and Tamoxifen on tumour heterogeneity on luminal A breast cancer cell lines, MCF7 and T47D.

1.9 Objectives

- a) To isolate and propagate stem cells (CD44+CD133+) and non-stem cell subpopulations (CD44-CD133-) within breast cancer cells (MCF7 and T47D) using magnetic cell sorting
- b) To verify features of the isolated populations using stem cell markers: CD44+, CD133+ and CD24-expression in breast cancer subpopulations using flow cytometry
- c) To examine protein expression for breast cancer diagnostic markers; ER α , ER β , PR and Ki-67 in isolated subpopulations following treatment with Tamoxifen and Anastrozole using Immunocytochemistry

Chapter 2: Methods and material

2.1 Ethical Clearance

An ethical waiver (W-CBP-210729-01) was obtained from the University of the Witwatersrand Human Research Ethics Committee (HREC) (Appendix A)

2.2 Cell Culture

2.2.1 Standard cell culture procedure for parent populations (P0)

Breast cancer cell lines representing luminal A subtype; MCF7 (p19-p25) (cellonix) and T47D (p24-p30) donated from Prof Sharon Price at the University of Cape Town, South Africa were used. MCF7 cells were cultured in Dulbecco's Modified Eagles' Medium (DMEM) (Pan Biotech, P04-03590) supplemented with 10% fetal bovine serum (Pan Biotech, P30-3302,) and 0.1% penicillin/streptomycin. T47D cells were cultured in Rosewell Park Memorial Institute 1640 media (RPMI) (Pan Biotech, P04-18500) supplemented in 10% fetal calf serum, 0.1% penicillin/streptomycin and 0.2 U/ml human insulin. These media will henceforth be referred to as complete culture media.

2.2.2 Subculturing procedure

Cells were cultured in 75 cm² tissue culture flasks (Lasec, GRE658170) and incubated in a HERaCELL 150i incubator (Thermo Fisher Scientific) at a temperature of 37 °C, at 5% CO₂. Once cells were 70% -80% confluent, they were rinsed twice with phosphate buffered saline (PBS) pH 7.4 and detached using 0.25% trypsin/Ethylenediaminetetraacetic Acid (EDTA) (Thermo Fisher Scientific, 25300062) for 5 mins. Thereafter, complete culture media was added to each flask to neutralise trypsin/EDTA and cells were transferred into 15 ml centrifuge tubes (Lasec, 188261) and centrifuged for 5 mins at 400 xg using a Rotafix32A centrifuge (Hettich, Sigma-Aldrich). Cells were then cryopreserved in cryopreservation media composed of; 60% FBS, 30% complete culture media and 10% Dimethyl sulfoxide (DMSO), in a Forma Scientific freezer at -80 °C or plated at a cell density of 1 x 10⁵ for further experimentation.

2.2.3 Cell counting

For cell counting purposes, cells were resuspended in 1 ml of relevant suspension solution (complete culture media or buffer) and a volume of 10 µl of cell suspension was added to 10 µl of 0.4% trypan blue in a 1.5 ml Eppendorf tube (Axiology labs, D1010S). A volume of 10 µl of the cell suspension and 0.4% trypan blue mixture was pipetted onto a Dual-Chamber

cell counting slide (Bio rad, 1450011). To quantify the cell numbers and determine cell viability the TC20 automated cell counter (Bio-Rad, 145-0101) was used.

2.3 Modified essential 8 Media Optimization for propagation of sorted cell populations

2.3.1 Rationale for the modified essential 8 media

Essential 8 (E8) media (Gibco, A15169) was initially used to propagate the sorted cell populations because it has the required growth supplements for propagating stem cells while preventing differentiation; however, this media was discontinued by the supplier. The essential 8 media (Chen et al., 2011) contained the following: DMEM/F12, L-ascorbic acid-2-phosphate (64mg/l), sodium selenium (14 µg/l), fibroblast growth factor (FGF2) (100 µg/l), insulin (19.4 mg/l), sodium bicarbonate (NaHCO₃) (543 mg/l), transferrin (10.7 mg/l) and transforming growth factor-β1 (TGFβ1) (2 µg/l). A modified E8 media was made in our laboratory to replace the commercially bought E8 media (Gibo, A15169-01); however, human transferrin could not be obtained from most companies due to permit requirements. As such, iron was used as an alternative to transferrin (Keenan J et al., 1996)

2.3.2 Optimising Iron salts

In order to optimise the iron salts, the effect of each iron salt; ferrous sulphate (FeSO₄) (BDH Chemicals Ltd, 10112) or ferric nitrate [Fe (NO₃)₃] (BDH Chemicals Ltd, 10306) in media alone (without additional growth supplements) was tested in order to determine whether cell growth was hindered. FeSO₄ or Fe (NO₃)₃ were dissolved at various concentrations (0.5 µg/ml, 5 µg/ml & 10 µg/ml) in DMEM/F12. Breast cancer cells were seeded in a 24 well plate at 1x10⁵ per well and the respective iron media was added to a total of 200 µl per well and incubated at 37°C , 5% CO₂. As controls, cells were also cultured in complete growth media; DMEM supplemented with 10% FBS and 0.1% pen/strep (MCF7 cells) and RPMI1640 supplemented with 10% FBS, 0.1% pen/strep and 0.2 U/ml human insulin (T47D cells) (Appendix A). At 24-hour intervals, cells were imaged using the Olympus iX51 Inverted Fluorescent Microscope with CellSens Software and counted using the TC20 automated cell counter (Bio-Rad, 145-0101) for a period of six days in order to monitor changes in cell growth over a prolonged period. Ferric nitrate [Fe (NO₃)₃] was determined to be the optimal substitute for transferrin and 10 µg/ml was determined to be the optimal concentration (Refer to Chapter 3, section 3.1-3.3).

2.3.3 Formulation of the modified Essential 8 media

Following optimisation of the iron salts, the modified essential 8 media was composed of the following;

Table 1: Modified essential 8 media

	Component	Concentration*	Company and catalogue number
1	DMEM/F12	Not applicable	Pan Biotech, P04-41500
2	Sodium bicarbonate (NaHCO ₃)	1.2 g/l	(supplemented in DMEM media) (Pan Biotech, P04-41500)
3	L-ascorbic acid-2-phosphate	64 mg/l	Sigma-Aldrich, A8960
4	Sodium selenium	14 µg/l	Sigma-Aldrich, S5261
5	Human insulin	19.4 mg/l	Sigma-Aldrich, 19278
6	Recombinant human transforming growth factor-β1 (TGFβ1)	2 µg/l	Thermo Fisher Scientific, 2455165
7	Heat stable human fibroblast growth factor (bFGF)	100 µg/l	Thermo Fisher Scientific, 2110110
8	Ferric nitrate [Fe (NO ₃) ₃]	10 µg/ml	AnalaR, BDH Chemicals Ltd, 10306

*Concentrations were scaled down to the equivalent concentration in a final volume of 50 ml of media

2.4 Magnetic Cell Sorting

Positive selection of CD44⁺ and CD133⁺ (double enriched) cells within the MCF7 and T47D cell lines were conducted using CD44 MicroBeads (Miltenyi biotec, 130-095-194) and CD133 MicroBeads (Miltenyi Biotec, 130097- 049), and manufacturers' guidelines (with minor adjustments) were followed as stated below.

2.4.1 Sample preparation

Reagents were kept at 2-8°C or on ice, for preservation and optimal staining of CD44 and CD133 microbeads. A single cell suspension of MCF7 or T47D cells containing 1X10⁷ total cells with an average cell viability of 82% were used for magnetic labelling. The buffer used for sorting procedures was comprised of PBS (pH 7.2), 0.5% bovine serum albumin (BSA), and 2 mM EDTA, henceforth it will be referred to as miltenyi buffer. (Appendix A).

2.4.2 Primary selection (CD44⁺ enriched population)

Following cell counting (refer to Chapter 2, section 2.2.3), the 1 ml cell suspension containing 1X10⁷ total cells was transferred to a 15 ml tube and centrifuged at 400 xg using a

Hettich centrifuge (Labotech) for 5 mins thereafter the supernatant was aspirated completely. The cell pellet was then resuspended in 80 µl miltenyi buffer. CD44 microbeads (20 µl) (Miltenyi biotec, 130-095-194) were added to the cells and incubated for 15 mins at 2-8°C in the dark. Cells were washed by adding 2 ml of miltenyi buffer and centrifuged at 300 xg for 10 mins using a Hettich centrifuge (Labotech). After cell centrifugation, the media was aspirated completely, and cells were resuspended in 500 µl of miltenyi buffer.

For preparation of the LS separation column (Miltenyi Biotec, 130-042-201), it was placed in the magnetic field of a MidiMACS separator and rinsed with 3 ml miltenyi buffer. The flow-through (refers to the contents drained from the magnetic column) collected in a 15 ml tube, was discarded. A new 15 ml collection tube containing 500 µl modified essential 8 media was placed under the LS column to preserve cell viability upon collection of cells into the tube.

2.4.3 Magnetic separation and collection of CD44⁺ and CD44⁻ populations

The cell suspension (500 µl) was applied onto the LS column and the flow-through containing CD44⁻ (depleted) cells was collected into a 15 ml tube containing 500 µl serum-free media. The LS column was washed with 500 µl miltenyi buffer, nine times. The column was removed from the MidiMACS separator and placed on a new 15 ml collection tube containing 500 µl modified essential 8 media. To collect CD44⁺ (enriched) cells, 5 ml of miltenyi buffer was added into the column and the magnetically labelled cells (CD44⁺) were flushed out by pushing the plunger into the column and collected into the new 15 ml tube. The CD44⁺ and CD44⁻ cell suspensions were then centrifuged at 300 xg for 10 mins and the supernatant was discarded. Cells were resuspended in 1 ml miltenyi buffer each and a cell count was conducted as previously stated (Chapter 2, section 2.2.3). The cell numbers obtained are presented in Appendix IV. Following cell counting, the CD44⁺ and CD44⁻ cell suspensions were centrifuged at 300 xg for 10 mins and the supernatant completely aspirated. The cell pellet was resuspended in 300 µl miltenyi buffer.

2.4.4 Secondary selection of CD133⁺ population from CD44⁺ population

The CD44⁺ population was used for the secondary isolation of CD133⁺ and the CD133⁻ population, while the CD44⁻ population were stored at 2-8 °C until needed. A volume of 100 µl of FCR blocking reagent was added to the CD44⁺ (enriched) population followed by addition of 100 µl CD133 microbeads (Miltenyi Biotec, 130097- 049). The samples were incubated at 2-8°C in the dark for 30 mins. During this time, the stored CD44⁻ (depleted) cell

suspension was removed from 2-8°C and 100 µl of FCR blocking reagent was added to it. A volume of 100 µl of CD133 microbeads (Miltenyi Biotec, 130097- 049) was added to the CD44⁻ (depleted) cell suspension and samples were incubated at 2-8 °C in the dark for 30 mins. Following the 30 mins labelling period with CD133 beads, cell suspensions were washed separately by adding 2 ml of miltenyi buffer and centrifuging at 300 xg using the Rotofix 32A centrifuge for 10 mins. The supernatant was aspirated, and each cell suspension resuspended in 500 µl of miltenyi buffer.

2.4.5 Magnetic separation and collection of CD44⁺CD133⁺ and CD44⁺ CD133⁻ population

The LS column was prepared as previously described (Chapter 2, section 2.4.2). The cell suspension consisting of 500µl of CD44⁺(enriched) cells labelled with CD133 antibody was applied onto the LS column, then the LS column was washed with 500 µl miltenyi buffer, nine times. The flow- through containing CD44⁺CD133⁻ cells were collected in 15 ml tubes containing 500 µl modified essential 8 media. The column was removed from the MidiMACS separator and placed into a suitable collection tube containing 500 µl modified essential 8 media. The magnetically labelled cells (CD44⁺ CD133⁺) were flushed out with 5 ml of miltenyi buffer pipetted into the LS column by pushing the plunger into the LS column in the suitable 15 ml tube. The procedure described in this section 2.5.5 was repeated for magnetic separation and collection of CD44⁻CD133⁺ and CD44⁻CD133⁻ subpopulations.

2.4.6 Subpopulation propagation and cell imaging

Propagation: Following magnetic cell sorting, the following subpopulations were obtained:

- Population 1 (P1): CD44⁺CD133⁺
- Population 2 (P2): CD44⁺CD133⁻
- Population 3 (P3): CD44⁻CD133⁺
- Population 4 (P4): CD44⁻ CD133⁻

The magnetically sorted subpopulations were subsequently cultured in modified essential 8 media. The cells were cultured in either a 24 well plate (WhiteSci, 702001), 25cm² tissue culture flask (Lasec, PGRE690175) or 75 cm² tissue culture flasks (Lasec, PGRE658170) depending on the cell density. Cells were incubated in a Heracell 150i incubator (Thermo Fisher Scientific) (w221296) at a temperature of 37 °C, at 5% CO₂). Sorted subpopulations were passaged once cells were 70% -80% confluent, as previously described and either cryopreserved or used for subsequent experiments (Chapter 2, section 2.2.2).

Imaging cell growth: For each population (P1-P4), cells for both MCF7 and T47D cell lines were imaged every 48 hrs when cell culture media was replaced using the Olympus iX51 Inverted Fluorescent Microscope where the morphology changes and growth patterns between each population were noted and recorded. Images were captured and saved using the CellSens software.

2.5. Verification of stemness phenotype using stem cell markers: CD44⁺, CD133⁺ and CD24⁻

2.5.1 Optimization of detachment solution

2.5.1.1 Rationale

Cell detachment methods include the usage of trypsin-EDTA, Accutase or EDTA-serum free media. While maintaining cell viability, cells are detached via mechanical mechanisms combined with calcium chelator or the enzymatic cleavage of adhesion proteins. However, some detachment methods may alter or affect surface proteins and phenotypical changes of the cell. This could thus lead to increased levels of autofluorescence. Therefore, cell detachment methods could influence the flow cytometry results during verification. Thus, it is essential to test these various detachment methods.

2.5.1.2 Cell detachment and counting

Following the standard cell culture procedure as previously described (Chapter 2, section 2.2.1) tissue culture flasks (75 cm²) were retrieved from the incubator and media was discarded. Cells were rinsed twice with PBS (pH 7.4), then 5 ml of detachment solution was added to each flask (as indicated in Table 2) and cells were incubated at 37 °C. Following the incubation period, cells were transferred into 15 ml tubes and centrifuged at 1500 rpm for 5 mins. The supernatant was discarded and 2 ml of 10% FBS in PBS was added to each 15 ml tube to resuspend the cells, then centrifuged at 1500 rpm for 5 mins. The supernatant was discarded, and pellet resuspended in 1 ml of 10% FBS in PBS, then cells were counted as previously described (Chapter 2, section 2.2.3).

Table 2: Detachment methods tested and duration

Detachment media	MCF7 cell line	T47D cell line
Trypsin-EDTA	20 mins	10 mins
2.9 mM EDTA in serum-free media (DMEM/RPMI)	40 mins	30 mins
Accutase	12 mins	5 mins

2.5.1.3 Antibody labelling

Cells were not, labelled with antibodies; however, they were incubated for the same duration as with antibodies in order to mimic experimental conditions (Chapter 2, section 2.7.2) for better applicability of the outcome. As such, following cell enumeration, a cell suspension of 1×10^5 cells/100 μ l was added into 15 ml tubes and incubated on ice for 20 mins. Then 2 ml of 10% FBS in PBS was added into each tube and centrifuged at 1500 rpm for 5 mins using a Rotofix 32A centrifuge (Labotec).

2.5.1.4 Fixation and storage

The supernatant was discarded, and cells were fixed in 200 μ l of 1% paraformaldehyde (PFA) on ice for 10 mins. Thereafter, 200 μ l of 10% FBS in PBS was added and centrifuged (Rotofix 32A) at 1500 rpm for 5 mins. The supernatant was discarded, and pellet resuspended in 500 μ l of 10% FBS in PBS supplemented with 1 mM EDTA. Each sample was then transferred into a corresponding polystyrene flow tube (BD Biosciences, 352063) and stored at 4 °C overnight, protected from light until analysis on the LSR Fortessa (BD Biosciences).

2.5.1.5 Data acquisition and analysis

Prior to running samples through for each experiment, cytometer setting and tracking (CST) was conducted using CST beads (BD Biosciences, 641319) to ensure optimal functioning of the cytometer (Appendix V B). Samples were acquired on the LSR Fortessa flow cytometer using the Diva software. The threshold was set at 5000 and 1000 events were recorded. The following voltages were used to acquire data, forward side scatter (FSC): 147 v (for MCF7 cells) & 50 v (for T47D cells). Side scatter (SSC) was recorded using 200 v for both cell lines (MCF7 and T47D).

2.5.2 Titration of antibodies

Cells were detached and counted as stated in Chapter 2, section 2.2.3 and 2.9 mM EDTA in serum free media (DMEM for MCF7) and (RPMI for T47D) was used to detach the cells. Fixation and storage were carried out as stated in Chapter 2, section 2.5.1.4

2.5.2.1 Antibody labelling

Antibody labelling (Chapter 2, section 2.5.1.3) was conducted following cell enumeration, antibodies were added to the cell suspension containing 1×10^5 cells in 100 μ l as shown in the **Table 3**.

Table 3: Antibody labelling of various volumes for titration

Antibody and fluorochrome conjugate/stain	Titration volume 1	Titration volume 2	Titration volume 3
FITC Mouse Anti-Human CD44-FITC (BD Science, 555479)	20 μ l	10 μ l	7 μ l*
Alexa Fluor (AF) 647 Mouse Anti-Human CD24 (BD Science, 561644)	20 μ l	10 μ l	7 μ l*
BV421 Mouse Anti-human CD133 (BD Science, 566594)	0.36 μ l	0.24 μ l	0.12 μ l*
Zombie red (ZR) (Biolegend, 423110)	0.25 μ l (1:100)	0.50 μ l (1:200)	1.00 μ l* (1:400)

*Optimal antibody concentration (titration data presented in Appendix V).

2.5.2.2 Data acquisition and analysis

Samples were acquired on the LSR Fortessa flow cytometer using the Diva software. The threshold was set at 5000 and 1000 events were recorded. The following voltages were used to acquire data; FSC 147 v, SSC 243 v, CD44-FITC 571v, CD24-AF647 813 v, CD133-

BV421 440, and ZR-PE-Texas red 518 v. Data was managed on Microsoft 365 Excel. The stain index (Maecker & Trotter, 2008) was calculated as follows;

$$\text{Stain Index} = \frac{(\text{Median of positive}) - (\text{Median of Negative})}{(\text{SD of negative} * 2)}$$

2.5.3 Labelling cells for the verification of stemness phenotype

Following the standard cell culture procedure as previously described (Chapter 2, section 2.2.1) tissue culture flasks (75 cm²) were removed from the incubator and media was discarded. Cells were rinsed twice with PBS (pH 7.4), then 5 ml of accutase was added to each flask and cells were incubated at 37 °C; 12 mins for MCF7 and 7 mins for T47D. Following the incubation period, cells were transferred into 15 ml tubes and centrifuged at 1500 rpm for 5 mins. The supernatant was discarded and 2 ml of 10% FBS in PBS was added to each 15 ml tube to resuspend the cells, then centrifuged at 1500 rpm for 5 mins. The supernatant was discarded, and pellet resuspended in 1 ml of 10% FBS in PBS, then cells were counted as previously described (Chapter 2, section 2.2.3). Following cell enumeration, a cell suspension of 1x10⁵ cells/100 µl was added into 15 ml tubes and antibodies were added as follows; 7 µl FITC Mouse Anti-Human CD44-FITC (BD Science, 555479), 7 µl Alexa Fluor (AF) 647 Mouse Anti-Human CD24 (BD Science, 561644), 0.12 µl BV421 Mouse Anti-human CD133 (BD Science, 566594) and 1 µl Zombie red (ZR) (Biolegend, 423110). Samples were incubated on ice for 20 mins, then 2 ml of 10% FBS in PBS was added into each tube and centrifuged at 1500 rpm for 5 mins using a Rotofix 32A centrifuge (Labotec). Samples were fixed and stored as previously mentioned (Chapter 2, section 2.7.1.4). Prior to data acquisition, CST settings were conducted as previously stated (Chapter 2, section 2.2.1.5). Samples were acquired on the LSR Fortessa flow cytometer using the Diva software. The threshold was set at 5000 and 1000 events were recorded. The effects of spectral overlap in fluorescence emission for the different fluorochromes was accounted for by running compensation controls (Appendix V F). The voltages used to acquire data indicate in **Table 4**.

Table 4: Voltages used in Flow Cytometry verification in MCF7 and T47D cell lines.

	Voltage (v) used to acquire data in MCF7 cells	Voltage (v) used to acquire data in T47D cells
Forward side scatter (SSC)	147 v	50 v
Side scatter (SSC)	200 v	200 v
FITC	571 v	400 v
PE-Texas Red	518 v	518 v
APC	813 v	813 v
BV421	440 v	360 v

The optimal antibody volume for CD44 and CD24 was determined to be 7 μ l, whereas for CD133 it was 0.12 μ l. The Zombie red dye showed optimal exclusion of dead cells at 1:100. Refer to Chapter 3, section 3.5.2 for titration results.

2.6 Protein expression of key diagnostic markers in parent and sub-populations of breast cancer cells (MCF7 and T47D) using immunocytochemistry (ICC)

2.6.1 Cell preparation

Parent populations (P0) of MCF7 and T47D cells were prepared as previously stated (Chapter 2, section 2.2.1-2.2.3). The sorted populations of MCF7 and T47D cells (P1, P2, P3 & P4) were prepared as previously stated (Chapter 2, section 2.5). All cell populations (P0, P1, P2, P3 & P4) were counted as stated in Chapter 2, section 2.2.3. To facilitate immunolocalization, cells were seeded onto glass coverslips (Lasec, 111500) placed within 24 well plates (WhiteSci, 702001). Glass coverslips used for T47D cells were coated with poly-D-lysine (Sigma-Aldrich, P6407) to facilitate adhesion of T47D cells onto a glass surface (Appendix B). Cell counting and viability was determined (Chapter 2, section 2.2.3) and 1×10^5 cells per well were seeded and incubated for 2 days to allow cell adhesion and confluency at 37 °C, at 5% CO₂.

2.6.2 Cell treatment with hormone therapy

Cells were treated with 1.616×10^{-5} mg/ml Anastrozole (Pather et al., 2019) and 2 μ M Tamoxifen (Xulu et al., 2020) for 24 hrs, as previously demonstrated in our laboratory. Subsequently the treatment solution was removed and cells were rinsed twice with 1ml of PBS.

2.6.3 Fixation, permeabilization and blocking

Cells were fixed in 200 μ l of 4% paraformaldehyde (Appendix A) for 10 mins at room temperature and washed three times with 200 μ l ice-cold 0.1% tween-20/PBS for 5 mins each. To permeabilize the plasma membrane, cells were incubated with 500 μ l 0.2% triton x-100 in PBS for 10 mins. Thereafter cells were washed three times with 200 μ l ice-cold 0.1% tween-20/PBS for 5 mins per wash. Non-specific binding sites were blocked with 500 μ l 1% BSA in 0.1% tween-20/PBS (blocking buffer) for 30 mins prior to incubation with antibodies.

2.6.4 Incubation with primary and secondary antibodies

2.6.4.1 Titration of primary antibodies

Antibody titration was conducted on untreated parent populations (P0) cells only for both cell lines (MCF7 and T47D). Cells were prepared as previously stated (Chapter 2, section 2.6.1 & 2.6.3) then to determine the optimal concentration of antibodies, cells were incubated with 50 μ l primary antibodies (as indicated in **Table 5**) diluted in 0.1% tween-20/PBS at 4 °C overnight.

Table 5: Primary antibodies dilutions tested

Primary antibody	Dilutions tested
Rabbit monoclonal anti-ER α antibody (Abcam, ab32063)	Dilution 1= 1:200 Dilution 2= 1:500 Dilution 3= 1:1000
Mouse monoclonal anti-ER β antibody (Abcam, ab288)	Dilution 1= 1:100 Dilution 2= 1:200 Dilution 3= 1:400

Mouse monoclonal anti-progesterone receptor antibody (Abcam, ab2765)	Dilution 1= 1:50 Dilution 2= 1:100 Dilution 3= 1:200
Rabbit monoclonal recombinant anti-Ki67 antibody (Abcam, ab16667)	Dilution 1= 1:250 Dilution 2= 1:500 Dilution 3= 1:1000

Bold= indicates the optimal dilution determined (Refer to Chapter 3, section 3.4)

Following antibody labelling, the secondary antibody was added, and cells prepared for imaging as stated in subsequent sections (Chapter 2, section 2.6.4.3 and 2.6.5). The optimal primary antibody dilution were determined, result presented in Chapter 3, section 3.4.

2.6.4.2 Image acquisition

For antibody titration images were acquired using the Olympus ix51 Inverted Fluorescent Microscope with the CellSens Software, School of Anatomical Sciences, University of the Witwatersrand. Images were obtained at 40x magnification. Filter U-MNU2 was used to detect 4',6-diamidino-2-phenylindole (DAPI) fluorescence staining, filter U-MWIBA3 was used to identify Alexa Fluor 488 (ER α and Ki67) expression, filter MWIY2 used to identify Alexa Fluor 594 (ER β and PR) expression with 3 images acquired per sample.

2.6.4.3 Labelling with optimised primary antibodies

For experimentation, cells were prepared as previously stated (Chapter 2, section 2.6.1, 2.6.2, & 2.6.3). The blocking buffer was removed and 50 μ l of the optimal primary antibody applied as follows; sections were double-labelled with rabbit monoclonal anti-ER α antibody (1:500) and mouse monoclonal anti-ER β antibody (1:200); or mouse monoclonal anti-progesterone receptor (PR) antibody (1:100) and rabbit monoclonal recombinant anti-Ki67 antibody (1:500) overnight at 4 °C.

2.6.4.4 Incubation with secondary antibody

Following overnight incubation, the solution containing the primary antibodies was decanted, and cells were rinsed three times with 500 μ l 0.1% tween-20/PBS, for 5 mins per wash. Cells were incubated with 50 μ l secondary antibodies (dilutions were previously optimised in our laboratory); Goat anti-mouse Alexa Flour 594 (Abcam, ab150116) at 1:1000 (for labelling

primary antibodies targeting ER β and PR), and goat anti-rabbit Alexa Fluor 488 (Abcam, ab150077) at 1:1000) for labelling primary antibodies targeted against ER α and Ki67), in 1% BSA in PBS for 2 hrs at room temperature in the dark. The solution containing the secondary antibodies was removed, and cells were washed three times with 500 μ l 0.1% tween-20/PBS in the dark for 5 mins each wash. The nuclei were counterstained with 50 μ l DAPI (Sigma-Aldrich, D9542) at 1:500 (Sigma Aldrich, D9542) in PBS for 5 mins. The cells were washed twice with 500 μ l 0.1% tween-20/PBS for 5 mins per wash. Glass coverslips (Lasec, 111500) were removed from 24-well plates using forceps, then mounted using Fluoroshield (Sigma-Aldrich, F6182) onto glass microscope slides (Lasec, 300F-02-1009) and sealed with nail polish. The mounted coverslips were left to dry for 30 mins in the dark, then stored at 4 °C until image acquisition.

2.6.4.5 Technical controls

The following technical controls were used to ensure the primary and secondary antibodies were correctly matched: omission of primary antibody (replacement with PBS) and omission of secondary antibody (replacement with PBS). These controls showed no fluorescence ensuring that non-specific binding was absent and that autofluorescence was not present.

2.6.4.6 Image acquisition

Images were acquired using the Olympus Bx63 OFM with the CellSens Software, Microscopy and Microanalysis Unit, University of the Witwatersrand. Nine images per sample were obtained at 40x magnification based on clustering of samples and different fluorescence applied. Filter U-MNU2 was used to detect DAPI fluorescence staining, filter U-MWIBA3 was used to identify Alexa Fluor 488 (ER α and Ki67) expression, filter MWIY2 used to identify Alexa Fluor 594 (ER β and PR) expression.

2.6.5 Data analysis

2.6.5.1 Cell profiler for quantification of mean fluorescence intensity (MFI)

The primary objective (nuclei) was selected to identify the nuclei through DAPI fluorescent. The nuclei identified was measured at a diameter in pixel units ranging between 28 and 100 (**Fig 3.**). All pixels outside the range and touching the borders were discarded. For intensity thresholding the Otsu method was applied and a threshold correction factor of 1. The lower bound was 0 and the upper bound was 1. Clumped objects were identified via the intensity method. Secondary objects were selected based on the cell, according to their size and shape.

The Otsu method was applied and a threshold correction factor of 0.8. The lower bound was 0 and the upper bound was 1. The image intensity is to measure the fluorescent intensity of each marker. Images were saved using the “SaveImages” application and data exported to excel spreadsheet using the “ExportToSpreadsheet” application.

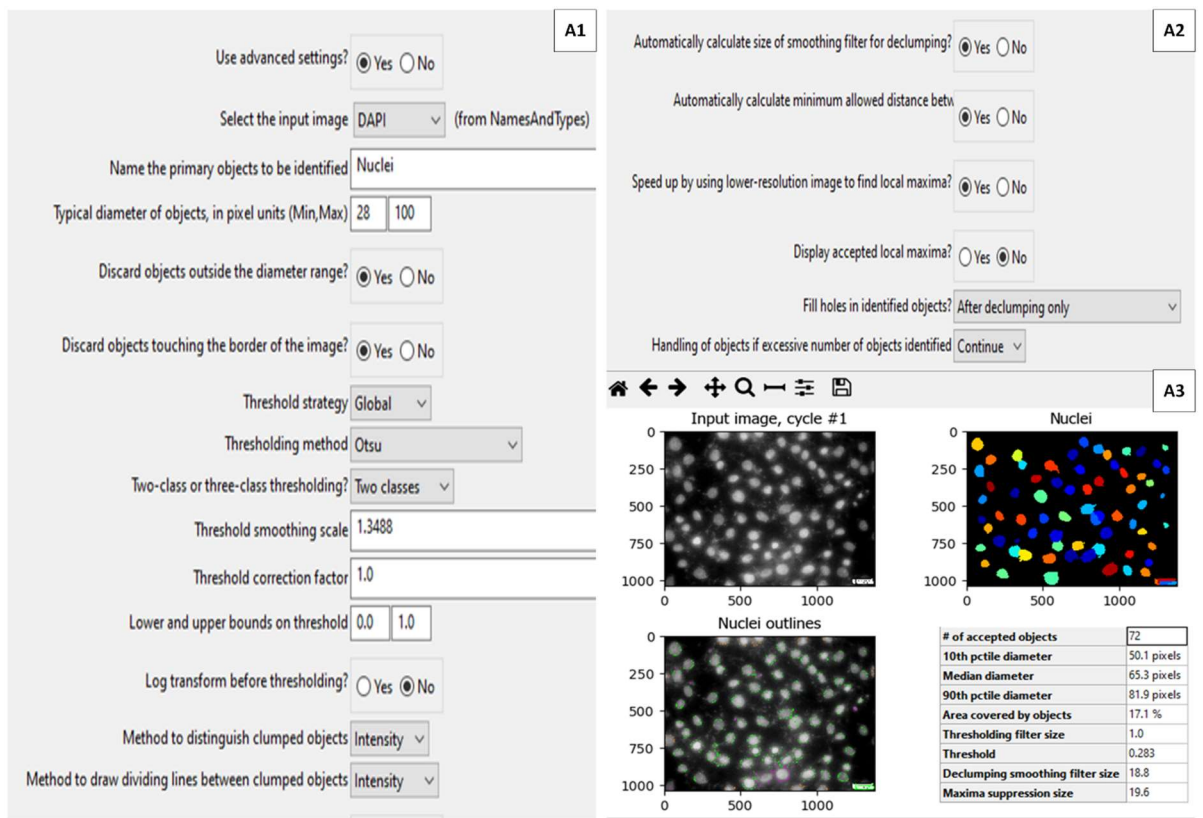


Figure 3: Representative images of cell profiler pipeline parameters. A1 and A2: Parameters of pipeline for the primary objective (nuclei). A3: Output of Nuclei being assessed.(Gallant, 2024)

2.6.6 Statistical Analysis

Data from CellProlifer was managed in Microsoft Excel and analysed using Statistica v23 software. Per cell data (Mean fluorescence intensity) was used in subsequent tests. As the number of samples ranged between 5 to 100, randomization was done to ensure a consistent sample size for each group. To test the normality of the data the Shapiro-Wilk test was used. The non-parametric Kruskal Wallis by Ranks ANOVA test, with a post-hoc, was used to determine the variation of MFI for each expression of ER α , ER β , PR and Ki67 in each population and treatment group per cell line. The Mann-Whitney U Test was performed based on comparing two matched independent groups. Significance was set at $p<0.05$.

Chapter 3: Results

3.1 Modified Essential 8 Media: iron salt optimization

Iron salts (ferrous sulphate and ferric nitrate) were tested for their efficacy as substitutes for ferritin. These iron salts were tested at varying concentrations (0.5 µg/ml, 5 µg/ml and 10 µg/ml) in DMEM/F12 culture media, and their effect on cell growth was as compared to cells grown in complete culture media (DMEM + 10% FBS). Cell growth was monitored daily for six days, and at each time point, cells were counted using the trypan blue dye, which stains cells with compromised cell membranes and allows for the enumeration of live cells with intact cell membranes (Chapter 2, section 2.2.3).

3.1.1 Ferrous sulphate (FeSO₄) induce growth in breast cancer cell lines (MCF7 and T47D)

Supplementation of FeSO₄ did not adversely affect cell growth in MCF7 and T47D cells (**Fig 4.1**). Each cell line generally followed a similar growth pattern in response to the different growth media. In MCF7 cells, lower cell counts were recorded during days 1-3 compared to days 4-6, indicating an increase in cell number (**Fig 4.1 A1-3**). In T47D cells, lower cell counts were recorded on day 1, and a steady increase between day 2-4 was observed; thereafter, cell counts generally decreased with relatively lower cell counts recorded on day 6 (**Fig 4.1 B1-3**). Higher cell counts were recorded in cells grown in DMEM with 10% FBS compared to cells grown in DMEM/F12 media, with or without FeSO₄ supplementation. The cell count was moderately higher with increasing concentrations of FeSO₄ in media; however, a similar growth pattern was observed at all concentrations except for MCF7 cells grown in 5 µg/ml of FeSO₄, which peaked earlier (at day 2) and higher cell counts were noted by day 6 (**Fig 4.2 A2**). Similarly, in T47D cells, the most variance was observed on day 2-3, with cells grown in 5 µg/ml of FeSO₄ at day 3 showing the highest cell count (**Fig 4.2, B2**). In MCF7 cells, days 1 and 2 showed the most uniformity among the different growth media, thus suggesting minimal differences at this time point. In T47D cells, uniformity was observed at later time points on days 5 and 6, which may indicate a plateau phase in the cell growth period, possibly due to contact inhibition. When images were assessed, normal cell morphology was observed; with a uniform cell monolayer, which suggests that cell numbers were increasing daily, thus indicating that cell growth was not impeded (**Figure 4.2 , Appendix V D**). These findings suggest that FeSO₄ supplementation did not hinder cell growth in relation to complete growth media.

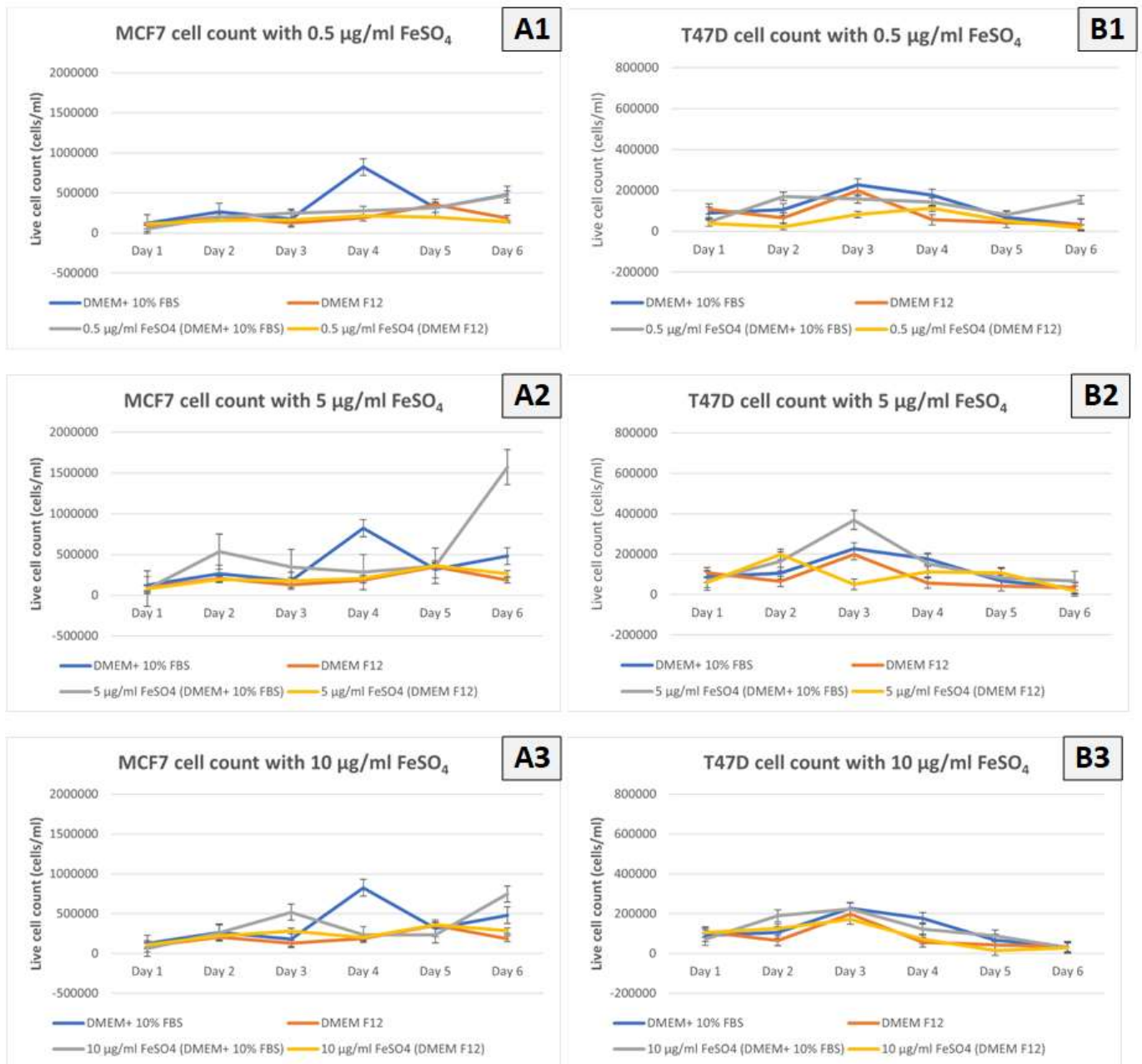


Figure 4.1: The effects of iron sulphate (FeSO₄) on breast cancer cell lines (MCF7 and T47D) in culture media (DMEM with 10% FBS and DMEM/F12). Cells were cultured with varying concentrations of FeSO₄; 0.5 µg/ml (A1 & B1), 5 µg/ml (A2 & B2) and 10 µg/ml (A3 & B3). Cell growth was monitored daily by counting cells using the trypan blue dye to exclude dead cells and enumerate live cells. Overall, a steady cell growth was maintained over the six days,

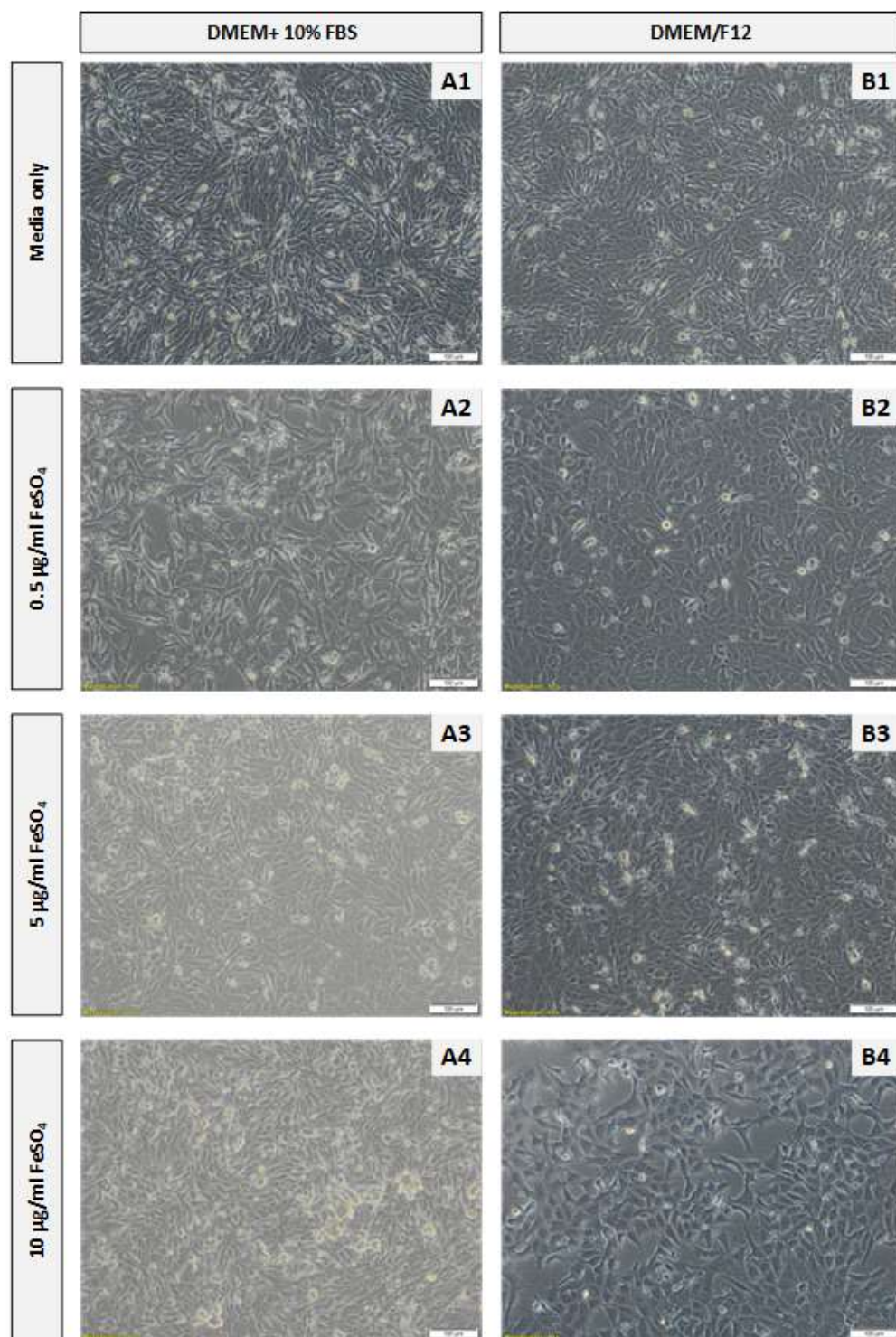


Figure 4.2: The effect of Fe SO_4 on MCF7 morphology. Micrograph showing MCF7 cell line (magnification x20, 500 μm), with different concentrations of FeSO_4 at day 2 MCF7 cells grown in 5 $\mu\text{g/ml}$ of FeSO_4 , peaked earlier (at day 2)

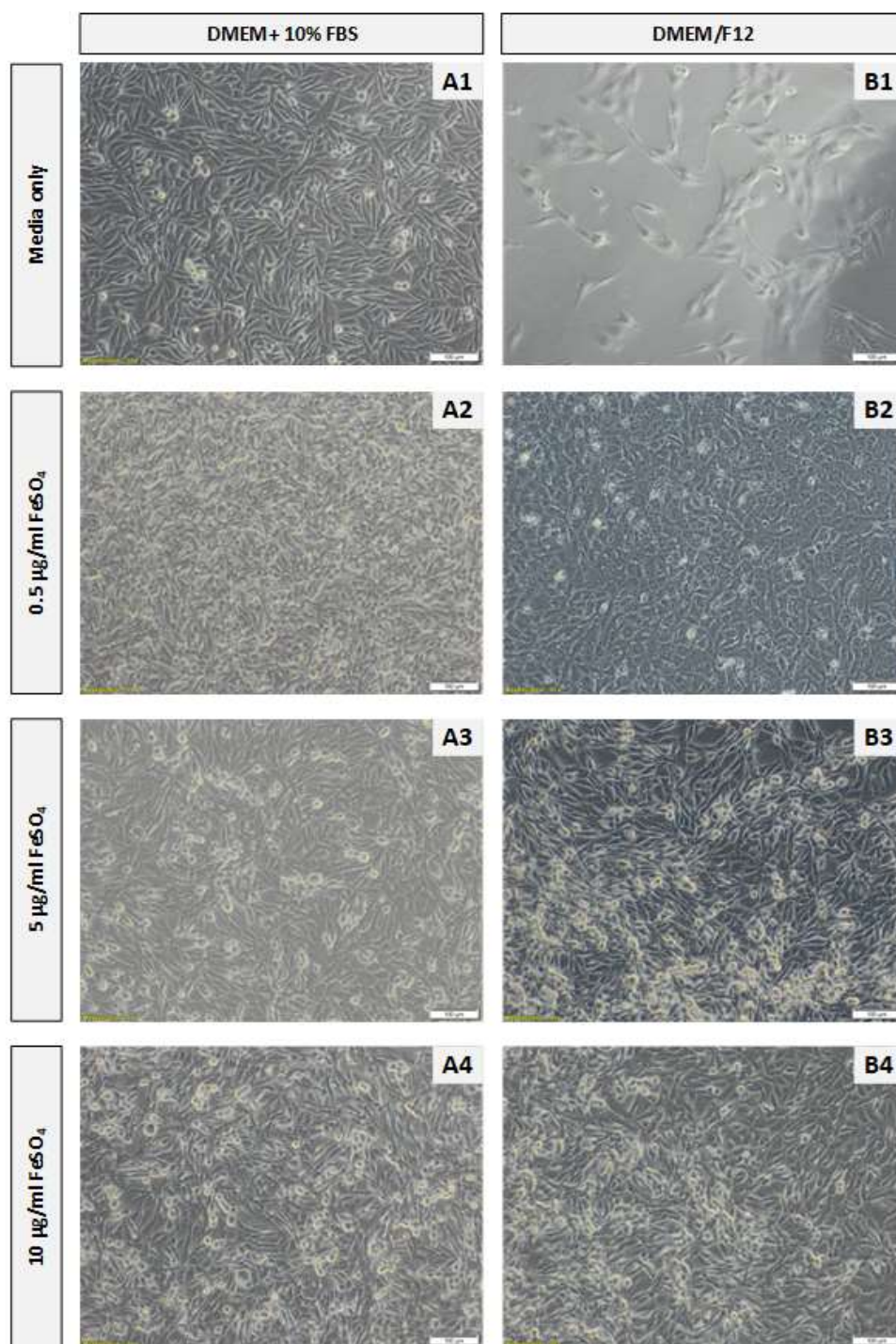


Figure 4.3: Effect of FeSo4 on T47D morphology. Micrograph showing T47D cells (magnification x20, 500 μm), treated with different concentrations of ferrous sulphate (FeSO_4) on day 2. T47D cells grown in 5 $\mu\text{g/ml}$ of FeSO_4 , peaked earlier (at day 2).

3.1.2 Ferric nitrate [Fe (NO₃)₃] induced growth in breast cancer cell lines (MCF7 and T47D)

Similar to FeSO₄ supplementation, Fe (NO₃)₃ did not adversely affect cell growth in MCF7 and T47D cells with each cell line generally showing a similar pattern of growth in response to the different media (**Fig 4.4**). In MCF7 cells, lower cell counts were recorded during days 1-3 compared to days 4-6, indicating an increase in cell number (**Fig 4.4** A1-3). In T47D cells lower cell counts were recorded on days 1-2, and the highest cell counts were recorded on day 3, followed by a decrease from 4-5 (**Fig 4.4**, B1-3). MCF7 cells showed a uniform response to the different growth media, with the most variation observed on day 4, whereby cells grown in DMEM with 10% FBS showed the highest cell count (**Fig 4.4**, A1-3). In addition, on day 6, cells grown in DMEM with 10% supplemented with Fe (NO₃)₃ at all concentrations showed high cell counts, this finding may be attributed to the effects of FBS which promoted faster levels of growth rate. In T47D cells, uniformity in cell counts across the different days was observed between cells grown in media containing 0.5 µg/ml Fe (NO₃)₃ and 5 µg/ml Fe (NO₃)₃. 10 µg/ml Fe (NO₃)₃ in DMEM with 10% FBS increased cell counts between days 2-3, suggesting higher levels of cell growth (**Fig 4.4**). In contrast, a decrease in cell counts was observed in T47D cells grown in 10 µg/ml Fe (NO₃)₃ in DMEM/F12 at day 3, then peaked thereafter. However, by day 6 all media showed similar levels of cell counts. Cell morphology indicated normal cell shape (polyhedral and spindle-shaped cells) with a uniform cell monolayer which suggests that cell numbers were increasing daily thus indicating that cell growth was not impeded (**Fig 4**, **Fig 5**, **Appendix V D**). These findings indicate that Fe (NO₃)₃ supplementation did not hinder cell growth in relation to complete growth media.

3.1.3 Optimal concentration and iron of choice

Overall, there were minimal differences in cellular response to Ferric nitrate and ferrous sulphate, indicating that either salt could be used to substitute ferritin in the modified E8 media; however, ferric nitrate was chosen as the optimal iron salt due to its stability in culture over prolonged period. Ferrous sulphate, while it did not adversely affect cell growth, has a tendency to oxidise which may become harmful to cultures when exposed for a prolonged period (Arigony et., 2013). Overall minimal differences were observed between the different concentrations tested for Ferric nitrate; therefore, 10 µg/ml was chosen as the optimal concentration because it is equivalent to the ferritin concentration used in E8 media (Chen et al., 2020). Considering that culture media would be changed every 48 hrs, this concentration (10 µg/ml) of ferric nitrate showed the most uniform effect on cell count on day 2 for MCF7 cells across the different media used, although some variance was observed with T47D cells at day 2, this concentration of ferric nitrate indicated elevated cell growth (**Fig 4.4**).

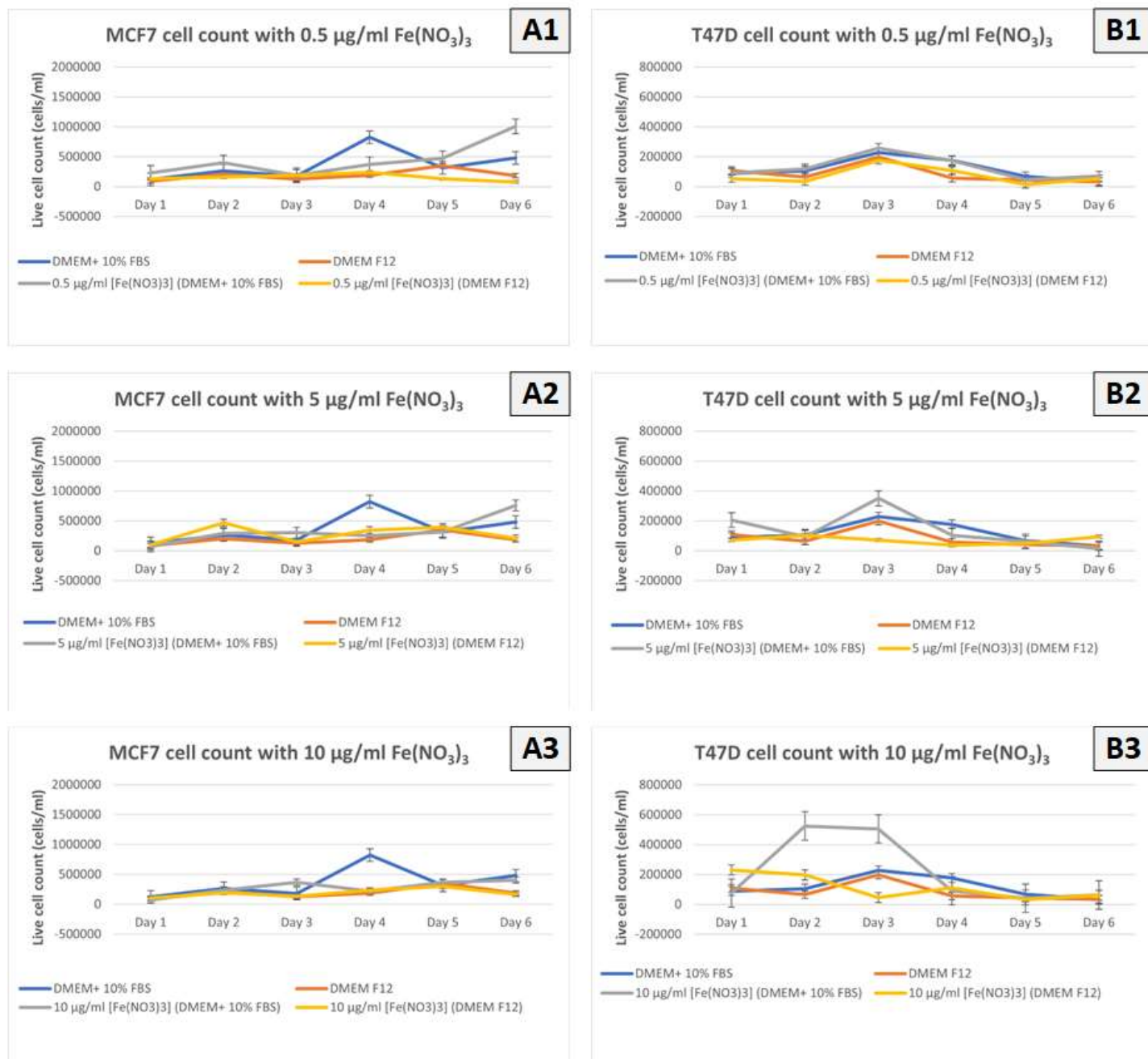


Figure 4.4: The effects of ferric nitrate $[\text{Fe}(\text{NO}_3)_3]$ on breast cancer cell lines (MCF7 and T47D). The effects of $[\text{Fe}(\text{NO}_3)_3]$ on breast cancer cell lines (MCF7 and T47D) in culture media (DMEM with 10% FBS and DMEM/F12). Cells were cultured with varying concentrations of FeSO_4 ; 0.5 µg/ml (A1 & B1), 5 µg/ml (A2 & B2) and 10 µg/ml (A3 & B3). Cell growth was monitored daily by counting cells using the trypan blue dye to exclude dead cells and enumerate live cells. Overall, a steady cell growth was maintained over the six days.

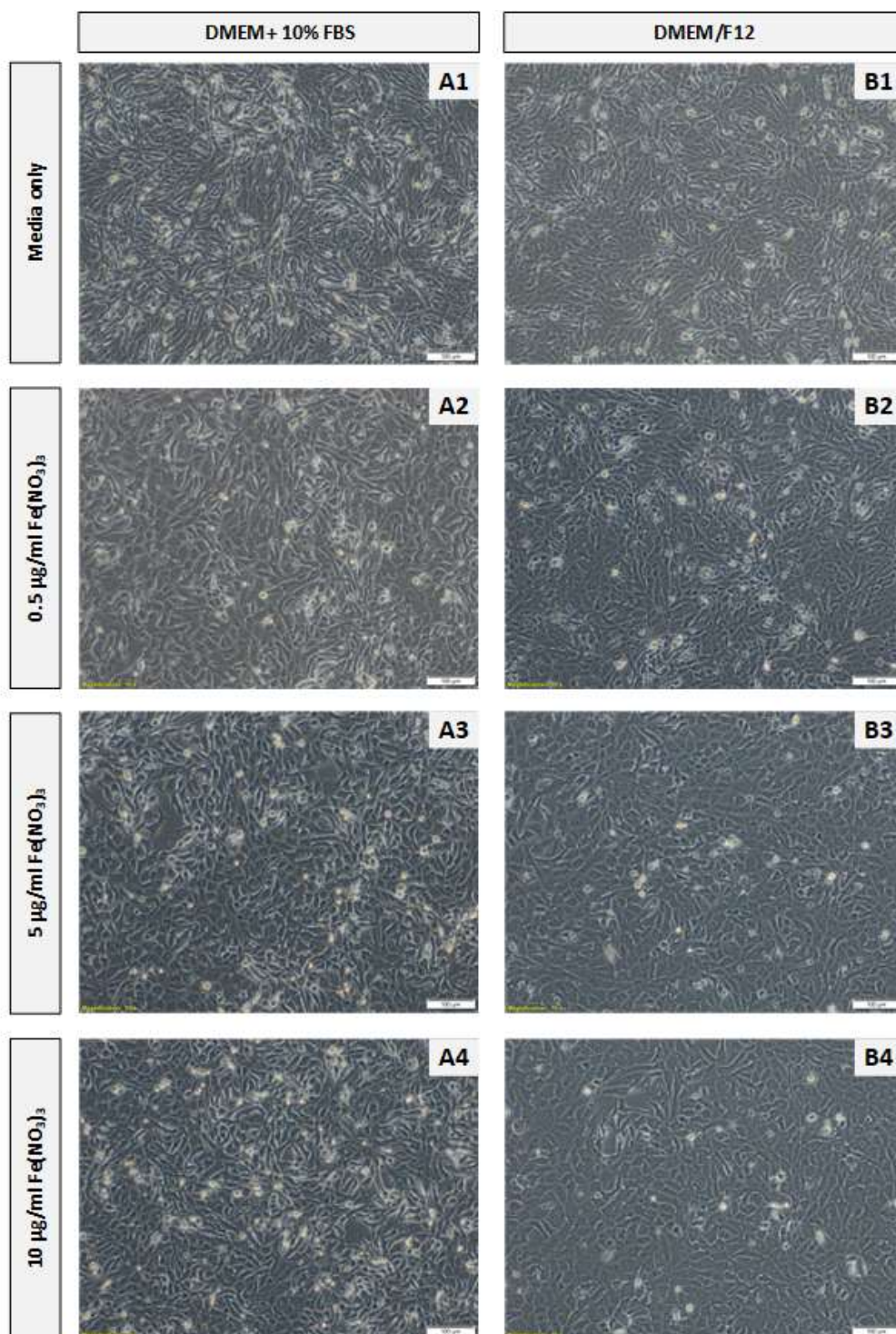


Figure 4.5: The effect of $[\text{Fe}(\text{NO}_3)_3]$ on MCF7 morphology. Micrograph showing MCF7 cell line (magnification x20, 500 μm), at different concentrations of $[\text{Fe}(\text{NO}_3)_3]$ on day 2.

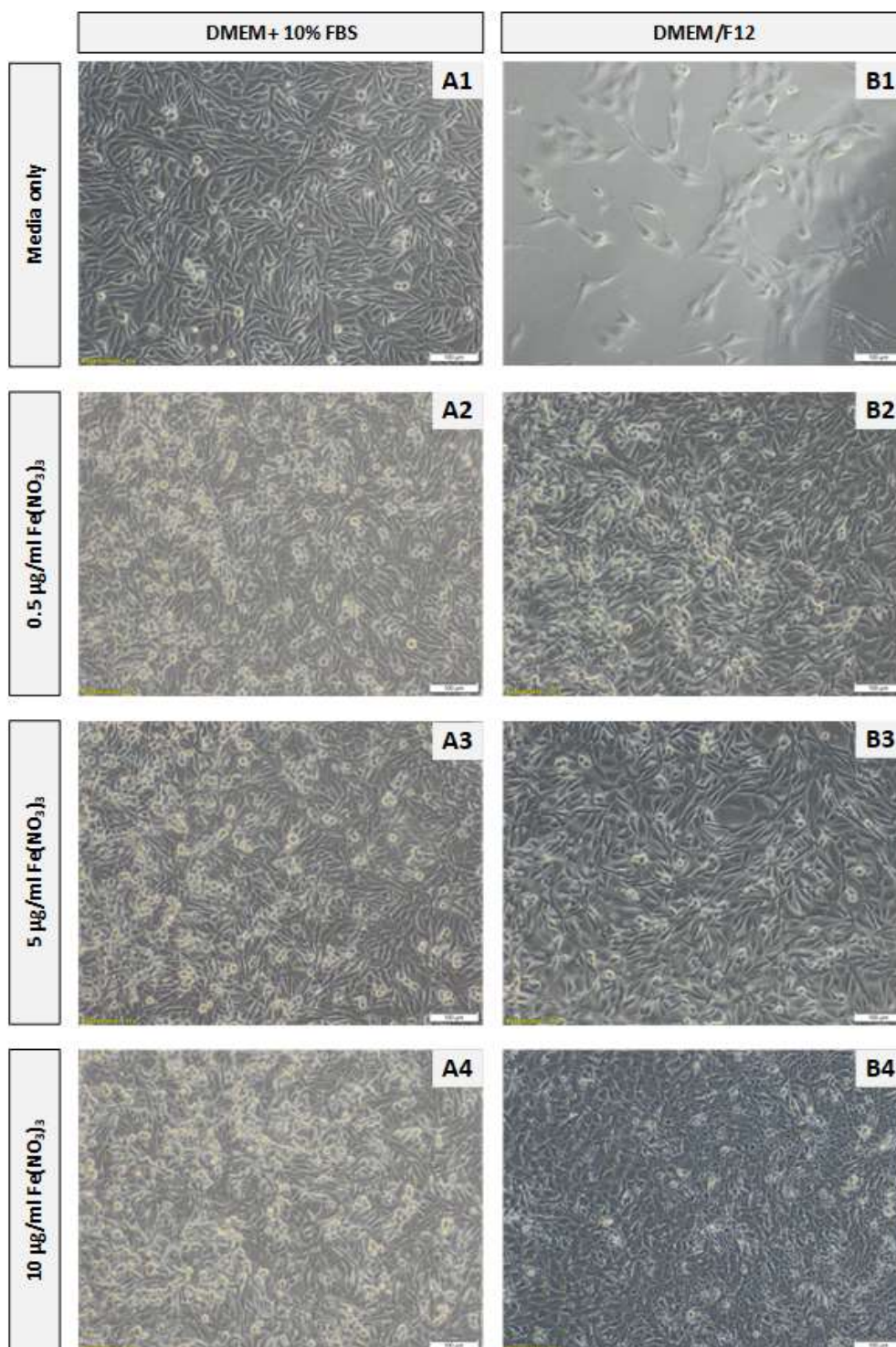


Figure 4.6: The effect of $[\text{Fe}(\text{NO}_3)_3]$ on T47D morphology. Micrograph showing T47D cell line (magnification x20, 500 μm), at different concentrations of $[\text{Fe}(\text{NO}_3)_3]$ on day 2. 10 $\mu\text{g/ml}$ $\text{Fe}(\text{NO}_3)_3$ in DMEM with 10% FBS increased cell counts between day 2-3, suggesting higher levels of cell growth.

3.2 Cell growth of magnetically sorted cell subpopulations based on CD44 and CD133

3.2.1 Parent populations (P0)

The P0 population of both cell lines showed a gradual increase in cell growth over the six days period, as the number of cells counted increased at each time point (**Fig 5.1**). MCF7 cells had a higher fold change at day 3 (9.4) and day 6 (4.3) suggesting that cell growth was occurring faster in these cells compared to T47D cells (**Fig 5.1**). Cell morphology supported these findings and indicated an increase in cell density at each time point (day 1-6) and higher cell density was observed in MCF7 compared to T47D cells (**Fig 5.2 & 5.3**). Cell clumps were observed between day 5 and day 6 as cells reached confluency (**Fig 5.2 & 5.3**).

3.2.2 CD44⁺ and CD133⁺ population (P1)

The P1 population showed a gradual increase in cell growth over the 6 days in both cell lines (**Fig 5.1, A & B**). The P1 subpopulation in MCF7 cells, showed a lower fold change compared to T47D cells at days 3 and 6, suggesting that cell growth in MCF7 P1 was slower than in T47D P1. Although the parent population for T47D cells displayed a slower growth rate compared to that of MCF7 cells, stemlike subpopulations of T47D cells followed a different trend. Cell morphology supported these findings and a rise in cell density was revealed at each time point (day 1-6) (**Fig 5.2 5.3**). Cell clumps were observed in MCF7 P1 on days 5 and 6 (similar to P0); however, these cell clumps were not observed in T47D P1, instead these cells expanded in cell monolayers (**Fig 5.2 & 5.3**).

3.2.3 CD44⁺CD133⁻ population (P2)

The P2 population showed a gradual increase in cell growth over the 6 day period for MCF7 cells; however, in T47D cells, cell growth was observed between day 3 and day 6 (**Fig 5.2**). The fold change in MCF7 P2 was higher between days 1-3 (3.3) but reduced between days 3-6 (2.9), thus suggesting that this population reached a log phase of cell growth indicated by rapid increase in cell numbers in earlier time periods then cell growth rate slowed down at later timepoints (**Fig 5.1**). T47D P2 showed a lower fold change between days 1-3 (1.1) suggesting that the rate of cell growth was slow and steady, a higher fold change was seen between days 3-6 (3.3) suggesting that cells were now growing at a faster rate (**Fig 5.1**). Cell morphology indicated an increase in cell density at each time point for each cell line; however, MCF7 cells displayed higher cell density and an affinity for clumping than T47D cells (**Fig 5.2 & 5.3**).

3.2.4 CD44⁻CD133⁺ population (P3)

The P3 population showed a gradual increase in cell growth over the 6 days in MCF7 cells and the fold change followed a similar pattern to that of MCF7 P2 showing that cell growth

rate was higher during days 1-3 and reduced during days 3-6 (**Fig 5.1**). Similarly, T47D P3 also followed a similar growth pattern to T47D P2; however, the fold change was slightly higher (1.3 between days 1-3 and 4.3 between days 4-6) suggesting that T47D P3 grew at a faster rate than T47D P2 (**Fig 5.1**). Cell morphology showed an increase in cell density at each time point in both cell lines (MCF7 P3 and T47D P3) (**Fig 5.2 & 5.3**). Large cell clumps were observed in MCF7 P3 and smaller cell clusters were observed in T47D P3 (**Fig 5.2 & 5.3**).

3.2.5 CD44⁺CD133⁻ population (P4)

Cell growth in MCF7 P4 followed a similar pattern at other subpopulations (P1-3); however, the fold change was higher (5.9) in this population (P4) between day 1-3 compared to that seen in P1-3 (P1=2.4, P2=3.3, P3=3.8) for the same time-period. This was followed by a reduced fold change (2.6) between day 3-6, similar to P1-3. This finding suggests that this population (MCF7 P4) grew at a faster rate compared to the other subpopulations and further cell growth at later time points may have been limited by cells reaching confluency in the growth area and a fast uptake of nutrients in the media thus they were depleted sooner (**Fig 5.1**). T47D P4 showed a similar growth pattern to other subpopulations (T47D P1-3; however, it displayed a higher growth rate between day 3-6 as indicated by a high fold change (8.4) (**Fig 5.1**). The cell morphology findings support these findings and show an increase in cell density, cell clumping and monolayers displayed (**Fig 5.2 & 5.3**).

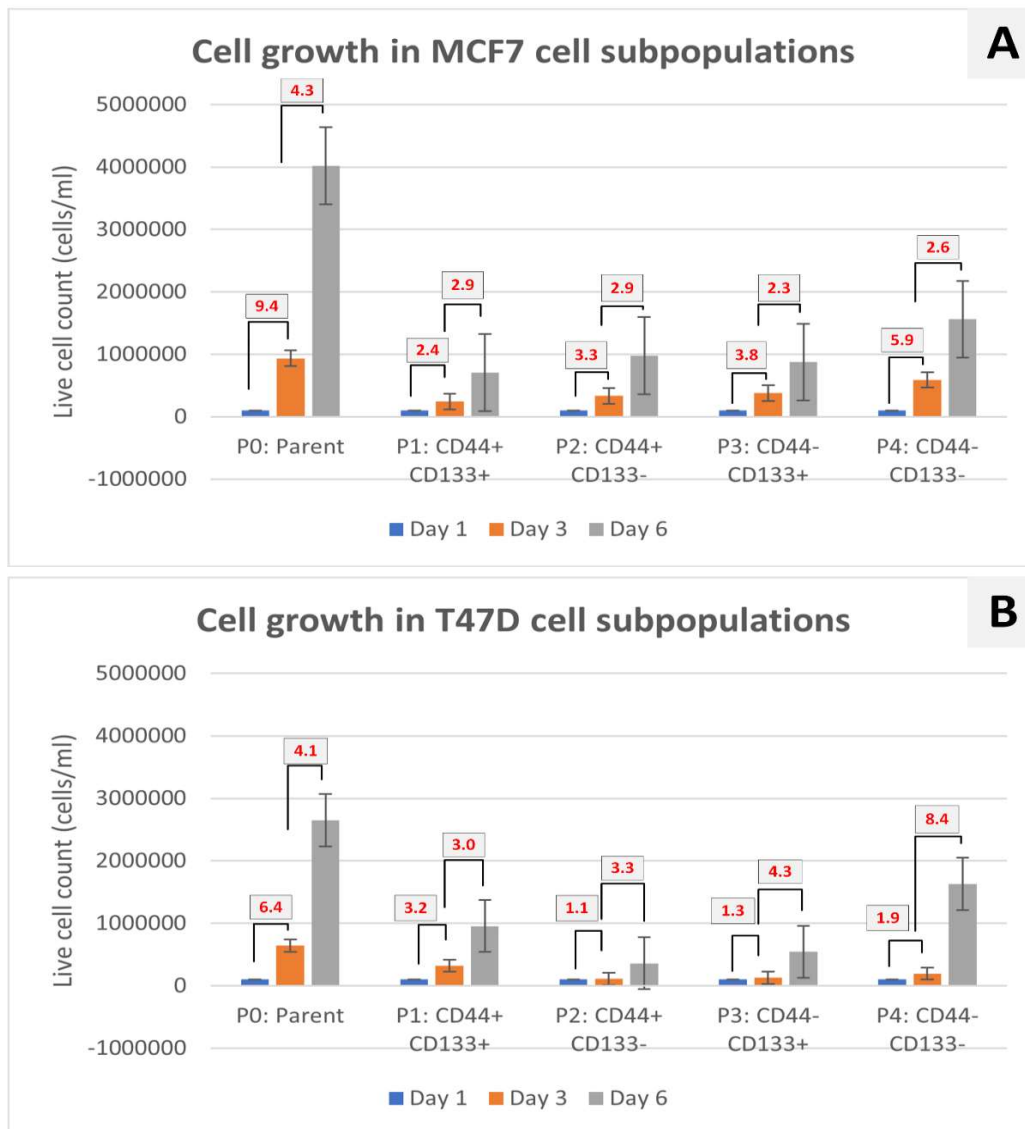


Figure 5.1: Bar graphs representing cell growth in MCF7 and T47D cell subpopulations over 6 days. MCF7 cell had a higher fold change at day 3 (9.4) and day 6 (4.3) suggesting that cell growth was occurring faster in this cell line compared to T47D cells in P0 (Parent).

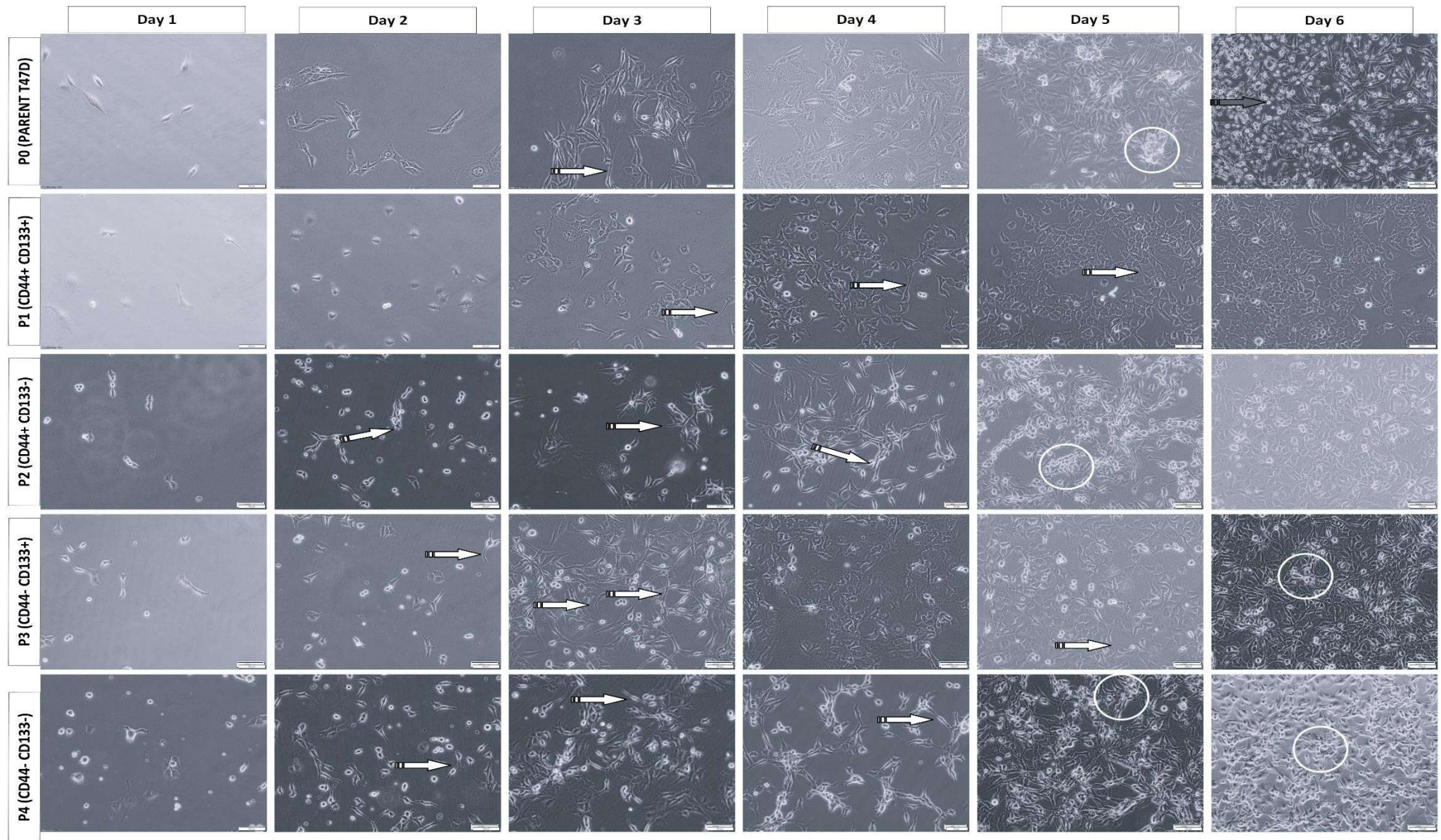


Figure 5.2: Photomicrographs showing T47D cell line (magnification X20, 500 μm), key: Grey arrow: blebbing, white arrow – filopodia, White circle-clumping. All populations grown over a period of 6 days. Increased filopodia indicates to cells potential to form more clustering structures, which could indicate to its metastatic qualities.

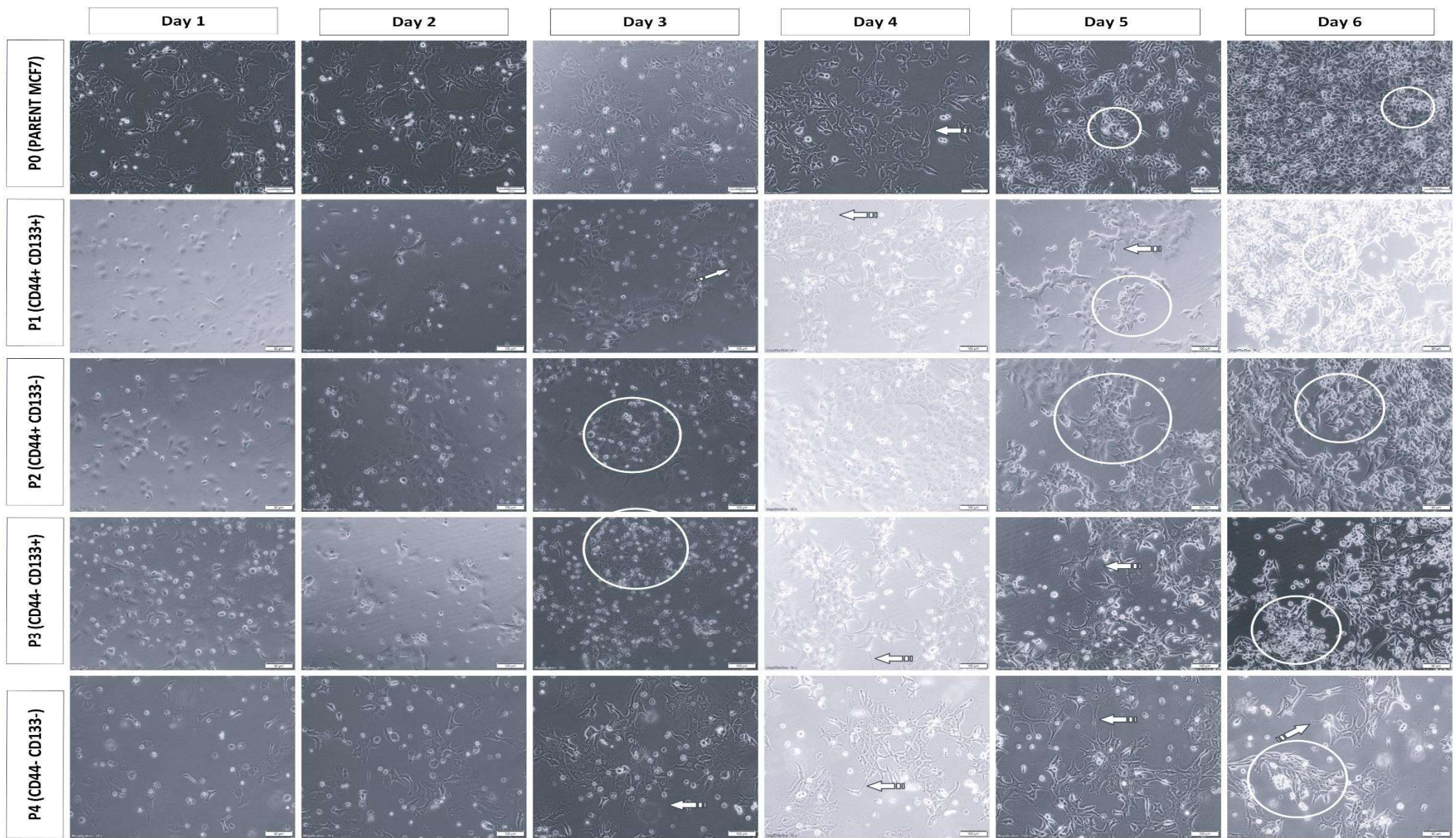


Figure 5.3: Photomicrographs showing MCF7 cell line (magnification X20, 500 μ m), key: white arrow – filopodia, White circle- clumping. All populations grown over a period of 6 days. Increased filopodia indicates to cells potential to form more clustering structures which could indicate to its metastatic qualities.

3.3 Verification of stemness phenotype with CD44, CD24 and CD133 expression using flow cytometry

3.3.1 Verification overview

The expression of stemness phenotype was investigated in cells following magnetic cell sorting and propagation of cell subpopulations. MCF7 cell subpopulations were cultured for 5 passages, whereas T47D cell subpopulations were cultured for 10 passages after sorting. Stemness is typically associated with the following phenotype $CD44^+ CD133^+ CD24^-$, as such, the presence and level of these markers (CD44, CD133 and CD24) were assessed in subpopulations. The main aim of this experiment was to determine whether the stemness phenotype was maintained after cells were magnetically sorted and subsequently cultured in modified essential 8 media. The parent population (P0) was also cultured in modified essential 8 media and used as a baseline to compare changes occurring in subpopulations. The number of events expressing each stemness marker was recorded (**Fig 6.1**, A1 & B1). Then the geometric mean fluorescence intensity (gMFI) for each marker was represented (**Fig 6.1**, A2 & B2). Overall, there was a discrepancy between the number of events and gMFI, indicating the need to assess these parameters dependently.

3.3.2 CD44, CD133 and CD24 expression within MCF7 cells

The number of events expressing CD44 was high in MCF7 P0 compared to all the subpopulations (P1-P4) in MCF7 cells (**Fig 6.1**, A1). In contrast, the gMFI for CD44 expression was generally low in parent and subpopulations; however, levels were slightly elevated in P1-P3 compared to P0 and P4 (**Fig 6.1**, A2). This finding suggests that although more MCF7 cells expressed CD44 in P0 and P4, these cells had very low levels of CD44 as indicated by a lower gMFI (**Fig 6.1**, A2). In contrast, MCF7 P1-P3 had a lower number of cells expressing CD44; however, the concentration of CD44 in these cells was slightly elevated compared to P0 and P4 (**Fig 6.1**, A2). The number of cells expressing CD133 was higher in parent (P0) MCF7 compared to subpopulations (P1-P4); however, CD133 expression in subpopulations was detected. The gMFI for CD133 was high in all populations, suggesting that there was a strong expression of this marker (**Fig 6.1**, A2). In addition, fewer cells for all populations expressed CD24 as indicated by a low number of events (**Fig 6.1**, A1). The gMFI for CD24 was very low for P0 and P2 suggesting that this marker was downregulated in these cells. Elevated gMFI for CD24 was observed in P1, P3 and P4 suggesting that CD24 was highly expressed in the few cells that expressed it. In summary, the

MCF7 cells maintained stemness phenotype in the modified essential 8 media for a prolonged time in culture, mainly through high expression of CD133⁺⁺ and low expression of CD44⁺.

3.3.3 CD44, CD133 and CD24 expression within T47D cells

The number of events expressing CD44 was generally higher in the T47D cell parent (P0) and subpopulations (P1-P4) than in MCF7 cells (**Fig 6.1**, A1-B1). In addition, the number of cells expressing CD44 was higher than those expressing CD133 and CD24 in T47D cells. P2 T47D cells had the highest number of cells expressing CD44, and the expression level in those cells was high as indicated by a relatively high gMFI for CD44 (**Fig 6.1**, B2). The number of cells expressing CD44 in P0, P1, P3 and P4 were comparable; however, P0 and P4 showed higher expression levels than P1 and P3 suggesting that although CD44 was detected in some cells within P1 and P3, its expression was very low in these populations (**Fig 6.1**, B2). The number of events expressing CD133 was generally low in P0 and P1, with even fewer cells expressing CD133 in P2. Expression of CD133 was undetected in P4. In contrast, a higher number of cells were expressing CD133 in P3 (**Fig 6.1**, B1). The expression intensity of CD133 was high in P0 and P1, despite fewer cells expressing this marker. P2 T47D cells showed the highest fluorescence intensity of CD133, despite, fewer cells expressing this marker, thus suggesting very high expression in these cells. In contrast, P3 showed low fluorescence intensity of CD133, despite the higher number of cells expressing this marker, thus suggesting a low to moderate expression in these cells (**Table 6**). Fluorescence intensity for CD133 was undetected in P4, suggesting the absence/loss of this marker. CD24 fluorescence intensity was high in P2 and P3 and moderate in P4, despite fewer cells detected expressing CD24 in these populations thus suggesting a concentrated expression of CD24 in these few cells. In summary, T47D cells maintained stemness phenotype in the modified essential 8 media through the expression of low levels of CD44 with low but concentrated expression of CD133. CD24 expression was even lower but highly concentrated in the few cells expressing it.

Table 6: Summary of verification results (gMFI) of stemness markers of each subpopulation

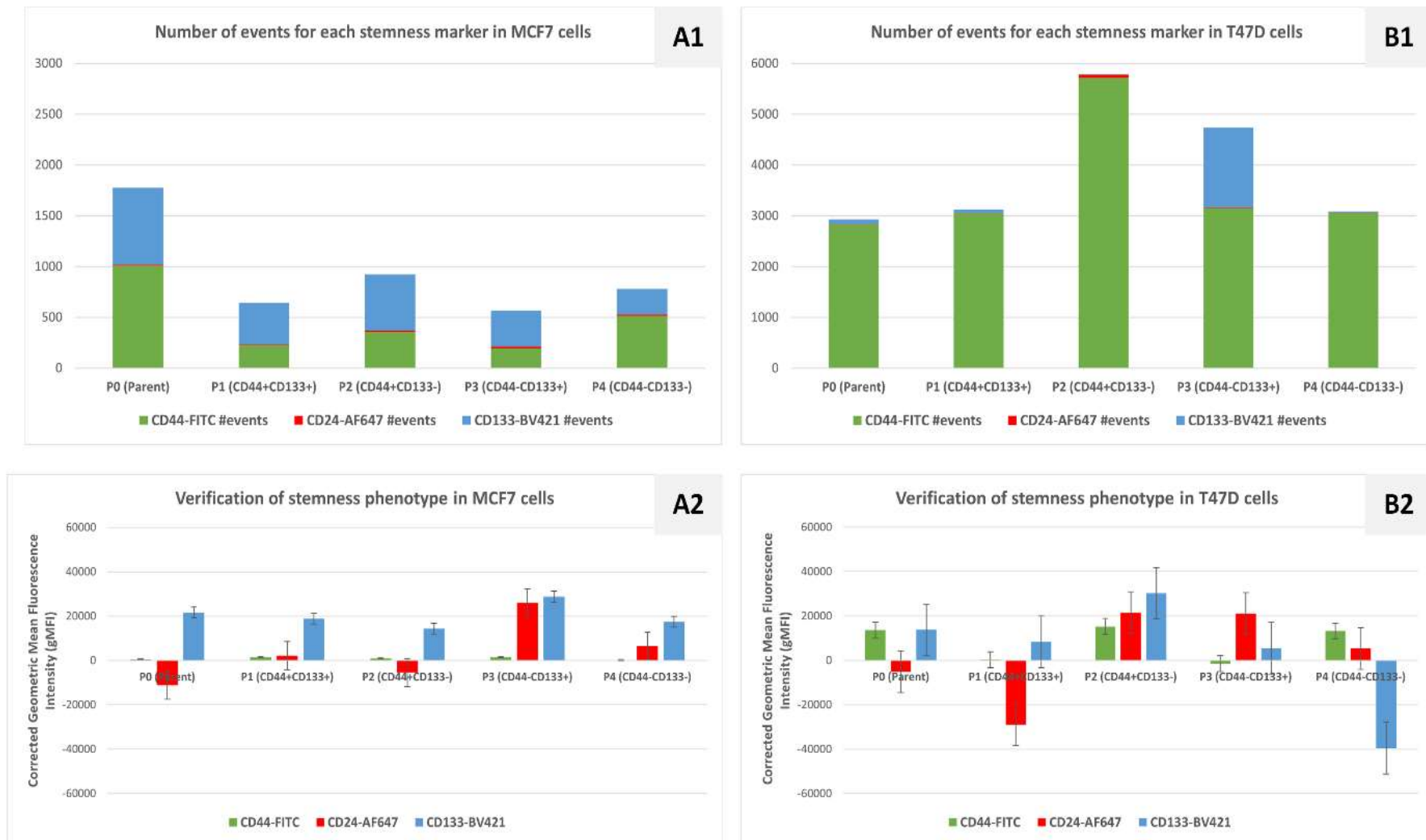


Figure 6.1: Bar graphs representing verified stemness in subpopulations of MCF7 and T47D cell lines. A1 and B1: Bar graph representing the number of events per stemness marker in MCF7 and T47D cell lines. A2 and B2: Histograms representing the verification of stemness phenotype in MCF7 and T47D subpopulation. An increase number of CD44 is seen in T47D cells in comparison to MCF7, particularly in P2. However showed greater expression of CD24 and CD133 once verified in P2

Population	Cell line	CD44 expression +=low, ++=moderate +++ = high	CD133 expression	CD24 expression
P0	MCF7 T47D	CD44 ⁺ CD44 ⁺⁺	CD133 ⁺⁺ CD133 ⁺	CD24 ⁻ CD24 ⁻
P1	MCF7 T47D	CD44 ⁺ CD44 ⁺	CD133 ⁺⁺ CD133 ⁺	CD24 ⁺ CD24 ⁻
P2	MCF7 T47D	CD44 ⁺ CD44 ⁺⁺	CD133 ⁺ CD133 ⁺⁺⁺	CD24 ⁻ CD24 ⁺⁺
P3	MCF7 T47D	CD44 ⁺ CD44 ⁺	CD133 ⁺⁺⁺ CD133 ⁺	CD24 ⁺⁺⁺ CD24 ⁺⁺
P4	MCF7 T47D	CD44 ⁺ CD44 ⁺⁺	CD133 ⁺⁺ CD133 ⁻	CD24 ⁺⁺ CD24 ⁺

3.4 Optimisation of antibodies for immunocytochemistry

For each marker of interest (ER α , ER β , PR and Ki67) different dilutions of the primary antibody were used to establish the optimal concentration (Figure 7.1 and Appendix VI B 1-4). Negative controls showed no non-specific binding (Appendix V1 B1-4.)

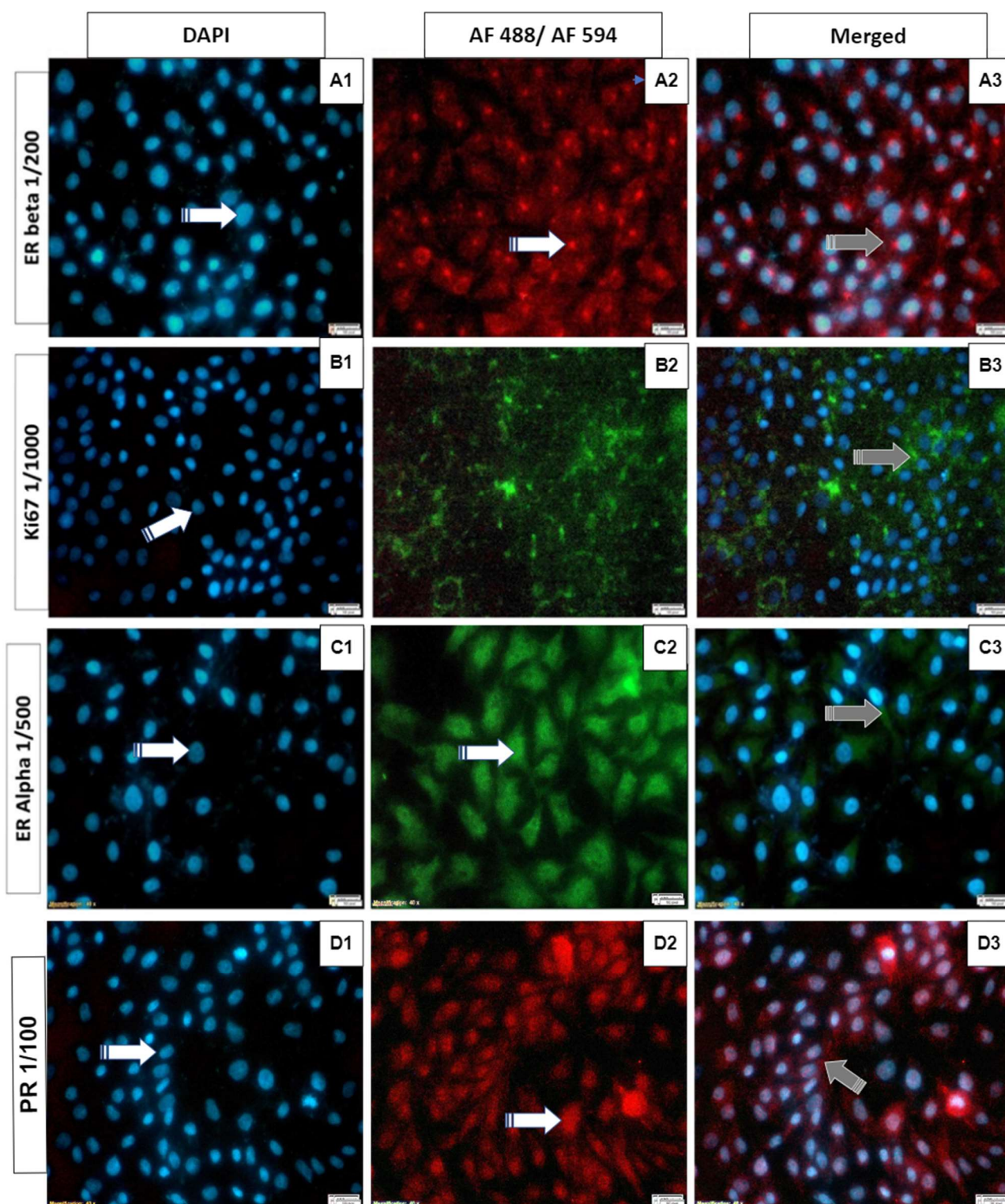


Figure 7.1: Antibody optimization. Optimal concentrations selected of primary antibodies for ER α , ER β , PR and Ki67: A1-D1 : DAPI, A2-D2 : Alexa flour 488/594, A3-D3: Merged images. White arrows indicating nuclear expression of receptors, grey arrows indicating cytoplasmic expression of receptors. PR reveals more expression in the cytoplasm of the cell. Ki67 expression is based on the stage of cell cycle.

3.5 Biomarker expression in breast cancer cell lines and subpopulations – the effects of hormone therapy

3.5.1 Alterations in the mean intensity of ER α and ER β MCF7 cells – the effects of hormone therapy

Untreated MCF7 cells revealed ER α and ER β expression primarily in the cytoplasm in the parent population P0 (Parent) (Appendix VI B and **Figure 7.2 A1**), with ER α dominantly expressed in comparison to ER β as seen by the yellow overlay (**Figure 7.2**). In derived populations, ER α expression was relatively similar in the nucleus and cytoplasm (**Figure 7.2**) as no significant changes were found ($p > 0.05$); however, overall ER β expression was altered greatly in certain populations. P1 (CD44⁺ CD133⁺) MCF7 cells revealed qualitatively bright nuclear ER α expression (**Figure 7.2**); with significantly reduced overall ER β expression ($p < 0.05$) (Appendix VI B) compared to the parent population (P0) (Figure 7.6). P2 (CD44⁺ CD133⁻) showed both markers concentrated in the cytoplasm (**Figure 7.2**) with no significant variation compared to the parent population. Both P3 and P4 revealed predominant expression of ER α and ER β in both the nucleus and perinuclear region (**Figure 7.2**). In addition, the subpopulations (P3 and P4) showed a greater expression of ER β in comparison to the P1 population (**Figure 7.2**) (Figure 7.6 A1, A2)). We investigated the impact of Anastrozole on MCF7 cells, no significant changes in either ER α or ER β were found in matched populations of the untreated group. However, it was found that the derived populations showed significant differences following treatment with Anastrozole. This was expected as the stemness markers could potentially interact with similar pathways that is initiated by Anastrozole. Specifically, in comparison to the parent population (P0) which showed cytoplasmic and bright nuclear expression of ER α and diffuse ER β expression (**Figure 7.2**), in P1 (CD44⁺ CD133⁺) Anastrozole treatment reduced the mean intensity of both biomarkers (ER α and ER β), ER β significantly so ($p < 0.05$) (Figure 7.6 A1, A2). In all other populations (P2 – P4), ER α and ER β mean intensity was higher than in P1, similar to levels seen in the parent population treated with Anastrozole, but lower than that of the untreated parent population (**Figure 7.2, Figure 7.6 A1, A2**).

When treated with Tamoxifen, MCF7 P0 (Parent) population revealed expression of ER α in the cytoplasm and perinuclear region (green-yellow) with low expression in the nucleus. On the other hand, ER β expression was seen to be dominant in the nucleus (orange). While not significant, the expression of both markers was lower than that of the untreated parent

population, rivalling that of the Anastrozole-treated parent population. Similar to all P1 (CD44⁺ CD133⁺) populations, whether untreated or treated with Anastrozole, ER α and ER β mean intensity reduced, and were mainly visualised in the cytoplasmic and nuclear regions, respectively (**Figure 7.2**). Similar to the trend seen in Anastrozole treatment, Tamoxifen treated P2 (CD44⁺ CD133⁻) and P3 (CD44⁻ CD133⁺) showed ER α and ER β mean intensity higher than in P1 (Figure 7.6 A1, A2). These expression levels were similar to that seen in the parent population (treated with Tamoxifen), but lower than that of untreated parent population (**Figure 7.6**). ER α expression was spread out across the cytoplasm as well as the nucleus showing a dominant co-localisation with ER β in some cells (yellow). In cells where ER β expression was dominant, a more orange colour was evident.

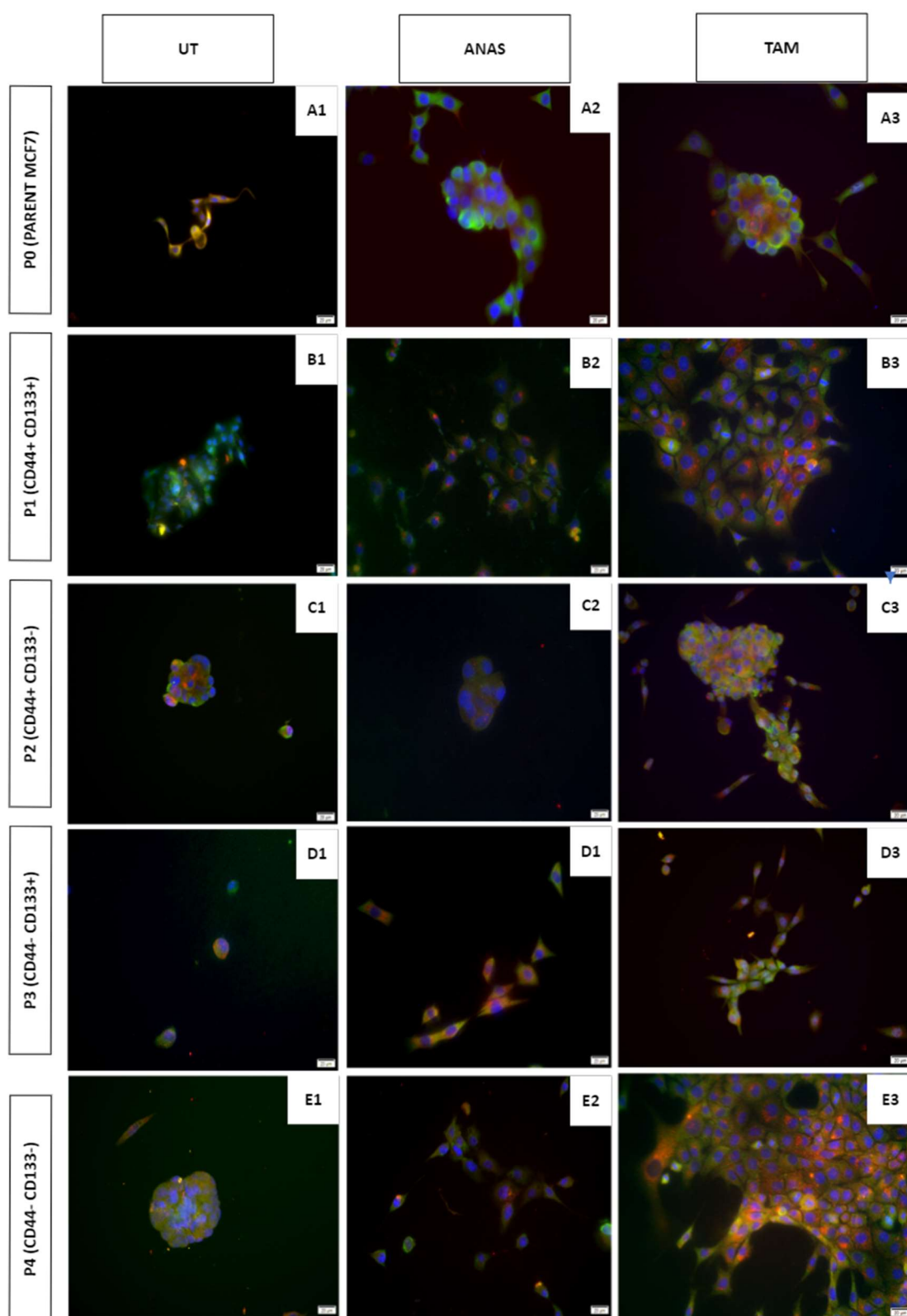


Figure 7.2. ER α (green) and ER β (red) expression in MCF7 cells. Nuclei are counterstained with DAPI (blue). Columns show untreated cells, Anastrozole and Tamoxifen treated cells. Rows indicate parent population and derived populations.

3.5.2 Alterations in the mean intensity of PR and Ki67 in MCF7 cells – the effects of hormone therapy

In untreated MCF7 cells, the P0 (Parent) showed low levels of Ki67 expression in the nucleus (Appendix VI B and **Figure 7.3**). This was similar across the populations, with the exception of P2 (CD44⁺ CD133⁻) which showed higher intensity Ki67 expression than the parent population. Notably, P4 (CD44⁻CD133⁻) had a significant reduction in Ki67 in comparison to P2, with only background evident (Figure 7.6 A3). PR expression was localised to the cytoplasm being of similar intensity in the P0 (Parent), P1 (CD44⁺ CD133⁺), and P3 (CD44⁻ CD133⁺) populations. In P2 (CD44⁺ CD133⁻), the mean intensity of PR was significantly higher than all populations (Figure 7.6 A4).

Under Anastrozole treatment, P0 MCF7 had low Ki67 nuclear expression and PR cytoplasmic expression was evident, with bright nuclear points noted. This trend was similar in P1 (CD44⁺ CD133⁺) and P2 (CD44⁺ CD133⁻) (Figure 7.6 A3). In P3 (CD44⁻ CD133⁺) few cells showed high intensity of Ki67 in the nucleus as opposed to the untreated parent (P0) population ($p < 0.05$) (Figure 7.6 A3). Similarly, in P3 (CD44⁻ CD133⁺), PR cytoplasmic expression was significantly higher than the Anastrozole-treated parent population, the untreated parent population (P0) and matched untreated P3 (CD44⁻ CD133⁺). While in P4 (CD44⁻ CD133⁻), Anastrozole treatment resulted in less PR and Ki67 expression, but the mean intensities remained higher than the treated parent population ($p < 0.05$), and more similar to the expression found in the untreated parent population.

Tamoxifen treatment resulted in no change in Ki67 or PR expression in the P0 (Parent) population compared to the untreated P0 (Parent) population; however, Tamoxifen treatment of P1 (CD44⁺CD133⁺), P2 (CD44⁺ CD133⁻) and P4 (CD44⁻ CD133⁻) showed a distinct reduction in Ki67 nuclear expression and PR cytoplasmic expression. Conversely, Tamoxifen treatment of P3 (CD44⁻ CD133⁺) induced significantly higher intensity expression of Ki67, compared to P2, with PR levels similar to the Tamoxifen-treated parent population (P0) (Figure 7.6 A3, A4).

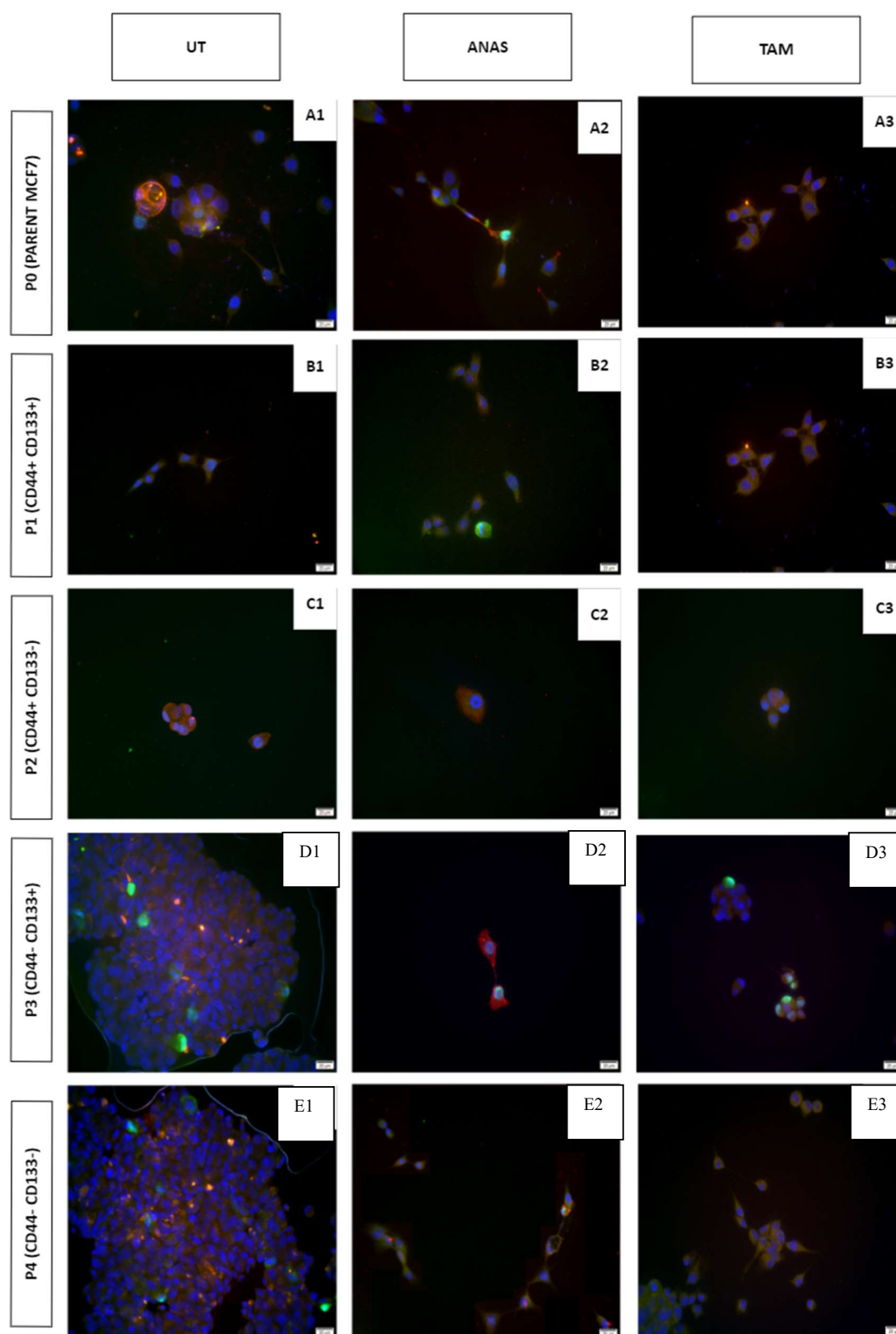


Figure 7.3: Ki67 (green) and PR (red) expression in MCF7 cells. Nuclei are counterstained with DAPI (blue). Columns show untreated cells, Anastrozole and Tamoxifen treated cells. Rows indicate parent population and derived populations.

3.5.3 Alterations in the mean intensity of ER α and ER β T47D cells – the effects of hormone therapy

The untreated parent population (P0) of T47D cells revealed similar mean intensity of overall ER α immunofluorescence, visually presenting as localised in the nucleus with less expression in the cytoplasm, and ER β , was concentrated in the nucleus (Figure 7.4 and 7.6). Despite similar levels of fluorescence intensity P1 (CD44⁺ CD133⁺), P2(CD44⁺CD133⁻) and P3(CD44⁻CD133⁺) populations showed primarily nuclear ER α and ER β , with P2 (CD44⁺CD133⁻) and P3(CD44⁻CD133⁺) showing a non-significant increase in ER α mean intensity compared to the parent (P0) and P1 population. Moreover, in comparison to the parent population (P0) for untreated samples, P1(CD44⁺ CD133⁺), P2 (CD44⁺CD133⁻) and P3(CD44⁻CD133⁺) showed ER β expression higher than ER α expression (**Figure 7.4**) and was visualised as orange (Appendix VI B). For population P4 (CD44⁻ CD133⁻) of the untreated sample, ER α immunofluorescence was found mainly in the cytoplasm, at the periphery whereas ER β immunofluorescence was more dispersed, being greater in some cells (orange fluorescence, **Figure 7.4**). Overall mean intensity of ER α in P4 (CD44⁻ CD133⁻) was significantly reduced ($p < 0.05$) compared to P1 (CD44⁺ CD133⁺) and P3 (CD44⁻ CD133⁺) (**Figure 7.4**), and ER β was also reduced, although not significantly (Figure 7.6 A5, A6).

The T47D P0 parent population treated with Anastrozole revealed immunofluorescence of ER α and ER β primarily in the nucleus, with no significant alterations compared to the untreated P0 parent population. Under Anastrozole treatment, all populations (P1 – P4) showed ER α to be dominant in the nucleus with little expression in the cytoplasm. P3 (CD44⁻ CD133⁺) showed significantly higher ER α expression than the Anastrozole treated parent (P0) population. Moreover, in P4 (CD44⁻ CD133⁻), Anastrozole treatment induced a significant increase in ER β compared to the P1(CD44⁺CD133⁺) population, visualised as orange.

Tamoxifen treatment of the P0 (Parent) again showed ER α and ER β immunofluorescence exhibited mostly in the nucleus than in the cytoplasm, with no significant variance to the untreated parent population. Qualitatively, ER α expression dominated the nucleus, seen as yellow-green. In all derived populations, expression of these markers remained primarily nuclear, with the exception of P4 (CD44⁻ CD133⁻), in which cytoplasmic evidence was found. Overall ER α mean intensity in P4 (CD44⁻ CD133⁻) was however, significantly reduced ($p < 0.05$) compared to P3(CD44⁻ CD133⁺) (Figure 7.6 A5), qualitatively seen as the orange dominance of ER β .

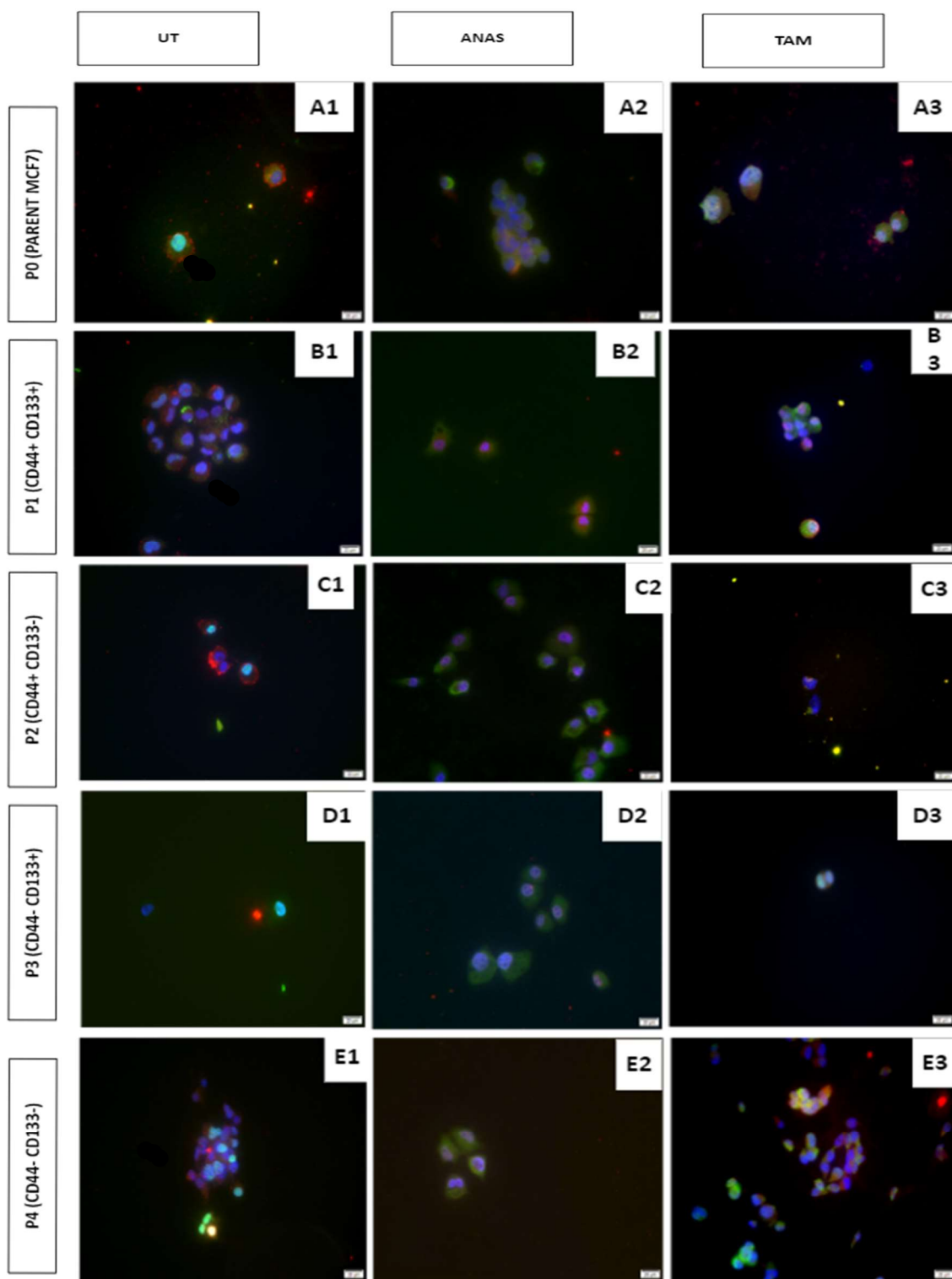


Figure 7.4. ER α (green) and ER β (red) expression in T47D cells. Nuclei are counterstained with DAPI (blue). Columns show untreated cells, Anastrozole and Tamoxifen treated cells. Rows indicate parent population and derived populations.

3.5.4 Alterations in the mean intensity of Ki67 and PR T47D cells – the effects of hormone therapy

Untreated P0 (Parent) revealed nuclear Ki67 found in some cells, particularly in the nucleolus. While there were no significant findings in terms of Ki67 mean intensity in the derived populations, a reduction in cell number (note blue nuclei) was evident in P2 (CD44⁺CD133⁻) and P3 (CD44⁻CD133⁺). Immunofluorescence of PR was found dominantly on the nuclear and cell border, and diffusely in the cytoplasm across all populations, with P4 (CD44⁻CD133⁻) showing a significant reduction in PR compared to the parent population and P1 (CD44⁺CD133⁺) (Figure 7.6 A8) (**Figure 7.5**, Appendix VI B).

Under Anastrozole treatment no significant alterations in Ki67 expression were seen across populations; however, the qualitative assessment showed, particularly in P2 (CD44⁺CD133⁻) and P4 (CD44⁻CD133⁻), a greater number of cells expressing the marker. PR expression in Anastrozole treated cells, was similar to the untreated populations whereby it was found on the nuclear and cell borders, in addition to the cytoplasm. PR expression was significantly reduced in Anastrozole treated P1 (CD44⁺CD133⁺) compared to their matched treated parent population (P0) and compared to the untreated parent population (Figure 7.6 A8). With Tamoxifen treatment, Ki67 expression while not significantly altered across population groups, was present in nuclei, particularly in the parent population (P0), P1 (CD44⁺CD133⁺), and P4 (CD44⁻CD133⁻), which demonstrated more cells. Immunofluorescence of PR was found in the cytoplasm and nucleus of the cell. P1 (CD44⁺CD133⁺) and P2 (CD44⁺CD133⁻) were significantly reduced compared to their matched counterparts in the untreated group (Figure 7.6 A8).

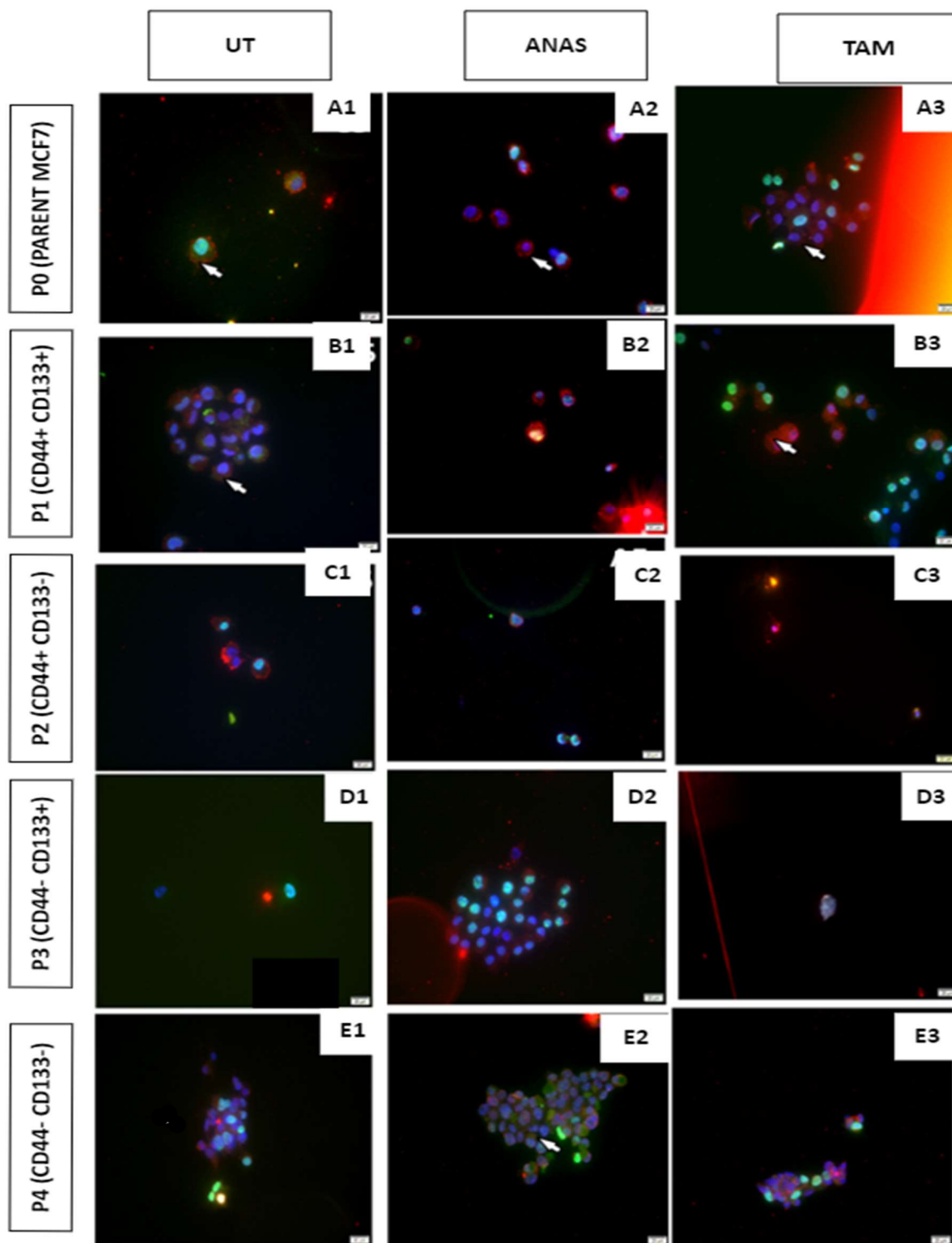


Figure 7.5: Ki67 (green) and PR (red) expression in T47D cells. Nuclei are counterstained with DAPI (blue). Columns show untreated cells, Anastrozole and Tamoxifen treated cells. Rows indicate parent population and derived populations. White arrow shows expression on the periphery of the cell.

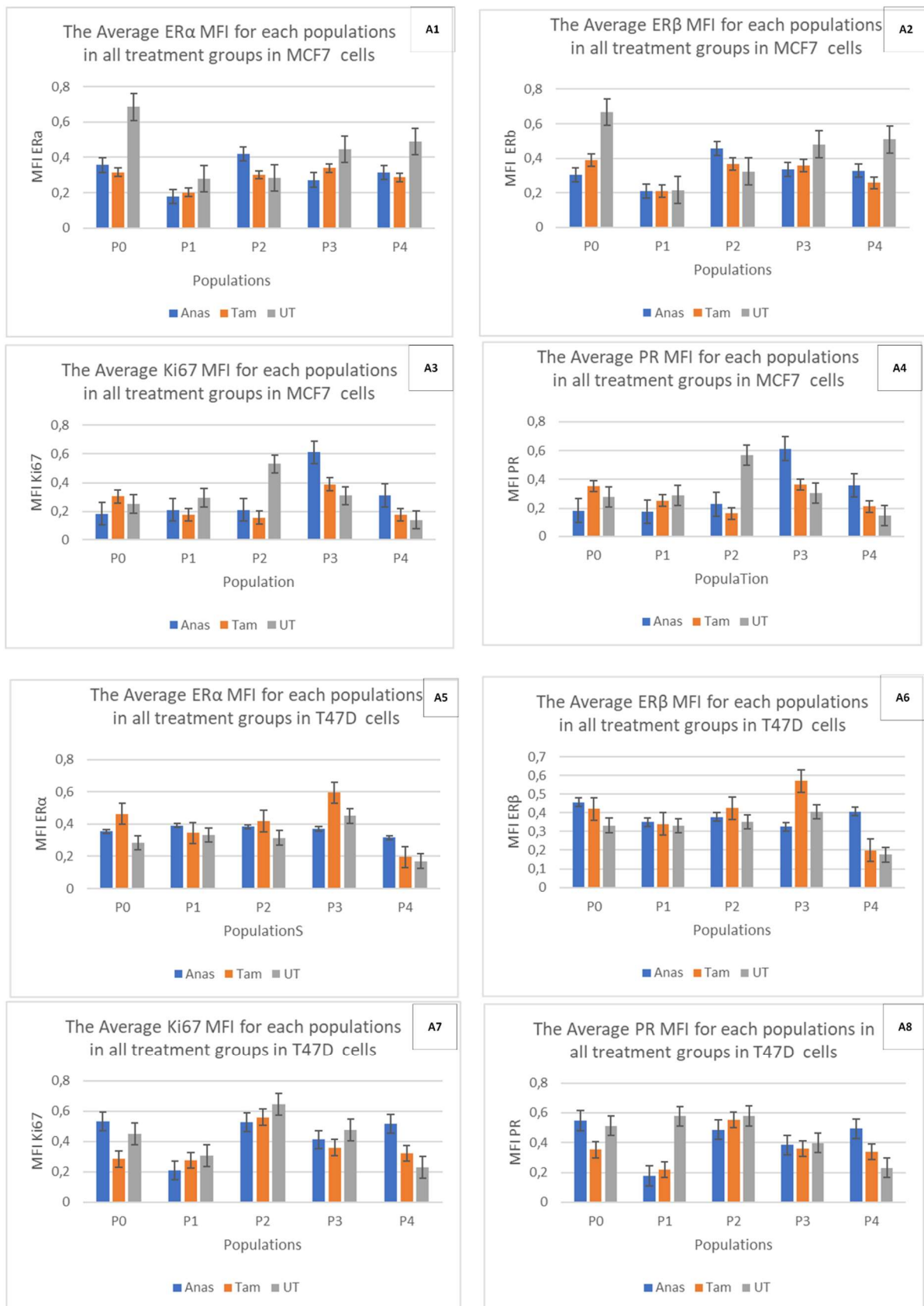


Figure 7.6: Bar graph representing the average MFI for each biomarker in subpopulations in each treatment group for MCF7 and T47D cell lines.

3.5.5 Matched analysis of populations between the MCF7 and T47D cell lines

The Mann-Whitney U test was run to establish whether populations derived from MCF7 and T47D cells had similar or different median biomarker expression. ER α expression median expression in the untreated parent populations (P0), was significantly higher in MCF7s, with no difference with ER β ; however, ER β expression in P1(CD44⁺CD133⁺) (untreated) was significantly higher in T47D cells. MCF7 cells also showed significantly higher expression of both markers on untreated P4 (CD44⁻ CD133⁻). In the untreated T47D cells, Ki67 expression was significantly higher in comparison to all populations derived from MCF7 cells. PR expression in untreated T47D cells was similarly significantly higher in all populations except for P2(CD44⁺CD133⁻), which showed little difference to the same population derived from MCF7 cells.

Under Anastrozole treatment, parent populations from T47D cells significantly reduced ER α expression, with the converse in P1 (CD44⁺CD133⁺) and P3 (CD44⁻ CD133⁺) (upregulation) compared to MCF7 cells. Only in P4 did T47D cells show a significantly higher median intensity of ER β . Anastrozole treatment significantly increased Ki67 and PR median intensity in T47D cells in P0 and P2 (CD44⁺CD133⁻), with a significant reduction in these biomarkers in P3 (CD44⁻ CD133⁺).

Tamoxifen, however, caused a significant increase in ER α median intensity in T47D cells in P1(CD44⁺CD133⁺), with the converse, a significant yet minor decrease in P4(CD44⁻ CD133⁻), with no differences in ER β or Ki67 median intensity. Tamoxifen treatment also caused a reduction PR median intensity in P1(CD44⁺CD133⁺) of T47D cells compared to MCF7 cells.

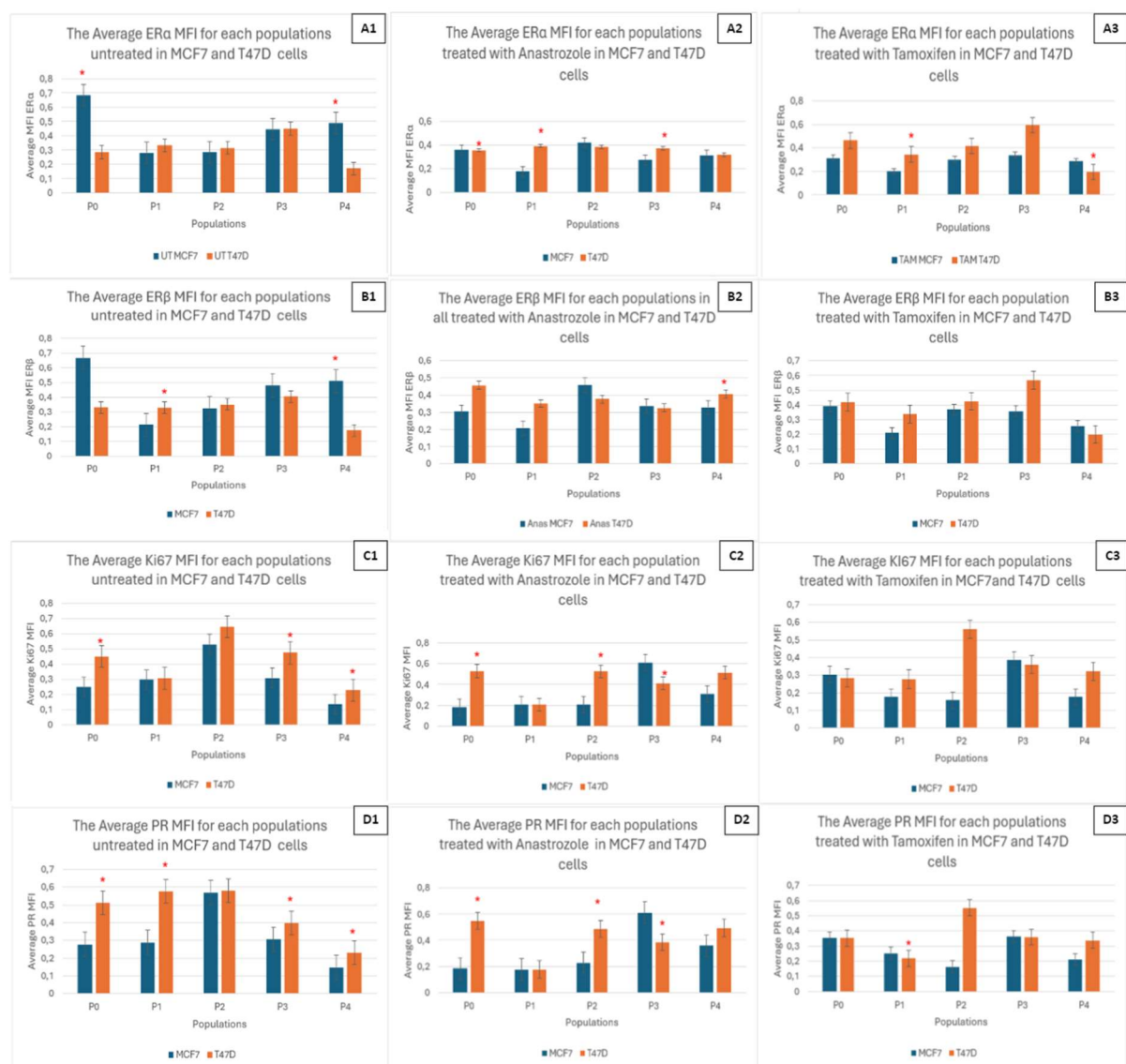


Figure 7.7: Bar graph representing the comparison between MCF7 and T47D average MFI for each biomarker in subpopulations in each treatment group. Key:* - significant differences between populations. A: Average MFI of ERα expression in subpopulations, B: Average MFI of ERβ expression in subpopulations, C: Average MFI of Ki67 expression in subpopulations, D: Average MFI of PR expression in subpopulations. 1- Untreated, 2- Anastrozole, 3 Tamoxifen

Chapter 4 Discussion

4.1 Overview

Tumour heterogeneity is a vital component of understanding the mechanisms involved in breast cancer in luminal A cell lines. CSC are an impacting factor that could potentially result in tumour heterogeneity, and as such the isolating of surface markers could aid in further understanding of how these cells grow. Therefore, our study aims to investigate stem cell markers, including CD44 and CD133 and their influence on tumour advancement. By magnetically sorting subpopulations ($CD44^+CD133^+$, $CD44^-CD133^+$, $CD44^+CD133^-$, $CD44^-CD133^-$). While there is research on stemness markers, its influence on luminal A breast cancer and its relation to hormone therapy is rare.

4.1.2 Modified essential 8 media

This study tested the substitutes for ferritin (ferrous sulphate and ferric nitrate) at various concentrations to evaluate cell growth in both DMEM/F12 culture media and complete culture media DMEM + 10% FBS. Between ferric nitrate and ferrous sulphate, no significant changes in cellular responses were observed, and both would be suitable as a substitute for ferritin. However, our study chose ferric nitrate at 10 $\mu\text{g/ml}$ due to its consistency in maintaining cell growth. Our sorted population was then cultured in this new modified essential 8 media.

4.1.2 Growth rate within subpopulations for MCF7 and T47D cell lines

This study isolated stem cell markers (CD44 and CD133), and 5 populations were assessed including the parent (P0). Cell growth in P0 (parent) over 6 days revealed that in comparison to T47D cell lines, MCF7 had a faster growth rate due to the increase of cell count seen on days 3 and 6. In P1 ($CD44^+CD133^+$) revealed a different trend to the parent population as T47D cell lines grew at a faster growth rate. An increase in cell density was also observed each day with increased cell expansion in T47D cell lines. However, in MCF7 cell lines, cell clumps were observed, which were not seen in T47D cells.

4.2 CD44 influence on cell growth and morphology

More insight into the relationship between CD44 and cellular growth is important in understanding what we see in our subpopulations. CD44 plays a role in cell plasticity regulation (Senbanjo & Chellaiah, 2017). A study showed that at the start of invasion of tumour tissues, leader cells characterised by $CD44^{\text{high}}$ arose from the $CD44^{\text{low}}$ cells at the

core of the tumour (Senbanjo & Chellaiah, 2017). CD44 ability to switch from epithelial to Mesenchymal induces a change from less invasive luminal breast cancer to more invasive (Senbanjo & Chellaiah, 2017). The variation in the amount of stemness markers seen across each population (Table 6), could potentially suggest the increased growth rate or changes to morphological structures.

CD44s and CD44v6 was observed as CD44 isoform representatives to regulate downstream signalling pathways (Chen et al., 2018). CD44v6 recruit ezrin/radixin/moesin (ERM) proteins that induce alterations in the cytoskeletal, potentially interacting with VEGFR, promoting angiogenesis, proliferation and, cancer cell division. In addition, CD44v6 is a co-receptor for tyrosine kinase c-Met that induces cancer cell invasion (Chen et al., 2018). On the other hand, CD44s can alter structures of the cytoskeleton promoting Snail/ β -catenin translocation to the nucleus, which induces transcription of urokinase-type plasminogen activator (uPA) expression or the matrix metalloproteinases (MMP) family, resulting in an increase of cancer cell invasion. CD44s can also promote induction of the PI3K/AKT pathway, and regulates Src/MAPK signalling pathway increasing invasion and proliferation in cancer cells (Chen et al., 2018). In Addition, CD44 induces Hypoxia-inducible factor 1 α (HIF1 α) binding to nuclear DNA causing a rise in glycolysis, producing a metabolic change in cancer cells (Chen et al., 2018). CD44 is thus responsible for tumour invasion and promotion. However, in our study, it is unclear if CD44 is responsible for the clumping of cells associated with tumour promotion as clumping was not observed in the T47D cell line containing CD44 expression. The branching and filopodia seen in T47D cell lines could, however, be the indication of CD44 effect in tumour promotion.

4.3 CD133 influence in cell growth rate and morphology

CD133 is involved in regulating signalling mechanisms at the plasma membrane via proteins responsible for cytoskeleton rearrangement, protein trafficking and signal transduction (Moreno and Robles, 2023). These mechanisms facilitate protrusion architecture and induce signalling pathways involved in cell proliferation, differentiation, migration and cell survival (Moreno and Robles, 2023). In addition, CD133 is not only found on the cell surface of CSC but also in the cytoplasm, facilitating cellular mechanisms involved in recycling surface proteins and the translocation to subcellular compartments (Moreno and Robles, 2023). Thus, suggesting a vast expanse of its various biological functions based on the cells' intrinsic state, external stimuli and other contexts of the cell. Based on the cell cycle, cell type and hypoxic condition and cytotoxic stress, CD133 expression can fluctuate (Moreno and Robles, 2023).

This could potentially be the reason for the variance in cell growth and morphology seen within each subpopulation (Moreno and Robles, 2023). Although P1(CD44⁺CD133⁺) should resemble attributes similar to the parent population (P0) we noticed a difference in the rate of cell growth, which could indicate the fact that the expression of the CD133, although present, could be either more or less in certain populations based on these factors. In breast cancer, non-small lung cancer and colon cancer, CD133 was observed in the nuclei (Moreno and Robles, 2023). Another study, according to Ross et al., 2019, observed CD133 expression intracellularly and lacking in surface expression in non-tumoral cells. The expression of CD133 was linked to revascularization potential (Moreno and Robles, 2023). In non-small cell lung cancer and hepatocellular carcinoma, CD133 expression was found in both cytoplasmic and nuclear levels and was linked to poor prognosis (Moreno and Robles, 2023). In addition, CD133 was also associated with PI3K/AKT signalling pathways that regulate phenotypic effects on cell surfaces (Moreno and Robles, 2023). As AKT signalling pathway induces proliferation, survival, migration, invasion and chemo-resistance via the downstream effects in particular cells (Moreno and Robles, 2023). CD133 regulates the AKT pathway via p85 regulation, which is a subunit of PI3K to its C-terminal domain that was phosphorylated by Src kinase at Y828 residue. Furthermore, the PI3K will be converted to PIP2 (phosphatidylinositol 4,5-biphosphate) into PIP3 (phosphatidylinositol 3,4,5-triphosphate), activating AKT in glioma cells, inducing self-renewal and tumor promotion (Moreno and Robles, 2023).

4.4 Filopodia/Pseudopodia and stemness markers:

Filopodia are finger-like plasma membrane protrusions responsible for a range of cellular processes such as cell migration, wound healing, chemoattractant guidance, extracellular matrix adhesion, remodelling and embryonic development (Kyykallio et al., 2020). In cancer cells, the amount of filopodia is typically abundant, which therefore increases the migration and invasiveness of cancer cells (Kyykallio et al., 2020). Actin bundling protein found in filopodia is commonly overexpressed in cancer and thus increases the length as well as the number of filopodia (Kyykallio et al., 2020). Therefore, there is an increased ability of cell migration and invasion leading to a poor survival prognosis (Kyykallio et al., 2020). According to Härkönen et al., 2019 CD44 expression is found accumulating on filopodia and binds HA on filopodia membrane (Härkönen et al., 2019). The study revealed the CD44 surface morphology contained high number of filopodia on the surface of the cell line

MKN74 (Härkönen et al., 2019). Populations P1 and P2 can be seen with many filopodia protrusions in both MCF7 and T47D cell lines.

4.5 Cell Migration and metastasis

Metastasis is the primary factor for mortality in breast cancer patients (Covert et al., 2019). Thus, evaluating these metastatic events can improve a patient's overall health (Covert et al., 2019). Tumour cells metastasize by changing phenotypically, an epithelial-mesenchymal transition (EMT) (Covert et al., 2019). EMT results in the intravasation of the tumor cell into blood vessels or lymphatic channel (Covert et al., 2019). Thus, once the primary breast tumour cells have reached the blood vessels, they spread to specific organs such as bones, lungs, liver and brain (Covert et al., 2019). The tumor microenvironment (TME) is involved in an important role in the metastatic cascade, signalling from cytokines found in the TME results in tumor cell phenotypic changes important for metastasis occurrence (Covert et al., 2019). Observing the phenotypical changes for each population revealed distinct characteristics, such as cells clumping together and others branching on top of each other. Since CD44 is a transmembrane protein that plays a role in cell adhesion and migration (Covert et al., 2019), analysing the growth patterns of populations P1 and P2 shows cells clumping together in MCF7. On day 6 for population P0, P1 P2 of MCF7 cell lines can clearly be seen forming more clumping, indicating cell migration and metastasis. According to studies EMT and the upregulation of CD44 are involved in breast tumour metastasis (Covert et al., 2019). However, in T47D cell lines, subpopulations containing CD44 markers (P0, P1 and P2) did not show as much clumping on day 6 as opposed to MCF7. This could be as a result of the slower growth rate observed in T47D cell line which is needed before cells decide to clump. Since CD44 is a molecule responsible for adhesion that regulates cell-cell and cell-matrix interaction these characteristics seem consistent with the findings for this population (P0 and P2) on day 5 (Zhang et al., 2014). According to Härkönen et al., 2019 CD44 cells were found growing on top of each other joining together in tight clusters. Thus, also supporting the implications of CD44⁺ involvement in metastasis functions as these growth clustering patterns are consistently seen in cells ready to migrate (Bonnet et al., 2021).

Studies have also revealed that CD133 could regulate tumourgenesis, self-renewal, angiogenesis and tumour metastasis (Liu et al., 2016) (Liou, 2019). A study highlighted that the activation AKT signalling pathway promotes invasion and migration of CD133 stem cell in the brain (Liu et al., 2016). Tumor cells can initiate mechanisms that allow them to change their own shape (Krakhmal et al., 2015). Thus, creating an environment for moving and

remodelling surrounding tissues to produce migration pathways (Krakhmal et al., 2015). The progression of a single cell is seen in embryonic processes and inflammation and similarly, the dissemination mechanism is common among cancer cells during tumour progression and metastasis (Krakhmal et al., 2015). In addition to single cell migration, there is also collective cell migration which occurs when sets of interconnected tumor cells migrate (Krakhmal et al., 2015). Collective migration suggests tissue rearrangement, initiates the process of embryonic morphogenesis, and plays an important part in wound healing (Krakhmal et al., 2015). This form of migration is seen in the development and progression of breast cancer, endometrial cancer, prostate cancer, colorectal cancer, large cell lung carcinoma, rhabdomyosarcoma, melanoma and squamous cell carcinomas (Krakhmal et al., 2015). Collective migration is identified by whole groups of cells interconnected by adhesion molecules and other communication junctions that migrate (Krakhmal et al., 2015). According to studies, a moving cell group has a “leading edge” or “leading front” that uses integrins and proteases (Krakhmal et al., 2015). They suggest a distinct variation in the expression of genes and the morphology occurring between the “leader” cells forming the leading edge and the “follower” cell found behind them (Krakhmal et al., 2015). The leading cells appeared mesenchymal in shape and characterised by less prominent ordering and structure, whereas the “followers” formed more tightly packed rosette-like tubular structures with tight intercellular contacts (Krakhmal et al., 2015). With growth patterns found within the populations, there is some correlation to the potential for cell migration.

4.6.1 Biomarker ER α and ER β expression in untreated groups

The variation of biomarkers ER α and ER β was seen across populations in MCF7 untreated populations with ER α being dominantly expressed in the cytoplasm compared to ER β in P0. P1(CD44+CD133+) ER β was significantly reduced, with ER α showing a brighter expression in comparison to P0. This highlights the heterogeneity within breast cancer cells. Although both populations should have the same stemness markers (CD44+ CD133+), their biomarker expression for ER α and ER β varied, which could be a result of phases of the cell cycle. The stemness marker could potentially impact the expression of ER α and ER β in the cell. Our study revealed the expression of both markers found in the cytoplasm, whereas P3 (CD44-CD133+) showed dominant expression in the nuclear and perinuclear of the cell. However, in T47D untreated cells, ER α and ER β expression was observed primarily in the nucleus. T47D cells revealed a greater yield of CD44 expression across populations in comparison to

MCF7 and MCF7 yielding a greater expression of CD133 across populations (Fig 6), these stemness markers could potentially impact ER α and ER β localization.

4.6.2 Biomarker ER α and ER β expression in Anastrozole treated groups

According to Smollich et al., 2009, Anastrozole reduces ER α effects by decreasing the ratio ER α /ER β . Anastrozole inhibits proliferation, invasion and ER α -mediated signal transduction. Although no significant differences were observed between untreated groups and Anastrozole groups, our study did show expression levels change in all populations (P2 -P4) treated with Anastrozole; with both ER α and ER β reduced in comparison to the untreated groups. However, another study suggests that hormone therapy effects are dependent on the ratio of ER α and ER β expression (Madeira et al., 2013). According to Pather et al., 2019, T47D cells revealed an increase in ER β expression and sustained ER α expression when treated with Anastrozole (Pather ., 2022).

It was reported that ER β is present in over 60% of breast cancer cases (Zhou & Liu, 2020). The DNA binding domain and ligand binding domain protein of ER β are similar by 96 and 60% respectively with those of ER α , which therefore could indicate similarity in function (Zhou & Liu, 2020). ER α and ER β levels in breast cancer are suggested to be linked to different signalling pathways vital for cell proliferation and endocrine therapy (Zhou & Liu, 2020). This includes the PI3K/AKT pathway involved in cellular proliferation, invasion, apoptosis, and hypoxia-related protein upregulation (Zhou & Liu, 2020). The induction of PI3K/AKT pathway is a vital component in downregulating ER β in breast cancer (Zhou & Liu, 2020). Induced PI3K/AKT signalling and activation of coactivator cAMP response element-binding (CREB)-CBP promotes ER β ubiquitination and degradation (Zhou & Liu, 2020). CD44 is also associated with the AKT pathway, inducing cell migration and invasion through multiple mechanisms (Thanee et al., 2021). Making it a possible link to the regulation of ER α and ER β expression in these subpopulations (Thanee et al., 2021) (Herishanu et al., 2011).

4.6.3 Biomarker ER α and ER β expression in Tamoxifen treated groups

In MCF7 cells, in the parent population treated with Tamoxifen, there was a reduction in ER α and ER β expression, although not significant. No significant reduction in ER α and ER β was seen in T47D cells as well. However, in T47D cell lines P4 (CD44- CD133-) showed ER α expression reduced significantly in comparison to P3 (CD44- CD133+). The MCF7 cells treated with Tamoxifen revealed a similar trend with P2 and P3 mean intensity higher than

that of P1 of both ER α and ER β expression. According to Miller et al., 2006 Tamoxifen treatment was associated with reduced ER α expression. This supports what was observed in our study, although not statistically significant reduction was observed in the expression of ER α . Differential changes of both ER α and ER β were also observed in their study (Miller et al., 2006). Tamoxifen is responsible for the inhibition of oestrogen induced growth of breast cancer by binding to and blocking ER α (Gruvberger et al., 2007). ER β expression was found to be reduced in Tamoxifen resistant tumours, with higher levels linked to positive outcomes in ER α presenting breast tumours (Madeira et al., 2013).

Although there was no major significant differences to indicate the impact of stemness marker CD44, studies show that CD44 protein is associated with the regulation of receptor tyrosin kinases such as c-Met , VEGFR-2, Her2 and the EGFR (Hiscox et al., 2012). As CD44 interacts with ERbB receptors, it was suggested that this interaction could potentially support Tamoxifen-resistant phenotypes, aiding in diverse cellular characterization (Hiscox et al., 2012). This was seen as the study indicated in MCF7 cells, the overexpression of standard CD44 isoform (CD44s) at protein and gene levels of Tamoxifen- resistant (TamR) (Hiscox et al., 2012). CD44 suppression was found to reduce endogenous EGFR activity (Hiscox et al., 2012). The findings concluded that CD44 was associated with Tamoxifen resistance, influencing cellular reaction to ERbB ligands and HA (Hiscox et al., 2012).

CD44-ligand, HA enriches the tumour microenvironment, with HA having a significant impact in breast cancer enhancement and metastasis when induced by CD44 signalling (Hiscox et al., 2012). CD44 and HA binding produces actin cytoskeleton system alterations via the induction of Rac1 signalling with the development of membrane ruffles and projections of the cell and migration (Hiscox et al., 2012). Furthermore, the clustering of CD44, which may be activated by HA, has been suggested to sequester MMP9 at the cell surface which may influence cellular invasion (Hiscox et al., 2012).

4.6.4 Bio marker KI67 and PR in untreated group

Across populations in MCF7 cells, Ki67 was expressed in the nucleus of the cell except in P2 revealing significantly increased levels of Ki67 expression in comparison to the parent population. In addition, in population P4, Ki67 expression was significantly reduced in comparison to P2. This could indicate a potentially significant relationship between CD44 and Ki67. According to Ma et al., 2017, Ki67 and CD44 expression is linked to tumour risk and mitotic characteristics in Gastric Stromal Tumour (GST). The T47D cells also revealed

nuclear expression of Ki67. PR expression in MCF7 revealed in P2 a significant increase in expression in comparison to all other populations .

4.6.5 Bio marker KI67 and PR treated with Anastrozole

MCF7 cells treated with Anastrozole revealed in population P3 showed a significance in increase in KI67 in comparison to untreated population P3. A significant difference was observed in our study between P4 and P0 (parent). with an increased expression of both Ki67 and PR. According to Chagas et al., 2020 their study revealed female rats administered with Anastrozole revealed a significant reduction in Ki67 expression in vaginal epithelium revealing an anti-proliferative effect of Anastrozole.

4.6.6 Bio marker KI67 and PR treated with Tamoxifen

In MCF7 cells treated with Tamoxifen, P3 (CD44- CD133+ showed significant high intensity of Ki67 compared to p2 (CD44+ CD133-). Thus, indicating the possibility that the stemness marker could influence Ki67 once treated. In the T47d cells, P1 and P2 showed a significant reduction in PR when treated with Tamoxifen in comparison to matched untreated groups. As a result, patients treated with short-term therapy of aromatase inhibitor or Tamoxifen revealed a reduction in Ki67 levels (Mattar et al., 2010). Short-term treatment of Tamoxifen revealed that Ki67 was a good indicator for overall survival in patients (DeCensi et al., 2011). According to Spartz et al., 2023, CD44 is associated with Cancer associated fibroblasts (CAF) breast cancer co-cluster development. Interactions between CAFs and PR was expressed in T47D and MCF7L cells and revealed PR cluster formation. Fibroblast growth factor 2 (FGF2) was shown to induce PR activation and increased CD44 expression, inducing cell-cell interaction. In addition, CAF-derived FGF2 can act via PR to induce MAPK/ERK signalling increasing stemness and resistance to endocrine therapy (Spartz et al., 2023). This could be an indication as to why PR was observed to be expressed in the cytoplasm particularly around the periphery of the cell due to the increase induction of cell-cell interaction.

Recent studies revealed that Ki67 is not necessary for cell proliferation (Mrouj et al., 2021). Ki67 is induced by cell cycle regulator such as cyclin-dependent kinases (CDKs), the initiation of subunit of the ubiquitin ligase APC/C-CDH1, that's responsible for the destruction of mitotic cyclins and cell cycle transcription factor B-Myb (Mrouj et al., 2021). This is consistent with our findings, as Ki67 expression was notably observed in the nucleus and perinucleus of the cells, indicating its cellular function in those regions of the cell. Thus, Ki67 expression does involve major steps of tumorigenesis such as initiation, progression and

metastasis, immune responses and drug sensitivity (Mrouj et al., 2021). By maintaining the transcriptional processes involved in tumor cell adaptations Ki67 plays an important role in tumorigenesis. This was shown in a study performed on mutant mice, whereby the Ki67 knock-out cancer cells reduced the ability to induce angiogenesis (Mrouj et al., 2021). In addition, due to the knockout Ki67 cells, epithelial and mesenchymal characteristics of the cells were altered and were therefore unable to induce metastasis. Ki67 reduction also resulted in increased sensitivity to the drugs and decreased interaction with the immune system (Mrouj et al., 2021). According to Mrouj et al., 2021 human colorectal cancer cells DLD-1 revealed that Ki67 knockout decreased the number of cells expressing the antigen CD133 (Mrouj et al., 2021).

4.7 Limitations

The specialized Essential 8 media was discontinued, and as a result, alternative modified essential 8 media was used in its place. After observing how rapidly sorted populations grew in the specialized essential 8 media as opposed to the modified essential media, the rate of growth was slightly slower in both cell lines. This resulted in a longer cell culture period to be longer, potentially leading to changes in the stem cells. These changes could have affected the verification of these sorted markers as cells may have undergone alterations during passages, thereby impacting the accuracy of verifying these stemness markers. Due to time constraints and limited resources, analysis of ALDH activity was unable to continue but would be assessed in future studies. Contamination of cells due to a mould outbreak throughout our lab impacted the study greatly as sorted populations were lost, and the process of magnetic cell sorting had to be redone. Due to confocal microscope damage, images had to be redone on the Olympus BX to analyse immunofluorescence in subpopulations.

Chapter 5: Conclusion

5.1 Concluding Remarks

In conclusion, the growth rate in MCF7 cells was found to be greater in comparison to T47D cell lines. However, P1 (CD44+ CD133+) in T47D the growth rate was higher in comparison to the P0 parent population. MCF7 cell lines across all populations revealed more clumping in growth than T47D but could be due to the difference in growth rate as cells were only observed for 6 days. Our study revealed that it was unclear whether to see if CD44 is responsible for the clumping of cells associated with tumour promotion, as clumping was not observed in the T47D cell line containing CD44 expression. The branching and filopodia

seen in T47D cell lines could however be the indication of CD44 effect in tumour promotion. More research on CD44 effect within different cell lines is needed to further understand the mechanism its involved in. We observed a difference in rate of cell growth in both MCF7 and T47D between P1(CD44⁺CD133⁺) and P0 parent which indicated to variance in CD133 expression levels impacting cell growth rates.

MCF7 cells treated with Anastrozole revealed a reduction in ER α and ER β expression in populations P2 - in comparison to the untreated matched groups. T47D cell lines treated with Tamoxifen P4 (CD44⁻ CD133⁻) showed a reduction in ER α expression in comparison to P3 (CD44⁻ CD133⁺). In MCF7 cells treated with Tamoxifen revealed a similar trend with P2 and P3 mean intensity higher than that of P1 of both ER α and ER β expression. According to Miller et al., 2006 Tamoxifen treatment was associated with reduced ER α expression. This supports what was observed in our study, although the changes were not significant.

In untreated groups, in population P4, Ki67 expression was significantly reduced in comparison to P2. This could indicate a potentially significant relationship between CD44 and Ki67. Samples treated with Anastrozole showed a significant difference between p4 and P0 (parent) with an increased expression of both Ki67 and PR. This could suggest the association stemness markers have in the expression of KI67 and PR and possible pathways that influences the increase of KI67 and PR in a reduced CD44 and CD133 expression. In MCF7 cells treated with Tamoxifen, P3 (CD44⁻ CD133⁺ showed significant high intensity of Ki67 compared to p2 (CD44⁺ CD133⁻). Thus, indicating to the possibility that the stemness marker could influence Ki67 expression once treated with Tamoxifen. In t47d cell, P1 and P2 showed a significant reduction in PR when treated with Tamoxifen in comparison to matched untreated groups.

These findings reveal the variations between subpopulations in both MCF7 and T47d cell lines. The results of the variation between populations with different stemness markers indicate their interaction with pathways common in hormone therapy and may act as therapeutic targets in further studies. Stemness markers play an important role in the expression of biomarkers ER α , ER β , Ki67 and PR. Either increase expression of biomarkers as seen when treated with Anastrozole or decreasing it. More research would be needed to make a conclusive statement of the interplay CD44 and CD133 with each other as well as with pathways influencing biomarker expression.

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UNIVERSITY OF THE WITWATERSRAND V JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
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Appendix I: Ethic clearance certificate

A: Ethics clearance certificate (waiver- bigger project)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Drs K, K, T & P Xulu, Pather, Augustine & Fru
School: Anatomical Sciences
Department:
Medical School
University
E-mail: Kutlwano.xulu@wits.ac.za

cc: Supervisor: N/A <>and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252
E-mail: Iain.Burns@wits.ac.za

DATE: 08/02/2021

REF: R14/49
PROTOCOL NO: W-CBP-210218-01 (This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)

PROJECT TITLE: Characterising breast and cervical cancer heterogeneity in vitro and the impact on cancer outcomes following hormone therapy and chemotherapy

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JOHANNESBURG

HUMAN RESEARCH
ETHICS COMMITTEE
(MEDICAL)



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Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

08/02/2021

Ref: W-CBP-210218-01

TO WHOM IT MAY CONCERN

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

Investigator: Drs K, K, T & P Xulu, Pather, Augustine & Fru
Student No. (if appropriate):
Staff No. (if appropriate): A0045322

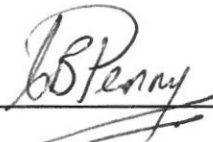
Supervisor: N/A

School: Anatomical Sciences

Department: Medical School
University

Project title: Characterising breast and cervical cancer heterogeneity in vitro and the impact on cancer outcomes following hormone therapy and chemotherapy

Reason: In vitro laboratory study
No human participants will be involved in the study



Dr CB Penny

Chairperson: Human Research Ethics Committee (Medical)

Research Office Secretariat:

Third Floor, Phillip Tobias Building, corner of St Andrews and York Roads, Parktown,
Johannesburg 2193

Postal address: Private Bag 3, Wits 2050

Tel Nos: +27 (0)11 717 1234/1252/2656/2700

Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za
Website:
<https://www.wits.ac.za/research/researcher-support/research-ethics/ethics-committees/>

B: Ethics clearance certificate (S. Gallant)
UNIVERSITY OF THE HUMAN RESEARCH ETHICS COMMITTEE

WITWATERSRAND,

JOHANNESBURG

Human Research Ethics Committee



(MEDICAL)

(Medical)

Research Office Secretariat:
Faculty of Health Sciences, Phillip Tobias Health Sciences Building, 3rd Floor, Office 301/2/4, 29 Princess of Wales Terrace, Parktown,
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Website: www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Ref: W-CBP-210729-01

29/07/2021

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical)

Investigator: Miss Simone Gallant

Supervisor: Dr Kutlwano xulu

School: Anatomical Sciences

Project title: Characterizing breast cancer heterogeneity in vitro and the impact of hormone therapy on tumour outcome

Reason: In vitro laboratory study using cell lines. No human participants will be involved in the study.

Dr CB Penny

Chairperson: Human Research Ethics Committee (Medical)

Copy — HREC (Medical) Secretariat: Ms Zanele Ndlovu, Ms Mapula Ramaila and Mr Rhulani Mkansi

Appendix II: Cell culture (buffer, reagents, procedures)

A: 50 ml Complete MCF7 media

- 45ml Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and 4.5g/L
- glucose
- 5ml Foetal Bovine Serum (FBS)
- 50ul Penicillin (10mg/ml)/Streptomycin (10mg/ml) (P/S)
- Sterile filter through a 0.2µM filter.
- Stored at 4°C.

B: 50 ml complete T47D Media

- 45ml Roswell Park Memorial Medium
- 80µl bovine insulin
- 5ml FBS
- 50ul Penicillin (10mg/ml)/Streptomycin (10mg/ml) (P/S)
- Stored at 4°C.

C: 0.1M Phosphate buffered saline (PBS) pH 7.4

- 8 g NaCl
- 1.15 g Na₂HPO₄
- 0.2 g KCl
- 0.2 g KH₂PO₄
- 1l distilled water

Appendix III: Modified essential 8 media (iron salt optimisations)

A: Stock solutions of ferrous sulphate and ferric nitrate

A volume of 0.002g of FeSO_4 was diluted in 10 ml of distilled water to make stock 1 of FeSO_4 .

A volume of 0.002g of $\text{Fe(NO}_3)_2$ was diluted in 10 ml of water to make stock 1 of $\text{Fe(NO}_3)_2$. Both solutions were filtered.

B: Working solutions of media containing ferrous sulphate and ferric nitrate :

Concentration of irons	MCF7 MEDIA: DMEM + 10% FBS + 0.1% P/S		T47D MEDIA: RPMI +10% FBS+ 0.1% P/S +0.2% INSULIN		DMEM F12 MEDIA	
	FeSO_4	$\text{Fe(NO}_3)_3$	FeSO_4	$\text{Fe(NO}_3)_3$	FeSO_4	$\text{Fe(NO}_3)_3$
C1: 0,5 $\mu\text{g/ml}$	55 μl stock 1 + 21945 μl media	55 μl stock 1 + 21945 μl media	55 μl stock 1 + 21945 μl media	55 μl stock 1 + 21945 μl media	110 μl stock 1 + 43890 μl media	110 μl stock 1 + 43890 μl media
C2: 5 $\mu\text{g/ml}$	550 μl stock 1 + 21450 μl media	550 μl stock 1 + 21450 μl media	550 μl stock 1 + 21450 μl media	550 μl stock 1 + 21450 μl media	1100 μl stock 1 + 42900 μl media	1100 μl stock 1 + 42900 μl media
C3: 10 $\mu\text{g/ml}$	1177 μl stock 1 + 20823 μl	1177 μl stock 1 + 20823 μl	1177 μl stock 1 +	1177 μl stock 1 + 20823 μl	2353 μl stock 1 + 41646 μl	2353 μl stock 1 + 41646 μl media

	media	media	20823µ l media	media	media	
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Appendix IV: Magnetic cell sorting

A: Buffers

A1: 1l PBS at Ph 7.2

- Measure:
- 8 g NaCl
- 1.15 g Na₂HPO₄
- 0.2 g KCl
- 0.2 g KH₂PO₄
- Into 800ml of distilled water on and stir
- Once dissolved top up with 200ml distilled water and Ph to 7.2

A2: PBS (pH 7.2), 0.5% bovine serum albumin (BSA), and 2 mM EDTA (Miltenyi buffer)

- Measure 0.25g of BSA and dissolve in 2mM EDTA

B: Cell counts of isolated subpopulations after magnetic cell sorting

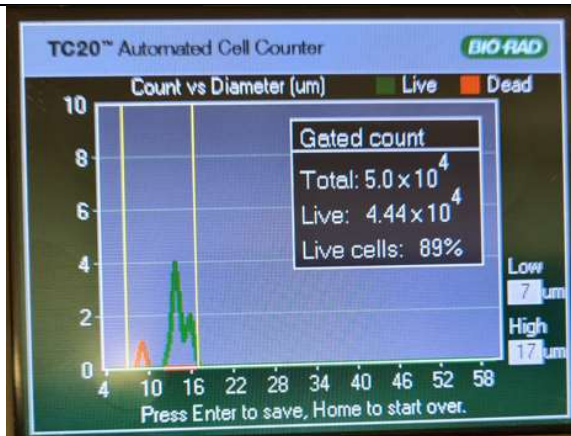
Cell line	Population	Total count	Live count	% live	Range
MCF7 sort 2	parent	1.04x10 ⁷	9.65x10 ⁶	92%	4-24 µm
	CD44+	2.54x10 ⁵	8.76x10 ⁴	75%	4-23 µm
	CD44-	1.1x10 ⁷	7.89x10 ⁶	72%	4-24 µm
	CD44+ CD133+	7.77x10 ⁴	6.66x10 ⁴	86%	4-20 µm
	CD44+ CD133-	4.44x10 ⁴	2.22x10 ⁴	50%	4-15 µm
	CD44- CD133+	3.89x10 ⁵	5.55x10 ⁴	14%	4-17 µm
	Cd44- CD133-	6.48x10 ⁶	5.9x10 ⁶	91%	4-21 µm
MCF7 Sort 3	parent	9.36 x10 ⁶	8.22x10 ⁶	88%	4-24 µm
	CD44+	1.22x10 ⁵	9.99x10 ⁴	82%	4-23 µm
	CD44-	1.4x10 ⁷	7.43x10 ⁶	71%	4-24 µm
	CD44+CD133	1.11x10 ⁴	1.11x10 ⁴	100%	11-15µm

	+				
	CD44+CD133 -	2.00×10^5	7.77×10^4	39%	4-42 μ m
	CD44- CD133+	5.22×10^5	1.44×10^5	28%	4-40 μ m
	CD44- CD133-	6.31×10^6	5.45×10^6	86%	4-25 μ m
T47D Sort 1	Parent	$3,52 \times 10^6$	3.08×10^6	88%	4-28 μ m
	CD44+	5.44×10^5	3.78×10^5	69%	4-31 μ m
	CD44-	4.33×10^6	3.39×10^6	78%	4-31 μ m
	CD44+CD133 +	2.05×10^5	1.78×10^5	86%	4-34 μ m
	CD44+CD133 -	2.39×10^5	1.72×10^5	72%	4-34 μ m
	CD44- CD133-	4.65×10^6	4.10×10^6	88%	4-29 μ m
T47D sort 2	CD44+ CD133+	9.77×10^5	8.33×10^5	85%	4-26 μ m
	CD44+ CD133-	4.05×10^5	3.78×10^5	93%	4-28 μ m
	CD44- C133+	6.55×10^5	5.22×10^5	80%	4-31 μ m
	CD44- CD133-	5.17×10^5	4.68×10^5	93%	4-31 μ m

CD44 ⁺ CD133 ⁺	CD44 ⁺ CD133 ⁻
--------------------------------------	--------------------------------------

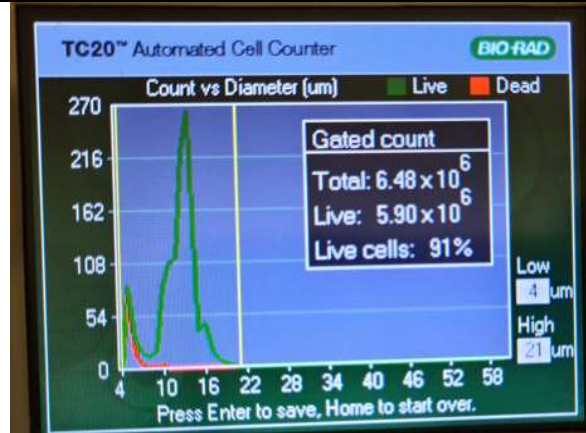
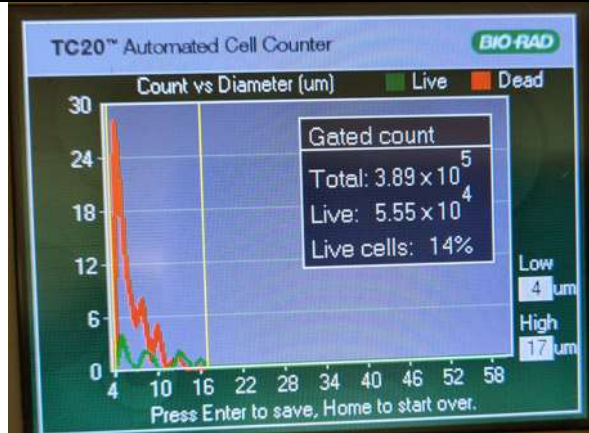
C: Cell counts from TC 20 automated cell counter showing gating

C1: MCF7 Sort



CD44⁻CD133⁺

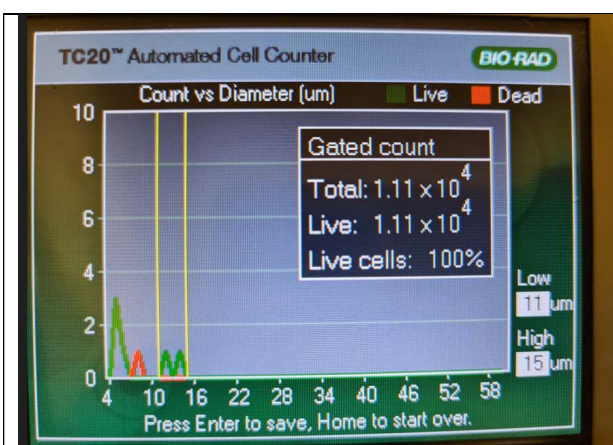
CD44⁻CD133⁻



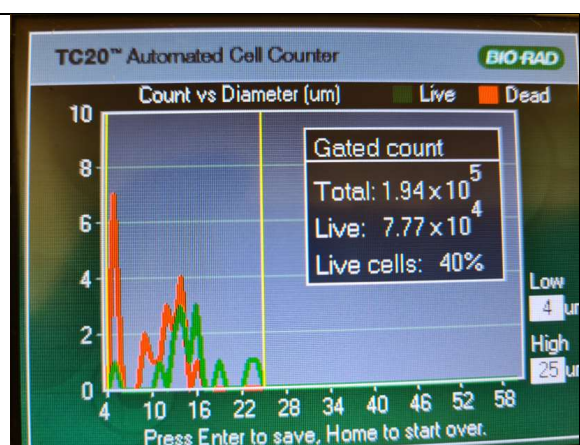
C2: MCF7 Sort 3

CD44⁺CD133⁺

CD44⁺CD133⁻

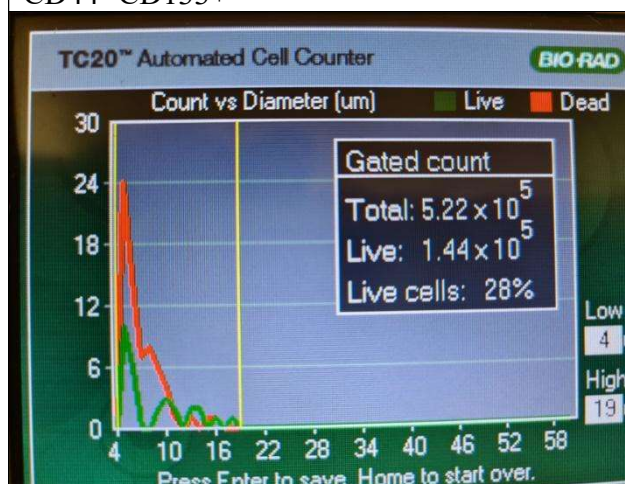


CD44- CD133+



CD44- CD133-

No image

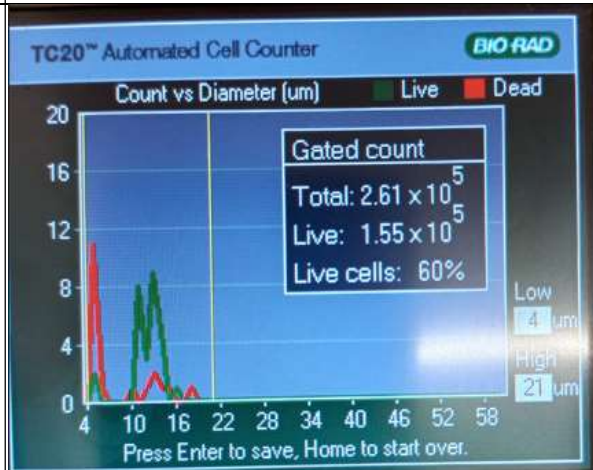


C3: T47D Sort 1

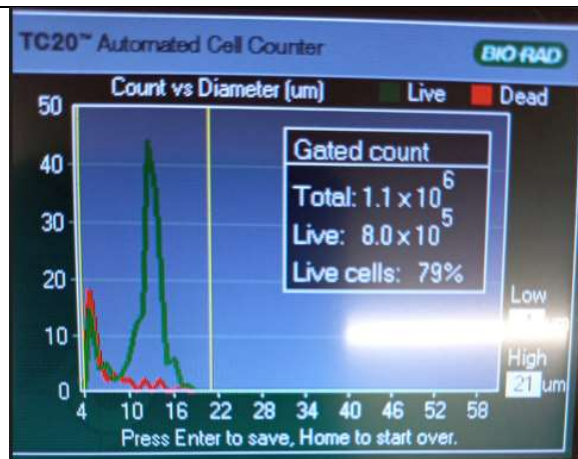
CD44+ CD133+



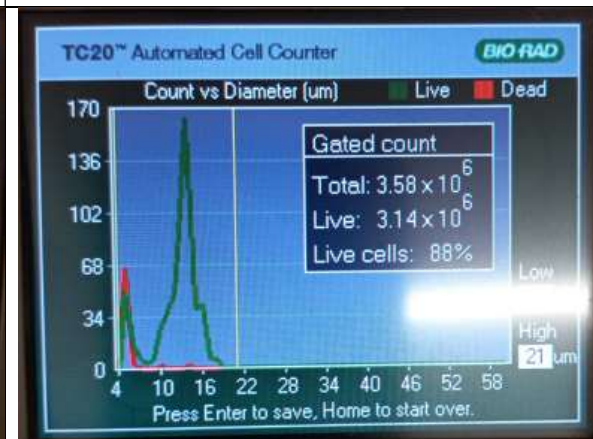
CD44+ CD133-



CD44-CD133+



CD44-CD133-



B4: T47D SORT 2

CD44+ CD133+



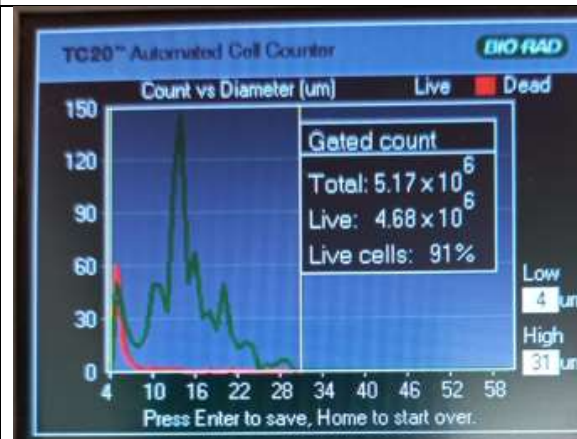
CD44+ CD133-



CD44- CD133+



CD44- CD133-



Appendix V: Flow verification of stemness phenotype

A: Buffers

A1: 10% FBS in PBS (Staining buffer)

- Add 10mls of FBS to 40mls PBS.

A2: 1% Paraformaldehyde (Fixative)

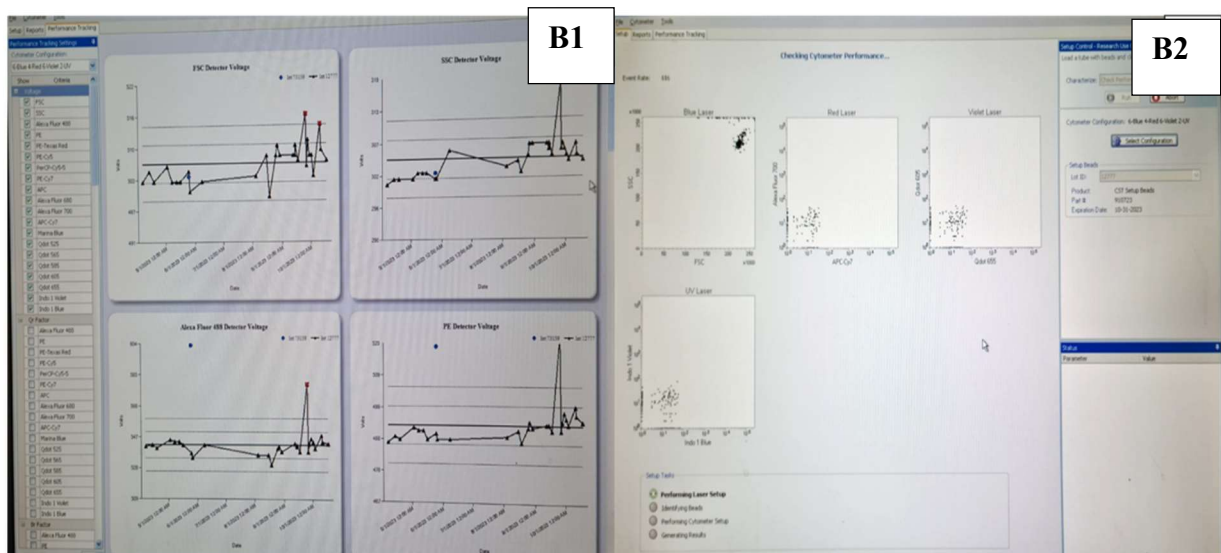
- Add 0.5g paraformaldehyde to 50ml of PBS and dissolve of heated plate.

A3: 10% FBS in PBS supplemented with 1 mM EDTA

- Dissolve 0.3g of EDTA in 80ml of PBS. This can be done with a magnetic stirrer on a warm plate.
- Add sodium hydroxide pellet.
- Wait till the solution is clear and top up to 20ml of PBS.
- Measure Ph to 7.4 and autoclave.
- 45mls of (10% FBS IN PBS) + 5mls of 1mM EDTA

B: Cytometer setting and tracking (CST) in the LSR Fortessa

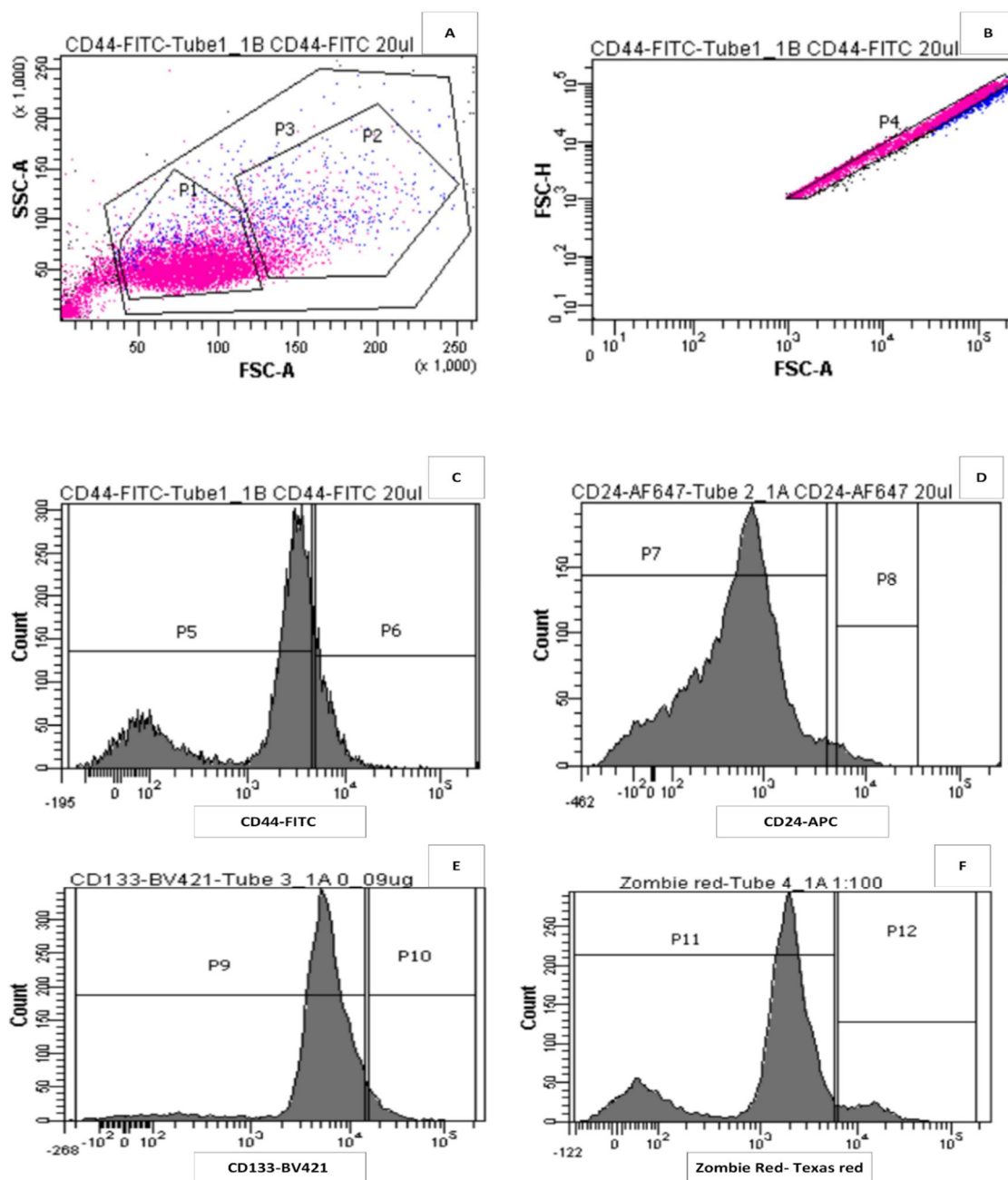
B1: representing cytometer settings and tracking (CST) B2: signal detections. CST Beads used Lot number 12777



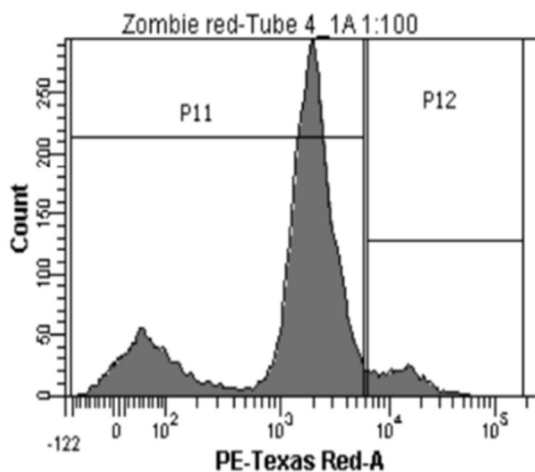
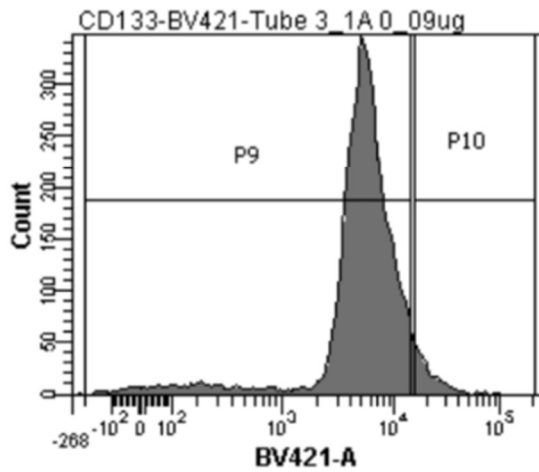
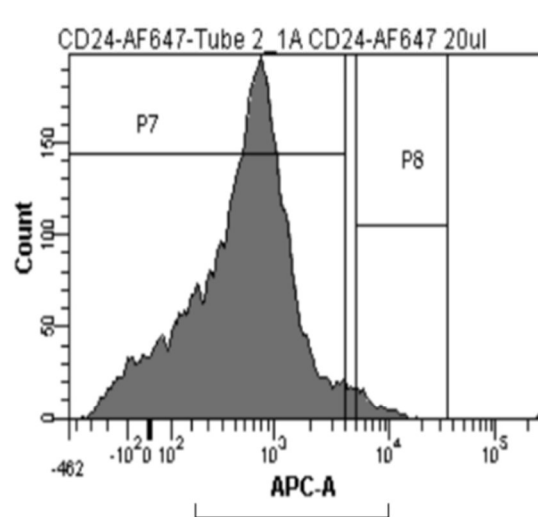
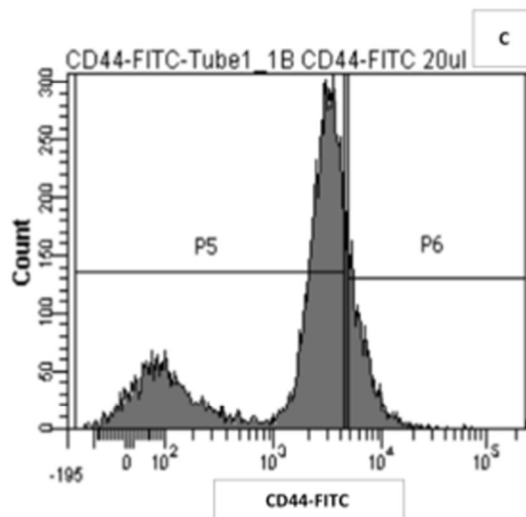
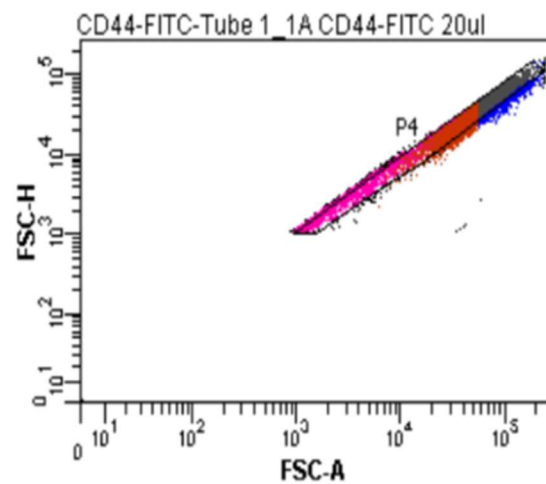
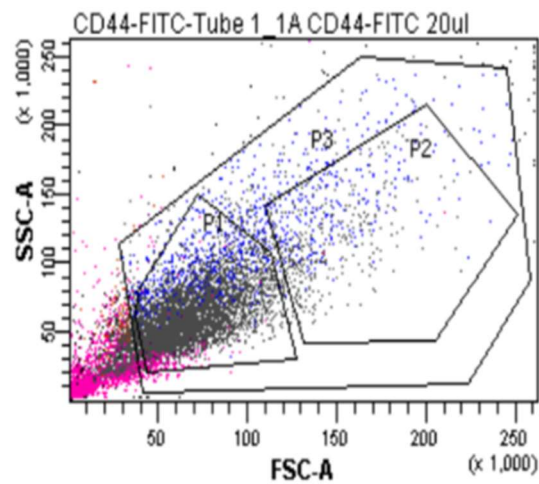
C: Checking for autofluorescence

Scatter plot A representing the forward and side scatter properties, gated as P1, P2,P3), Scatter plot B representing singlet population gated as P4 and histograms(C-F) representing the autofluorescence of MCF7 and T47d for each channel: C- FITC (20 μ l CD44), D- APC (20 μ l CD24), E- BV421 (0.09 μ L CD133) , F- Texas Red (1/100 Zombie Red).

C1: MCF7 Auto fluorescence



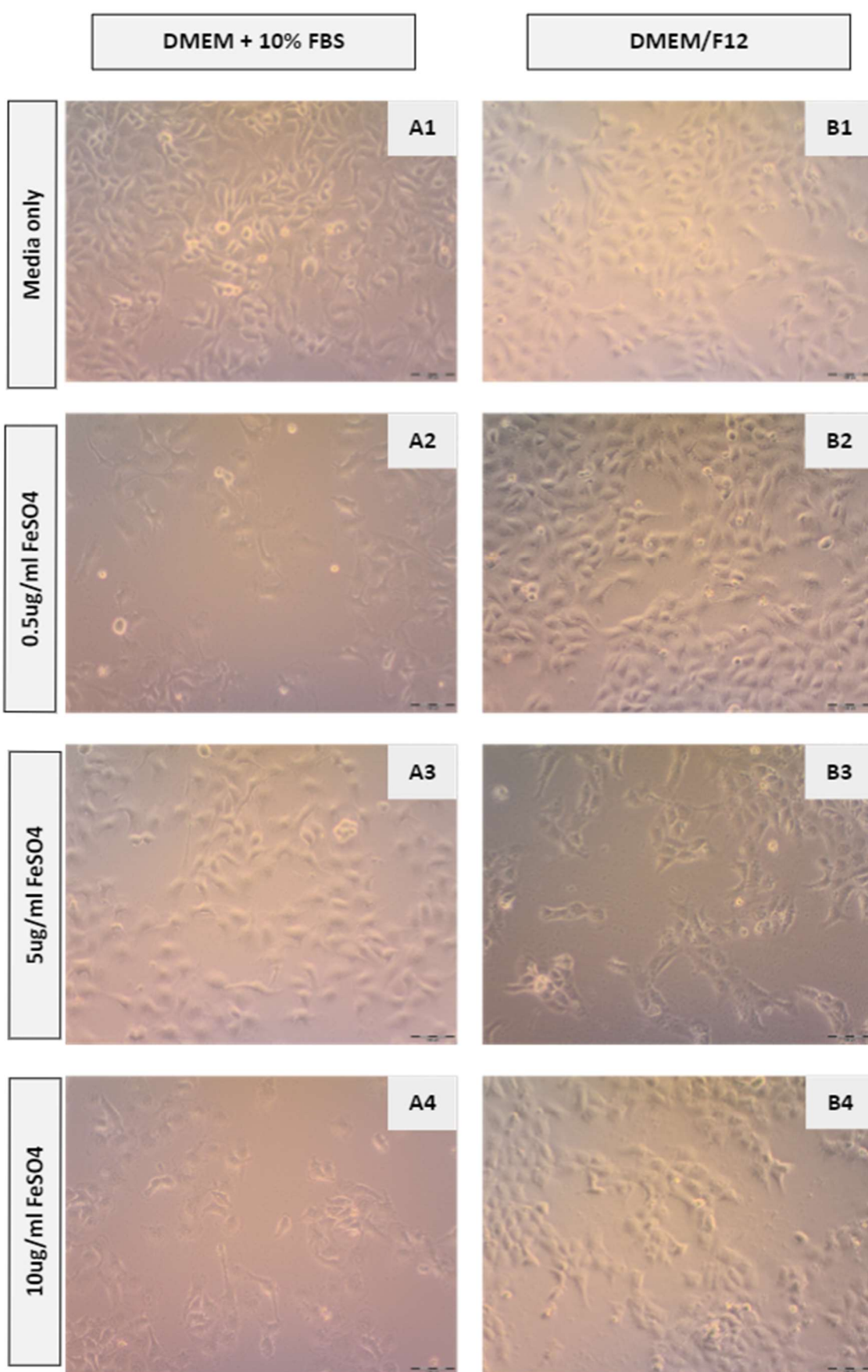
C2: T47D Auto fluorescence



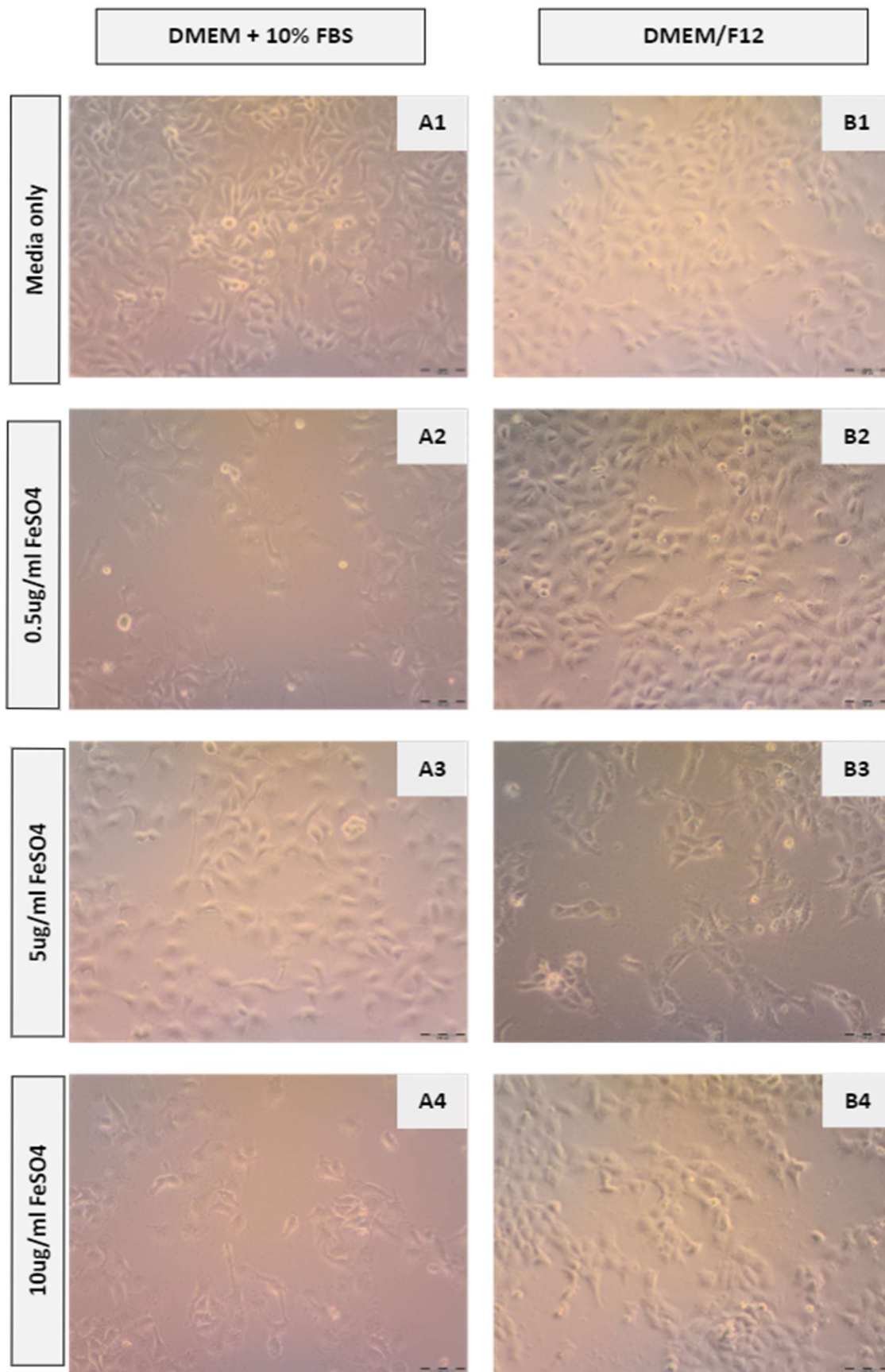
D: Optimising the detachment Media

Cell detachment methods include the usage of trypsin-EDTA, Accutase or EDTA-serum free media. While maintaining cell viability, cells are detached via mechanical mechanisms combined with calcium chelator or the enzymatic cleavage of adhesion proteins (Lai et al., 2022). However, some detachment methods may alter or affect surface proteins and phenotypical changes of the cell. This could thus lead to increased levels of autofluorescence (Lai et al., 2022). Therefore, cell detachment methods could influence the flow cytometry results during verification. Thus, it is essential to test these various detachment methods (Lai et al., 2022). Trypsin-EDTA detaches cell via peptide cleavage when lysin or arginine residues destroys cell surface proteins base on the incubation time. EDTA is a metal chelator and enhances detachment via the removal of calcium and magnesium from cell surface enhancing trypsin's ability to hydrolyse peptide bonds (Lai et al., 2022) (Lee T-J et al., 2021). Accutase is a gentle detachment method than trypsin. Accutase detachment solution consist of proteolytic and collagenolytic enzymes. EDTA is a calcium chelator that removes calcium ions essential for integrins to maintain cellular adhesion.

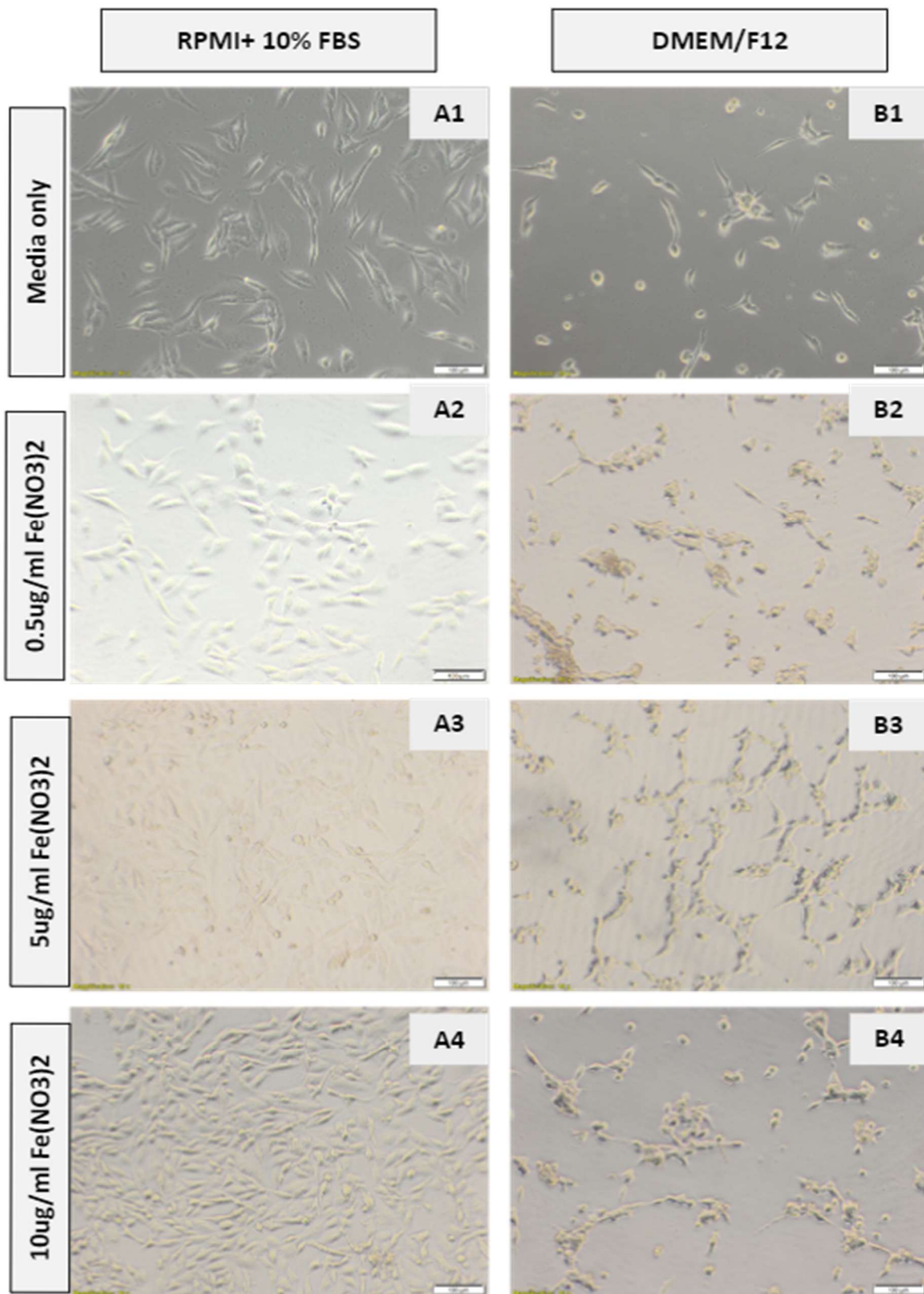
D1: Day 1 Fe(NO₃)₂ MCF7



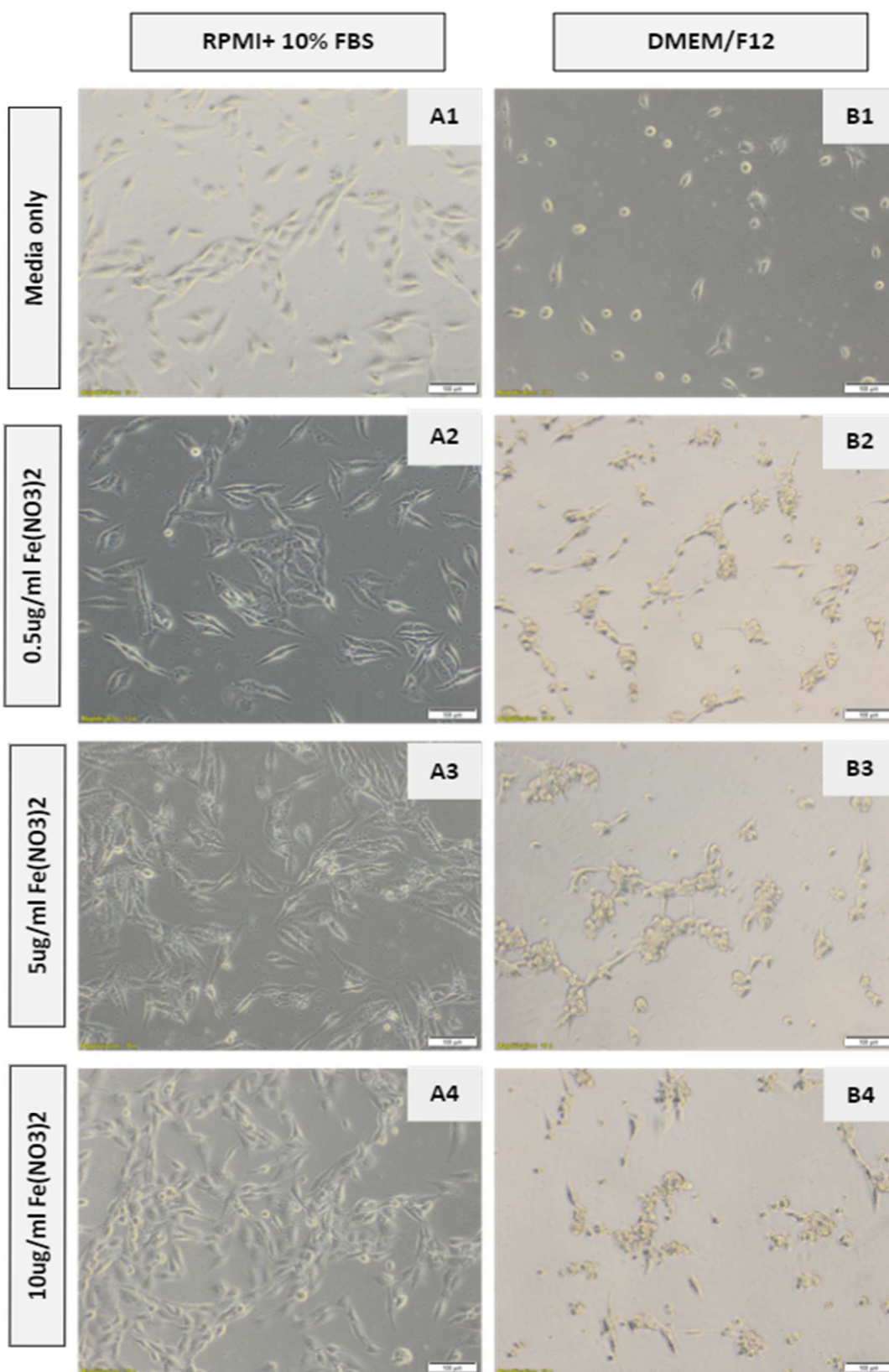
D2: Day 1 FeSO₄ MCF7



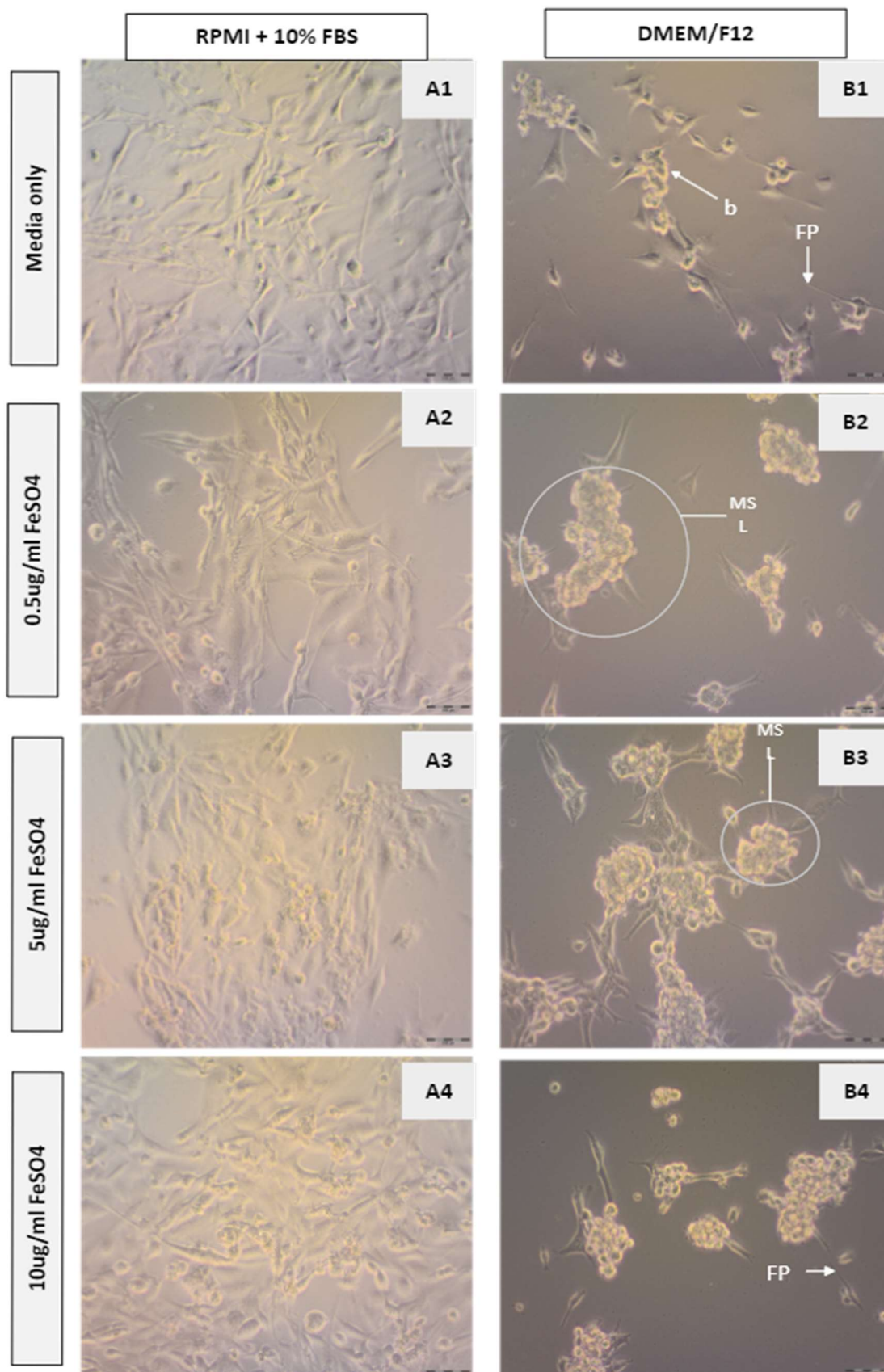
D3:Day 1 Fe(NO₃)₂ T47D



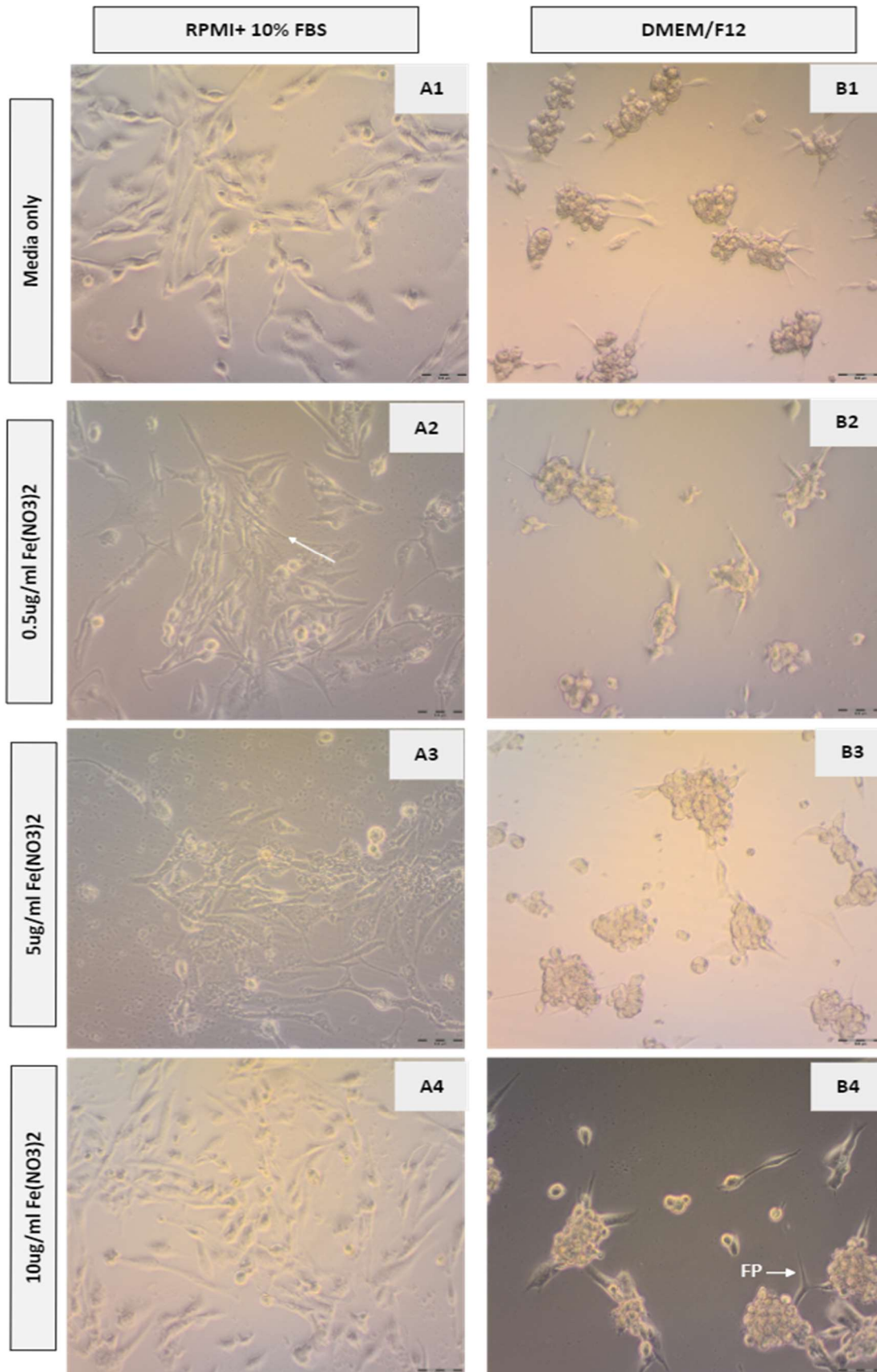
D4: Day 1 FeSO₄ T47D



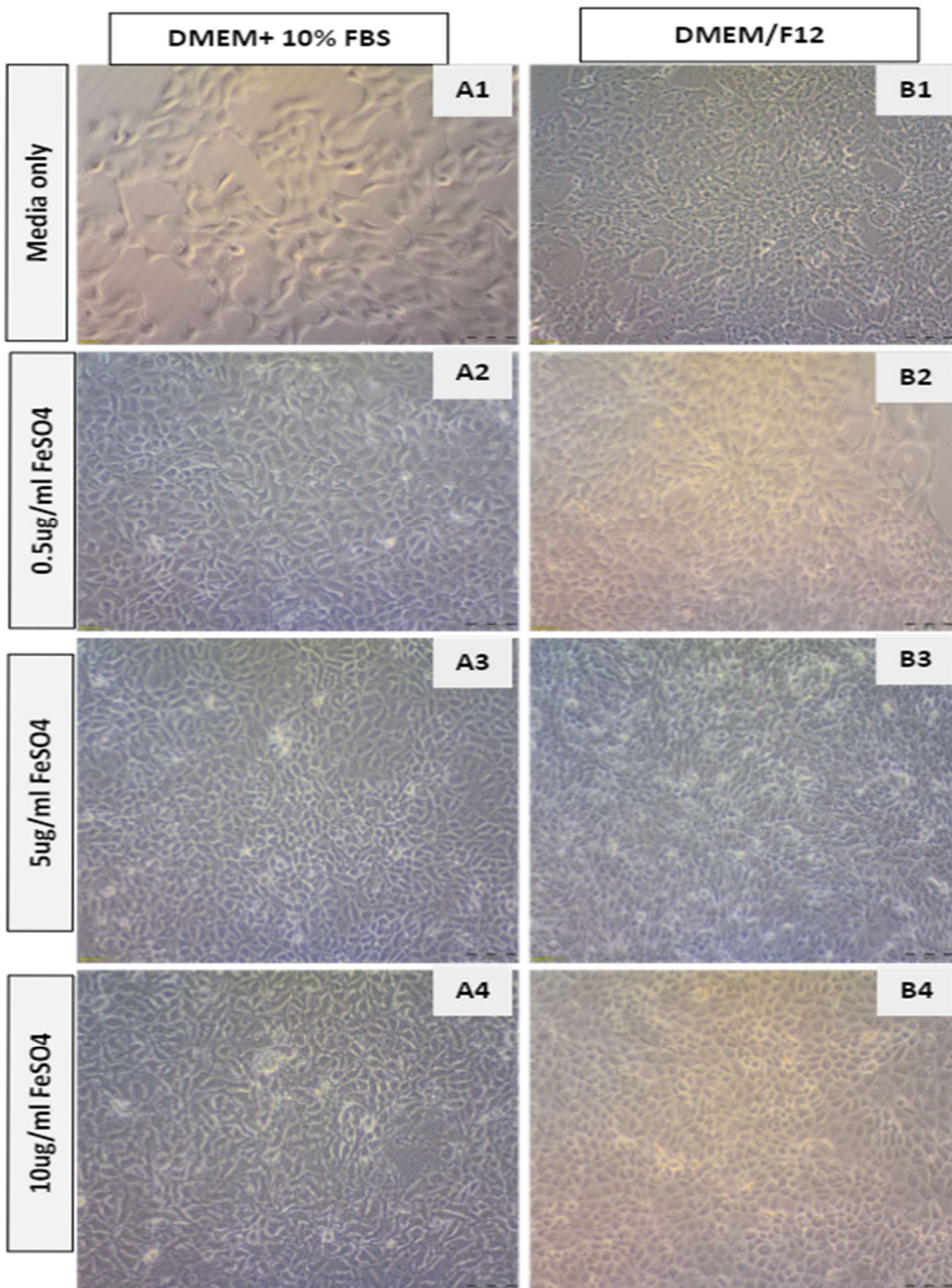
D5: Day 3 FeSO₄ T47D



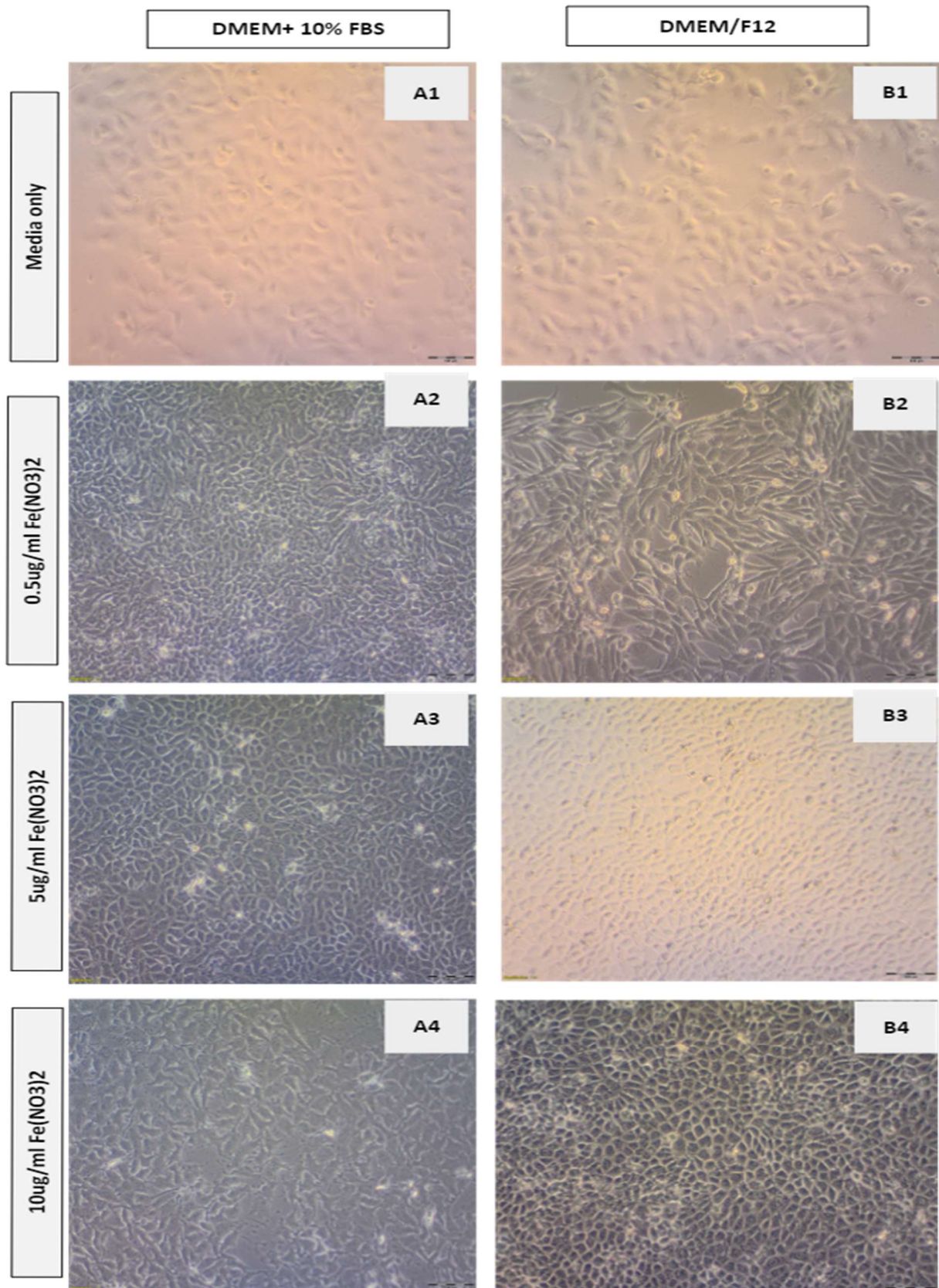
D6:Day 3 $\text{Fe}(\text{NO}_3)_2$ T47D



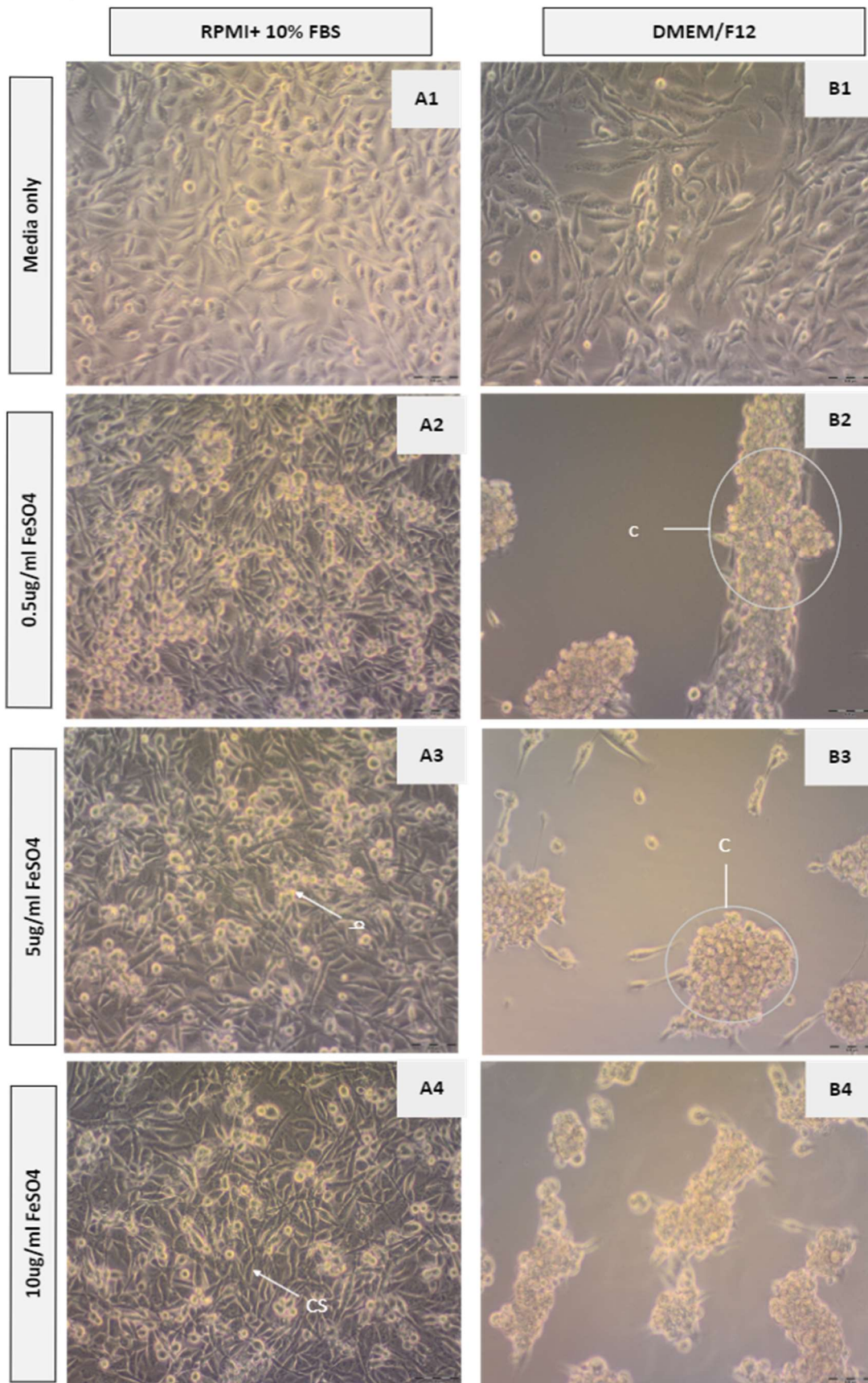
D7:Day 3 MCF7 FeSO4



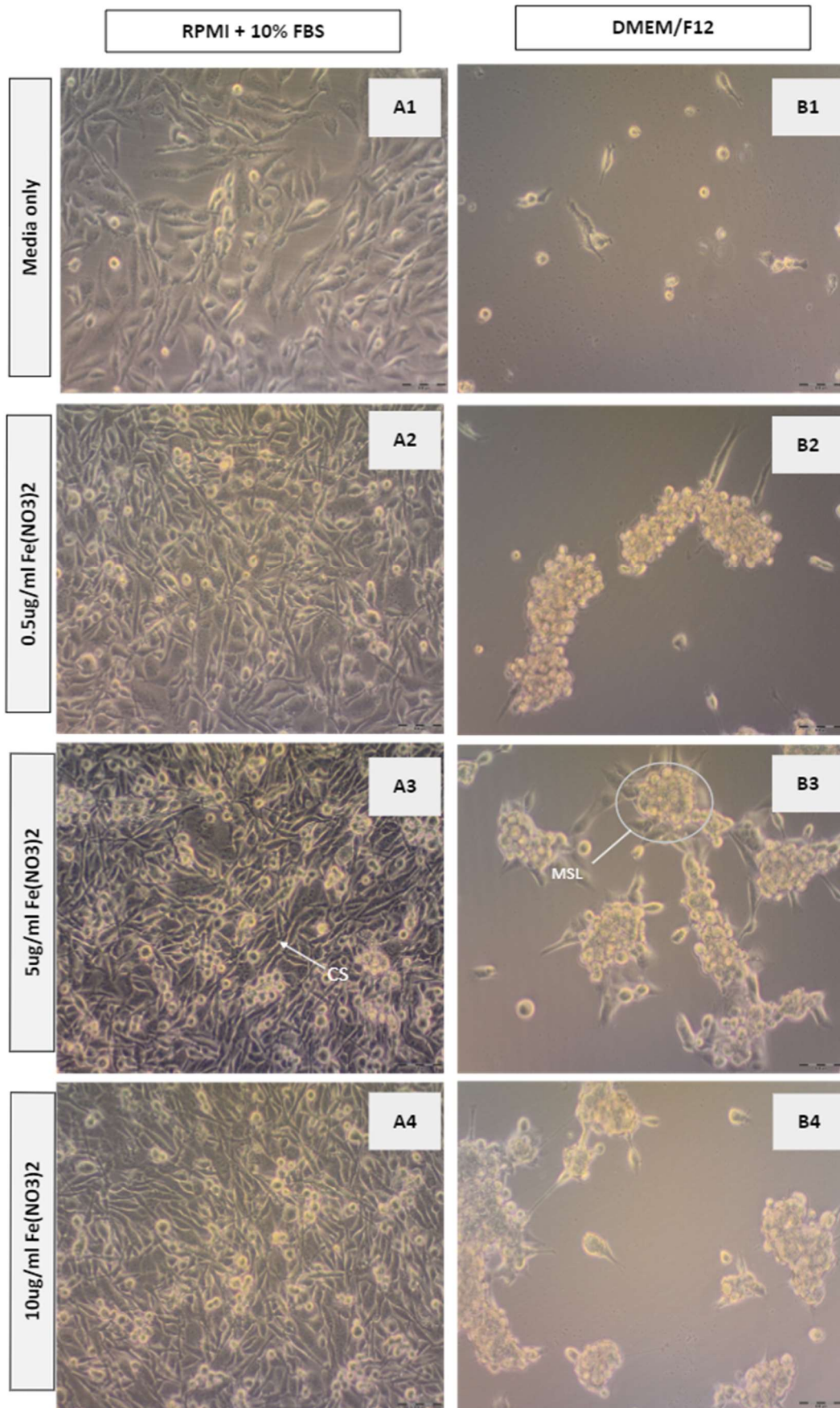
D8: Day 3 $\text{Fe}(\text{NO}_3)_2$ MCF7



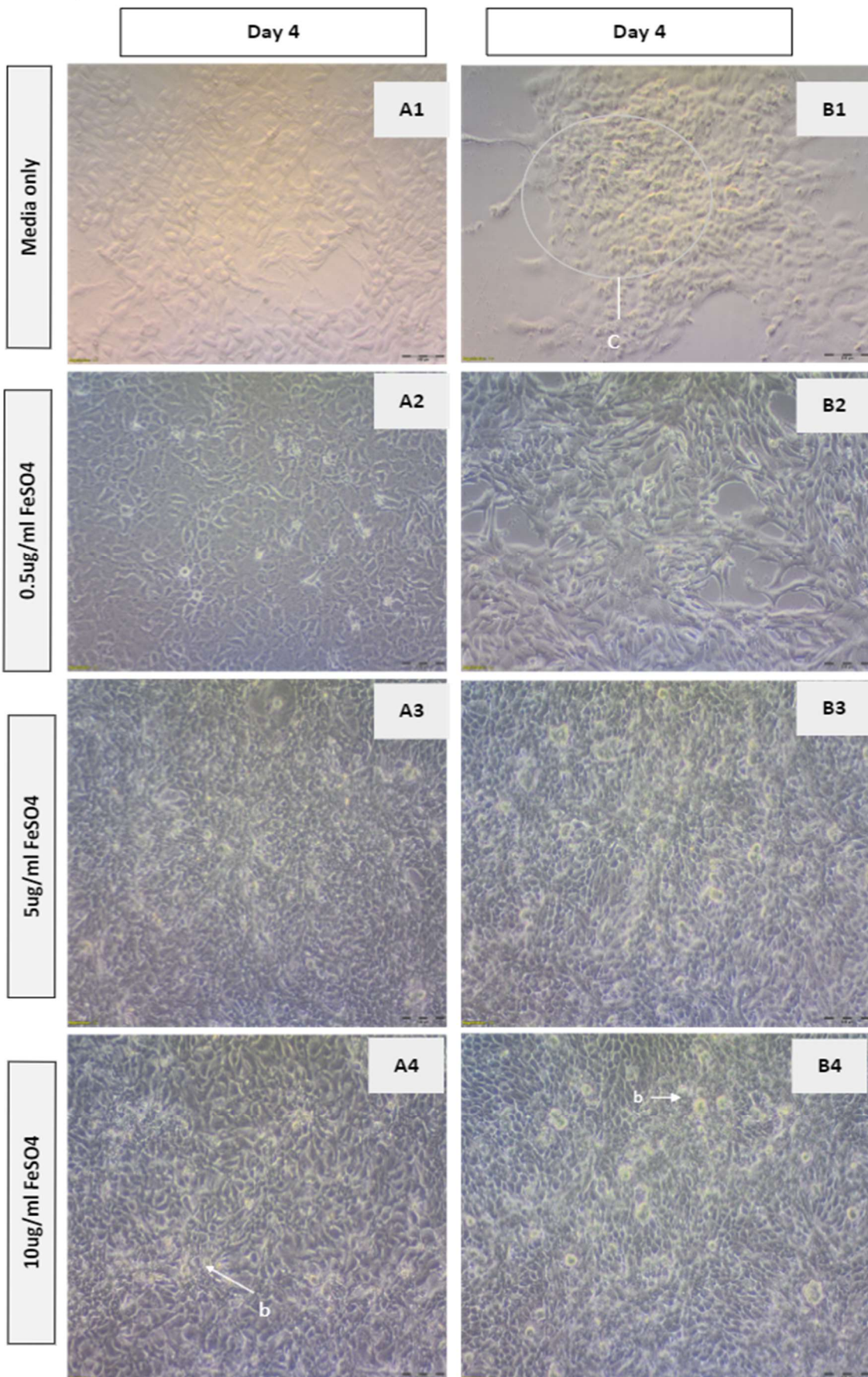
D9:Day 4 FeSO4 T47D



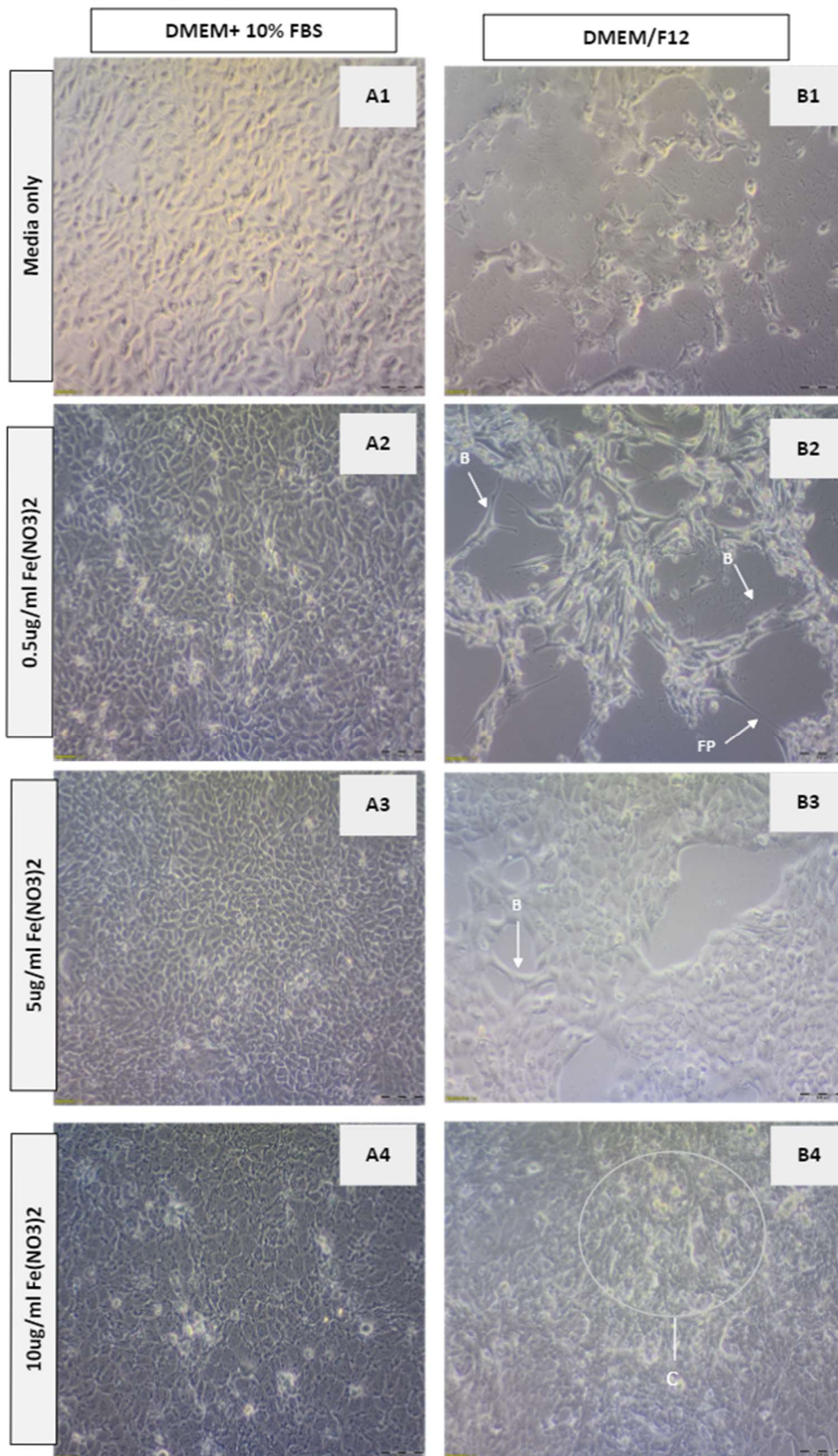
D10:Day 4 Fe(NO3)2 T47D



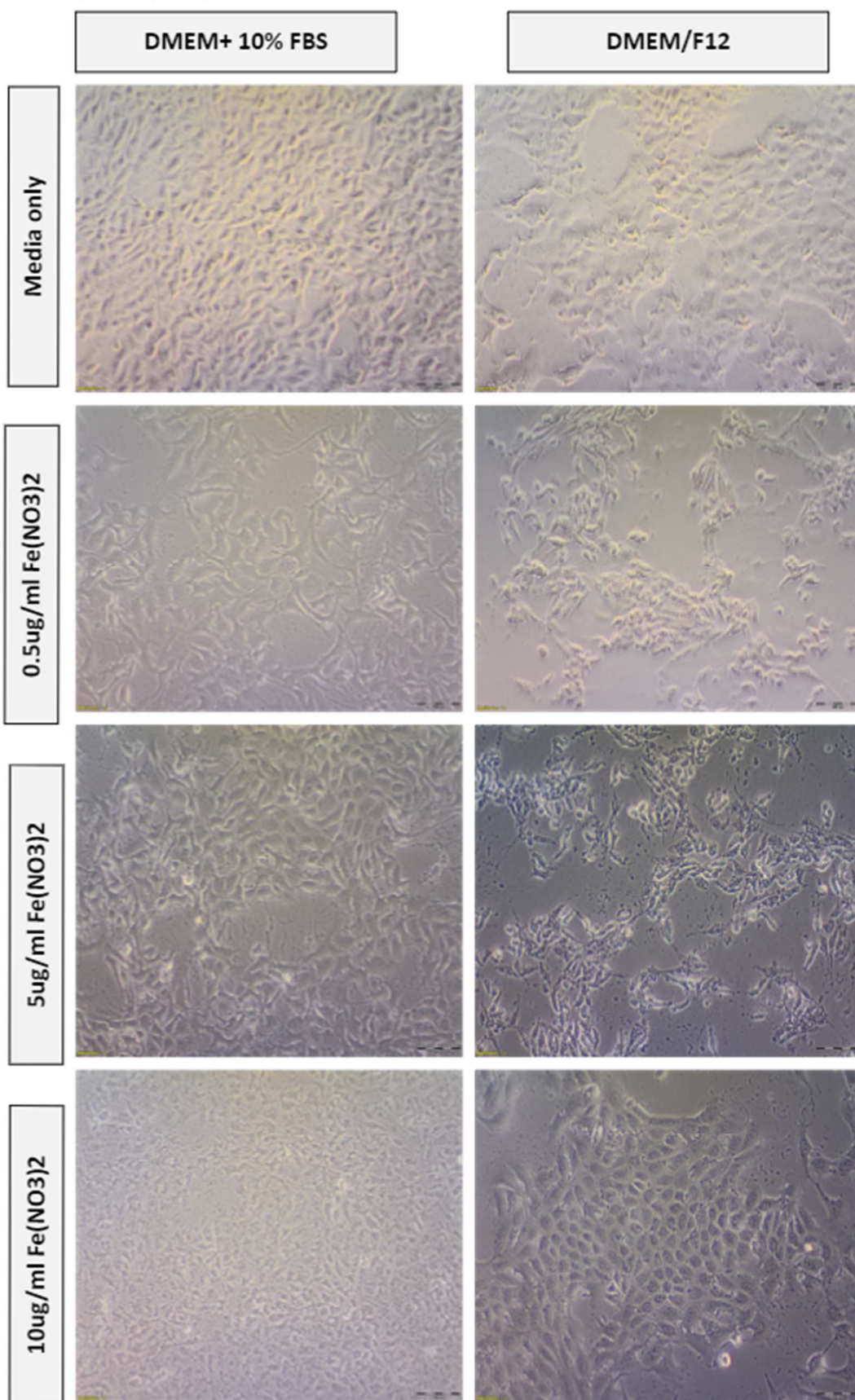
D11:Day 4 FeSO4 MCF7



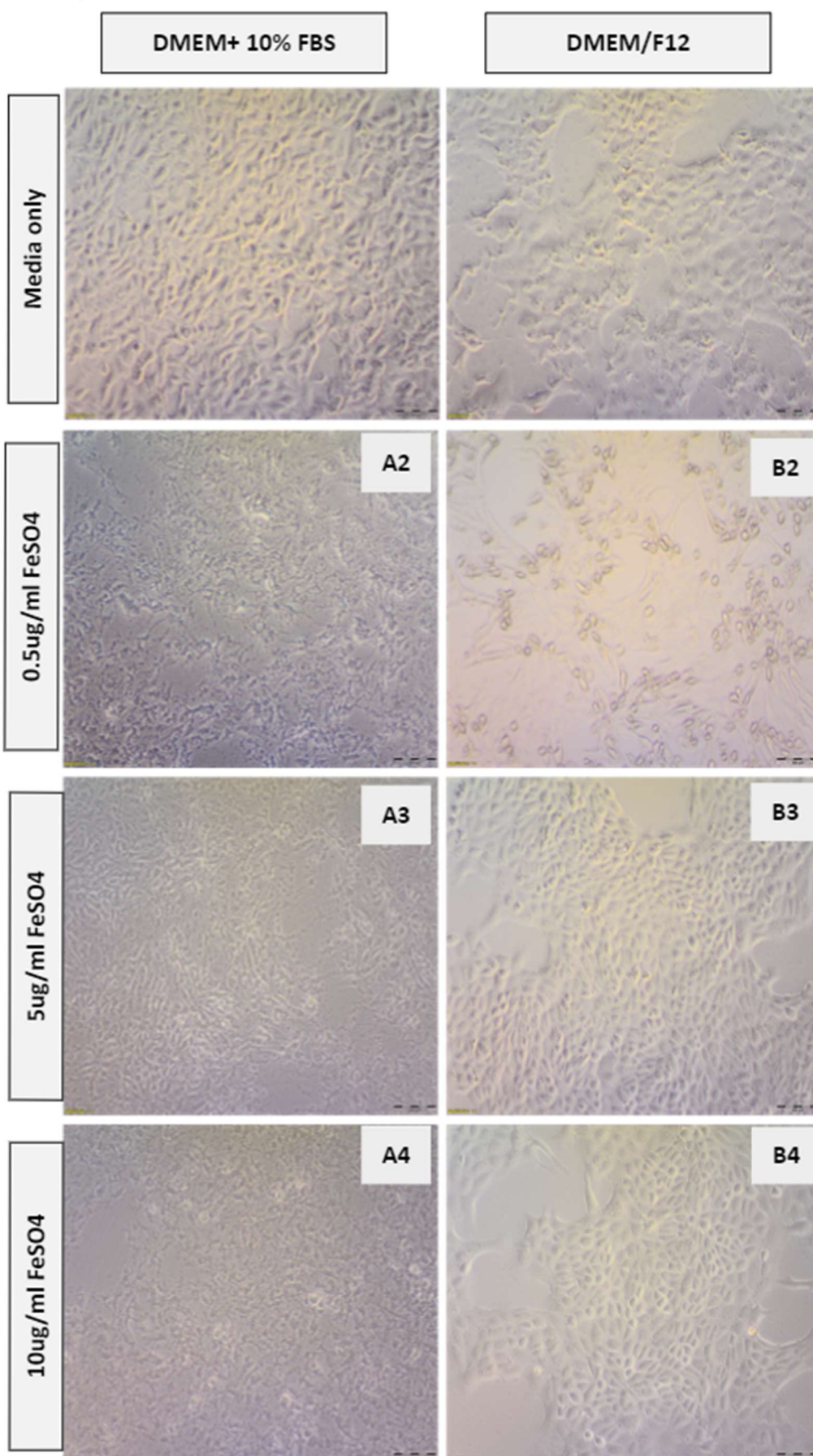
D12: Day 4 Fe(NO₃)₂ MCF7



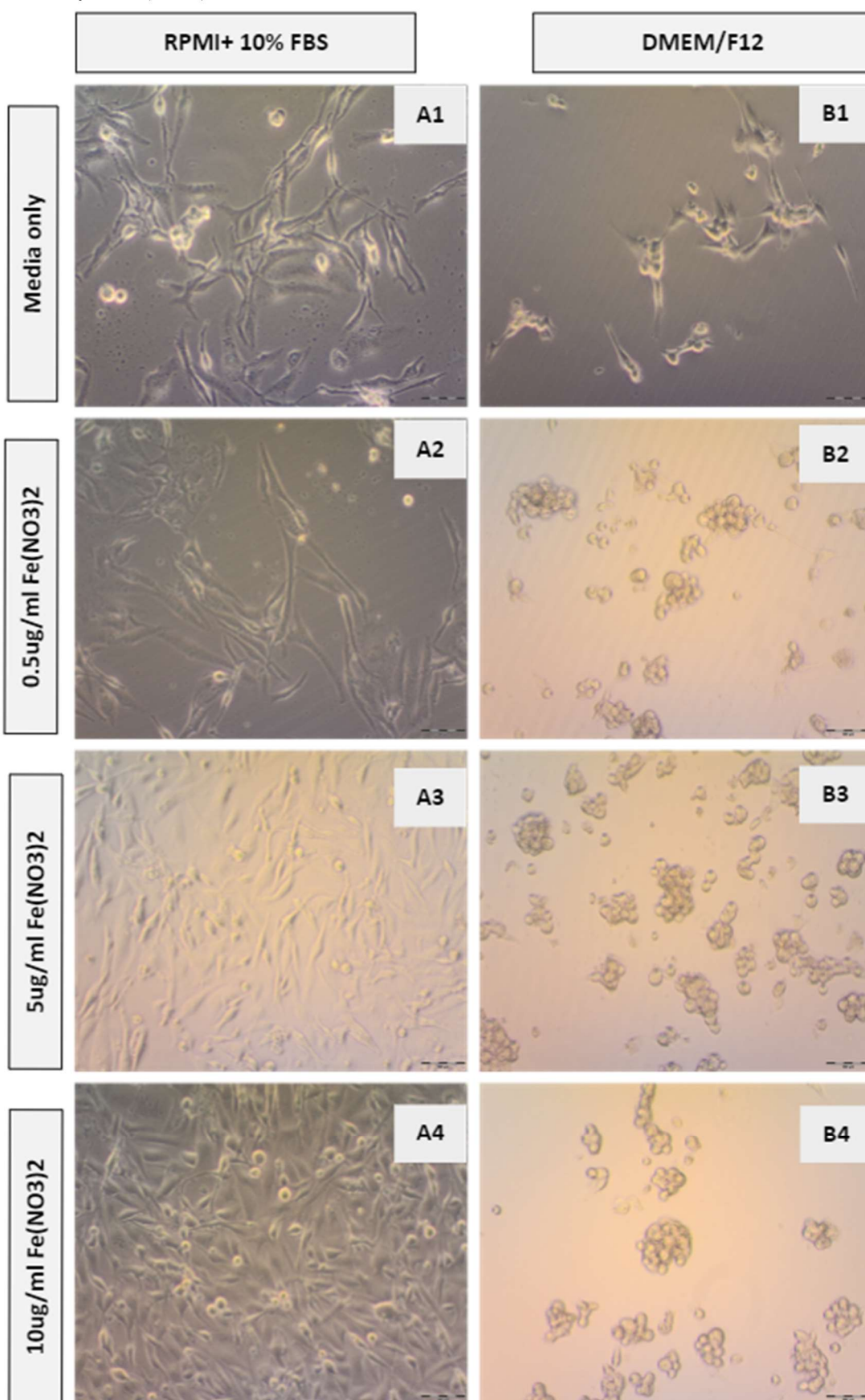
D13:Day 5 Fe(NO₃)₂ MCF7



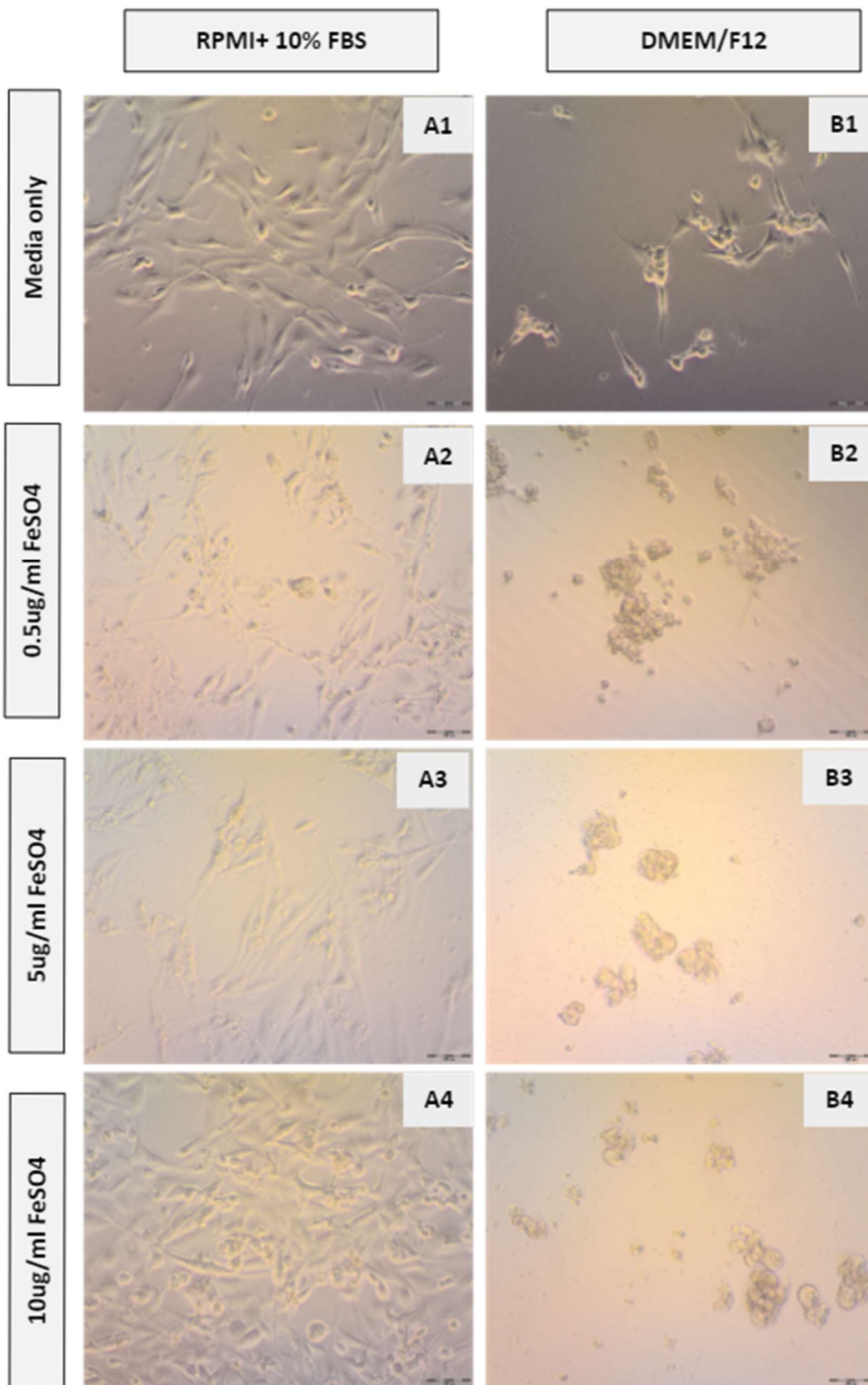
D14:Day 5 FeSO₄ MCF7



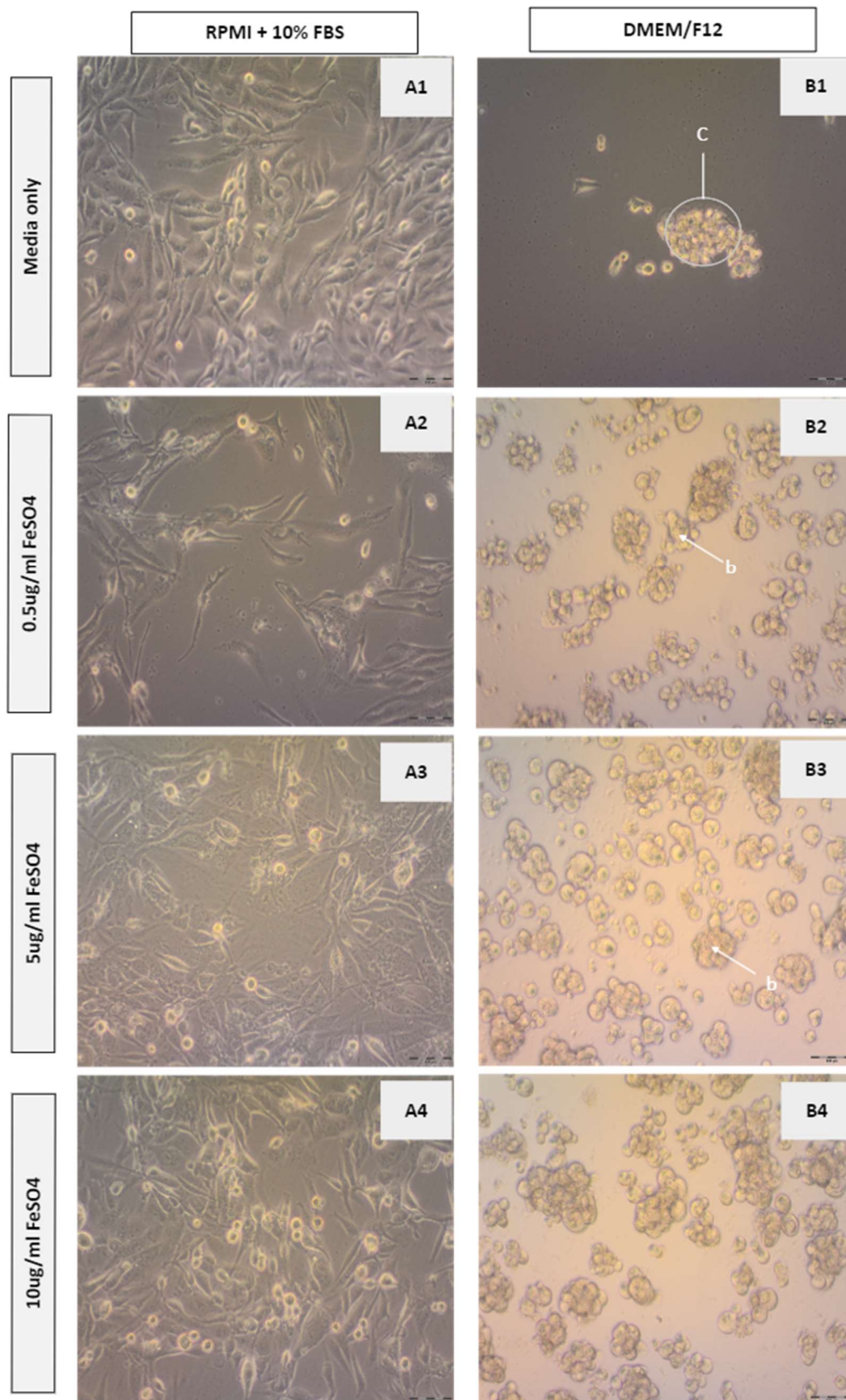
D14:Day 5 Fe(NO₃)₂ T47D



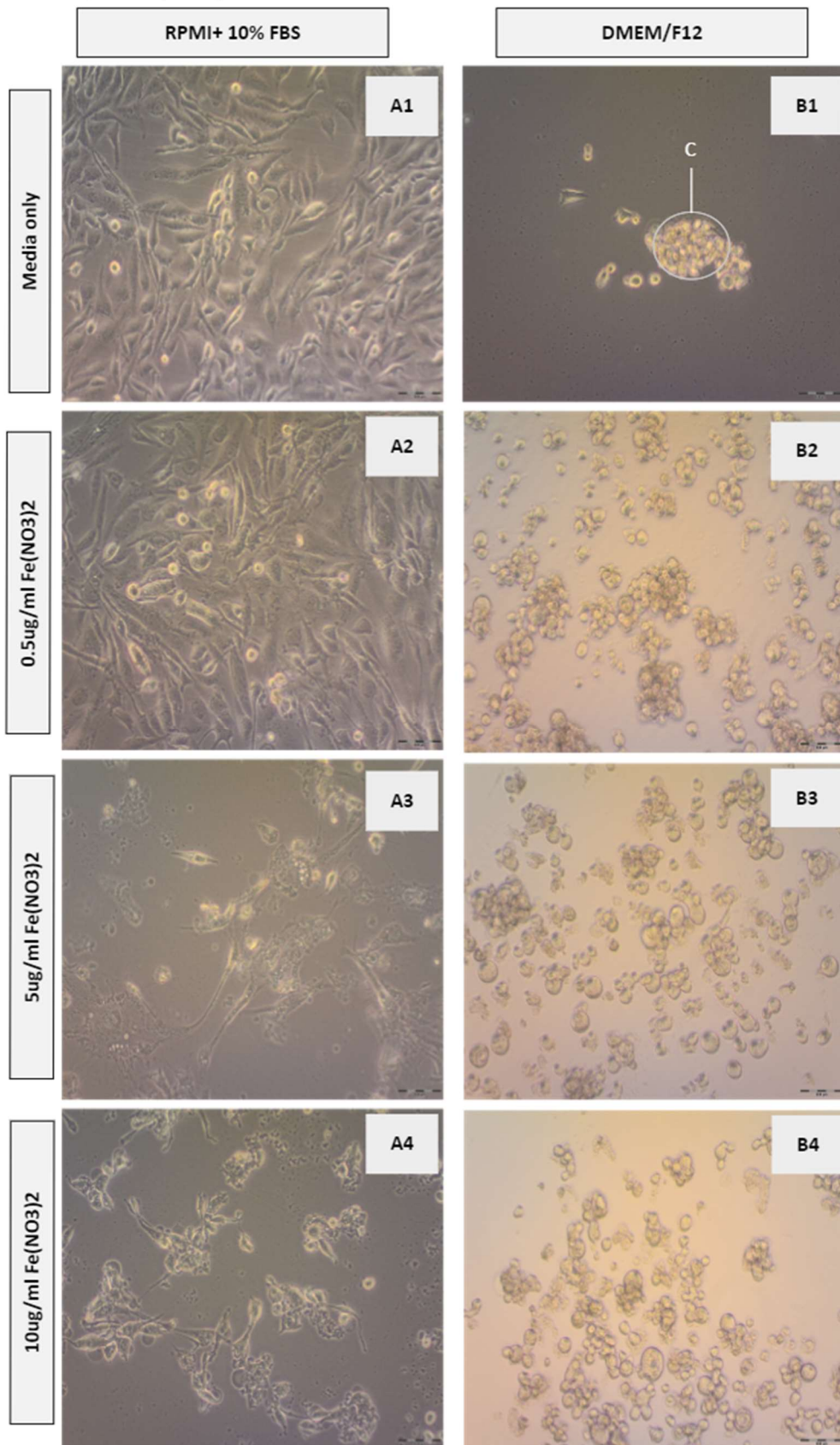
D15:Day 5 FeSO4 T47D



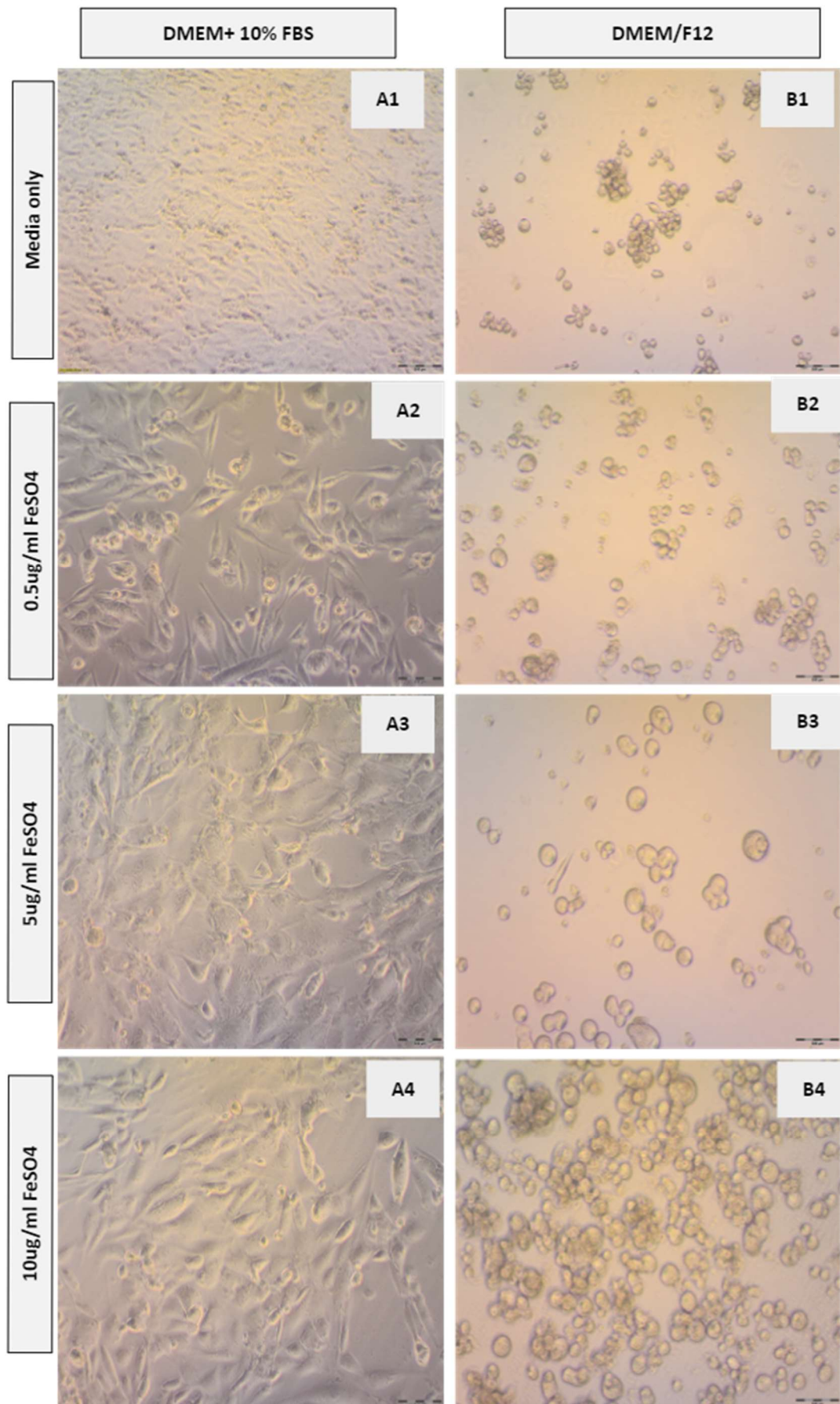
D16:Day 6 FeSO4 T47D



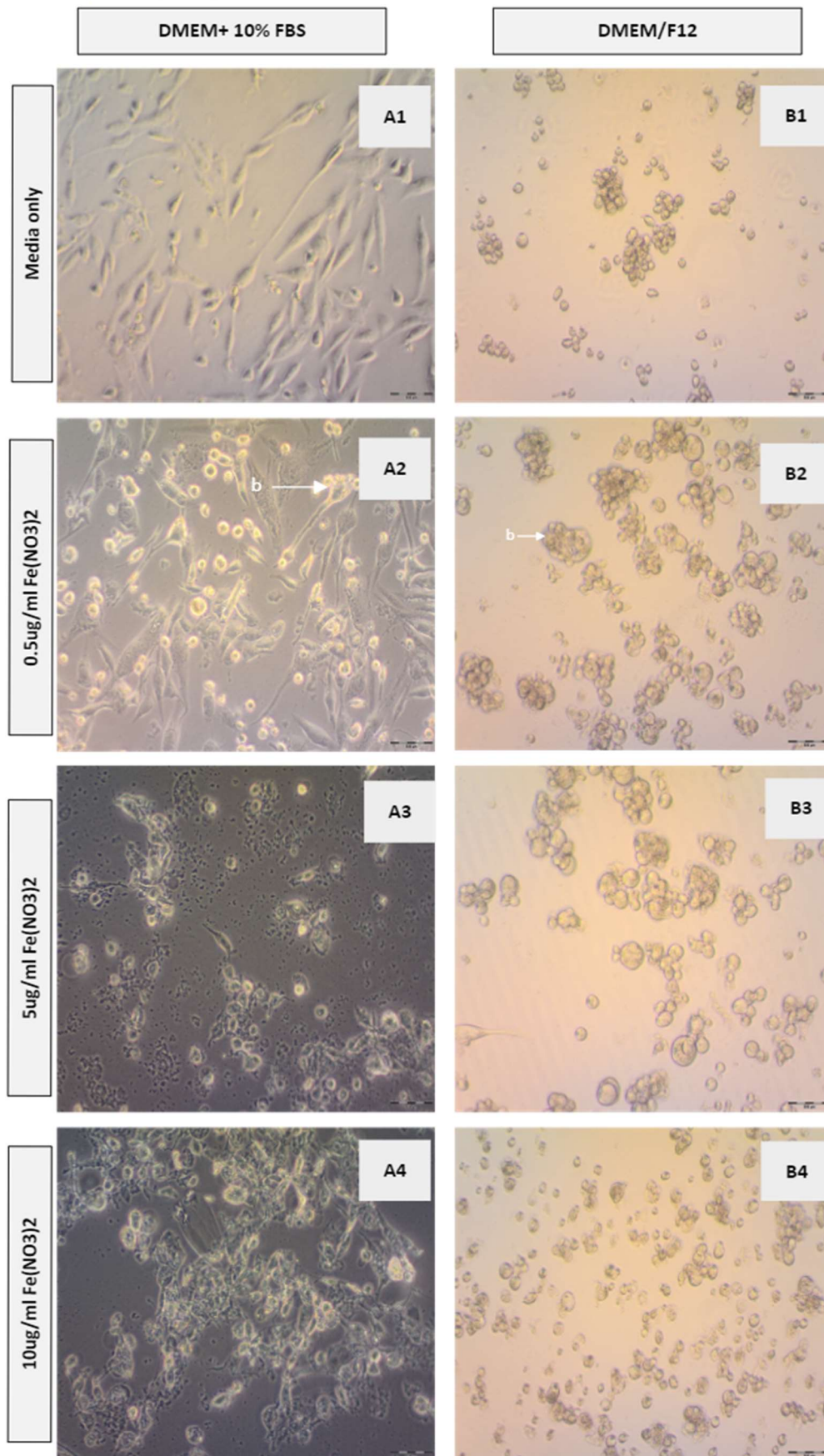
D17:Day 6 Fe(NO₃)₂ T47D



D18:Day 6 FeSO4 MCF7



D19:Day 6 Fe(NO₃)₂ MCF7



E: Titration of flow antibodies

Antibody titration is an essential factor for panel optimization. Its useful in optimizing resolution and achieving accurate results for population identification and expression level measurements. Its also important in sufficient staining and the reduction of non-specific binding. The Stain Index is used in titration to measure the brightness of different fluorochromes ensuring that optimal concentrations of the antibody is used. It is established by estimating the relationship between positive and negative background signals. The stain index is defined as the ratio of the separation between the positive and negative population divided by two times the standard deviation of the negative population. Once the stain index of each tested concentration is calculated using the formula: Stain index= (Median of positive-Median of negative)/ (SD of negative * 2), a graph of stain index against antibody concentration is then constructed and the concentration with the highest peak (highest stain index) is the optimal one.

$$\text{Stain Index} = \frac{(\text{Median of positive}) - (\text{Median of Negative})}{(\text{SD of negative} * 2)}$$

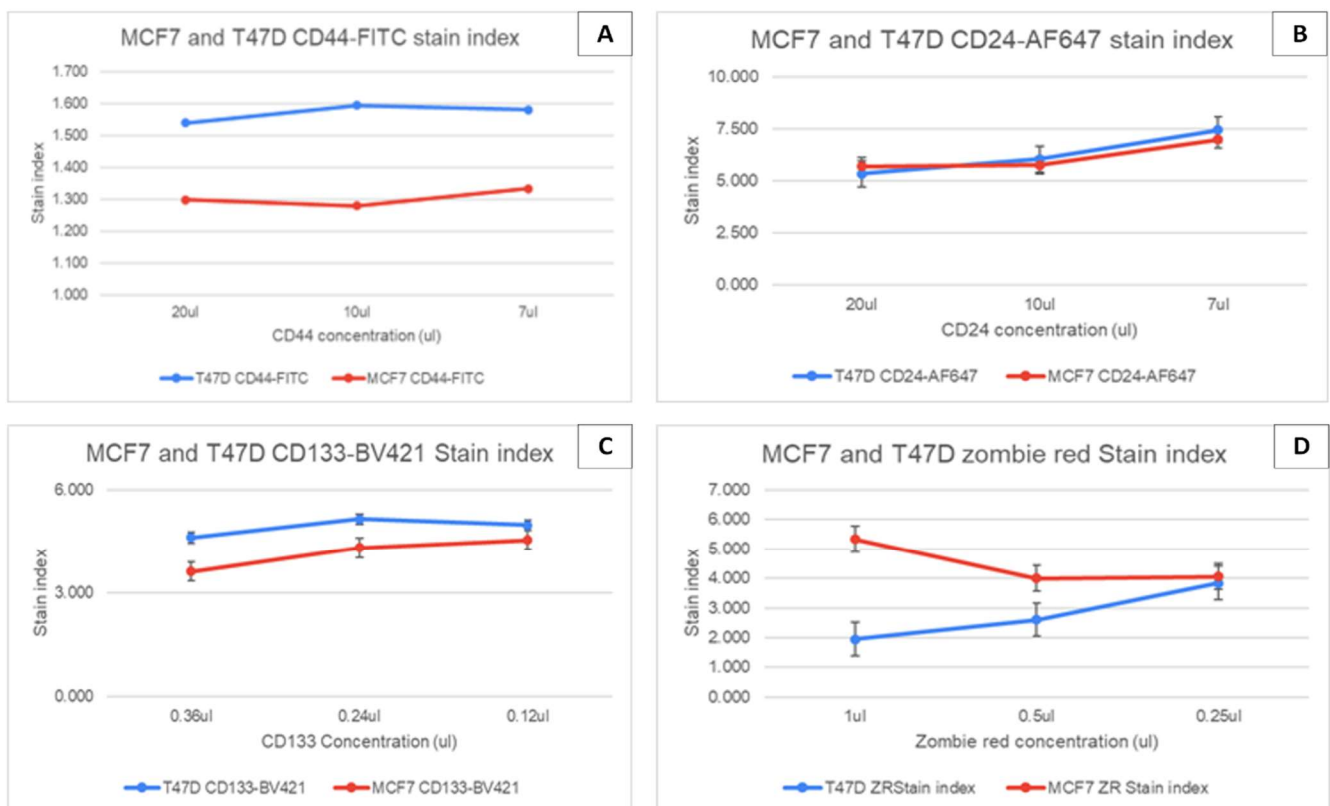


Figure E1: Concentrations 7µl were chosen for both CD44 an CD24 (A and C) as both concentrations for each marker revealed optimal stain index. Concentration 0.12µl was

chosen for CD133 and 1 μ l for zombie red. Concentrations were chosen based on whether is was optimal for both cell lines and trying to keep concentrations consistent throughout antibodies

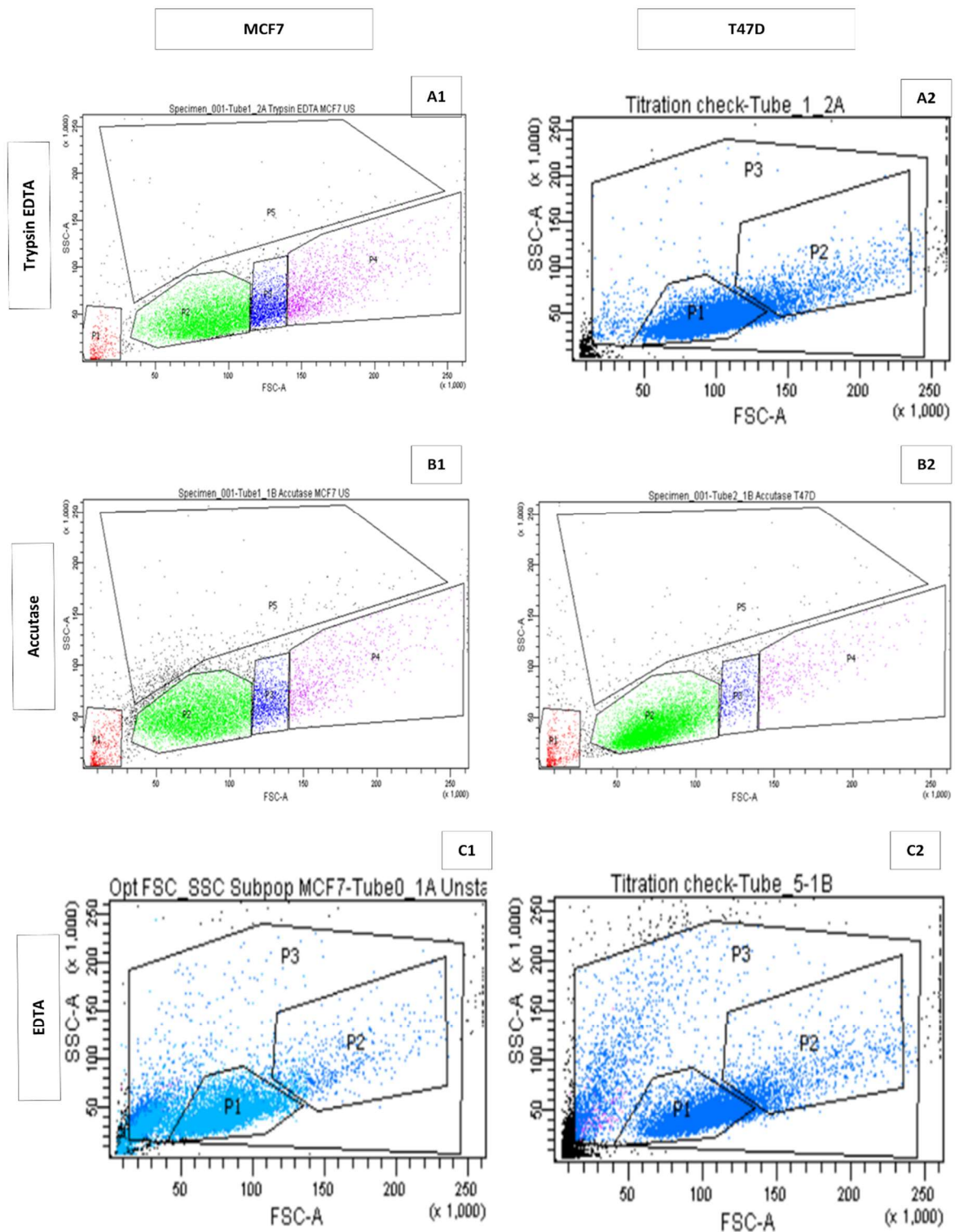
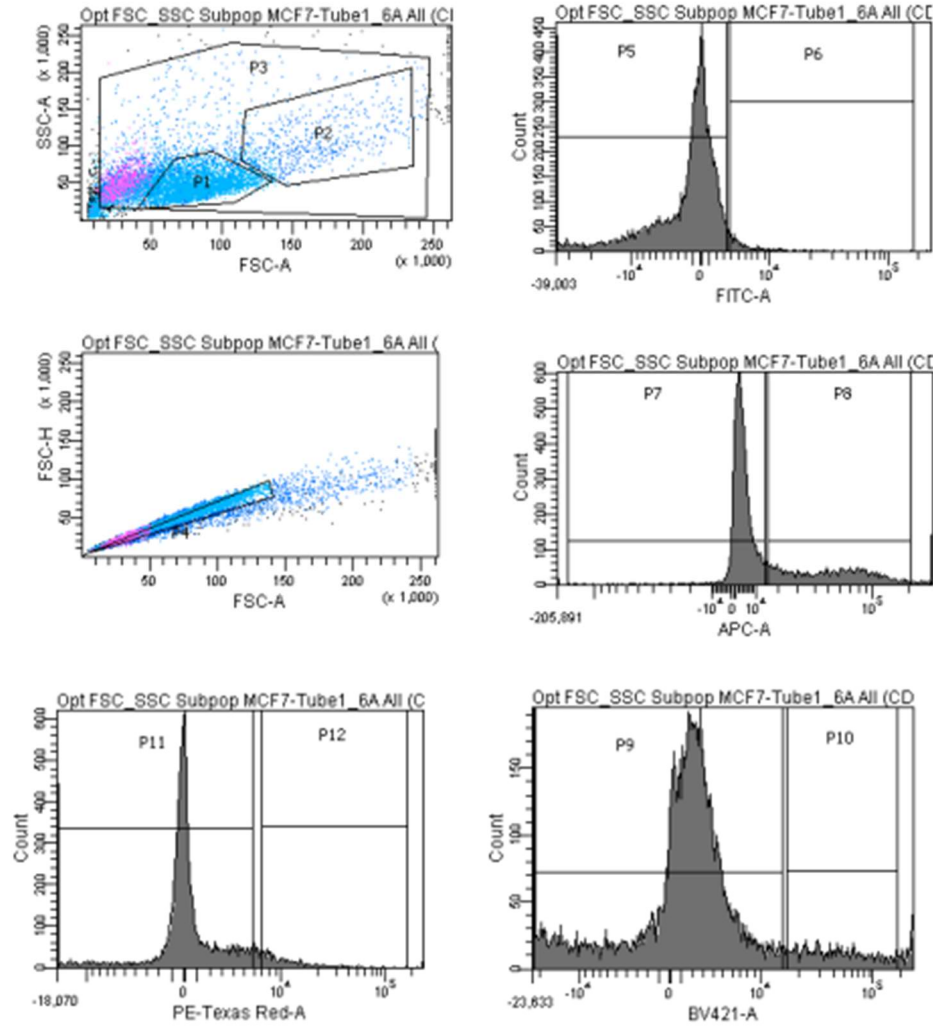


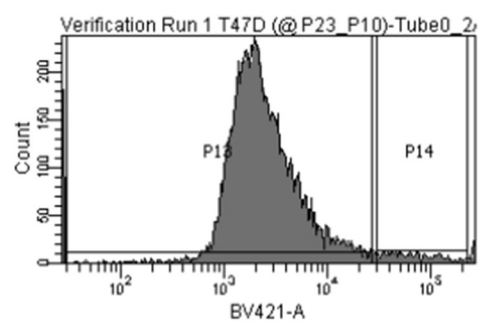
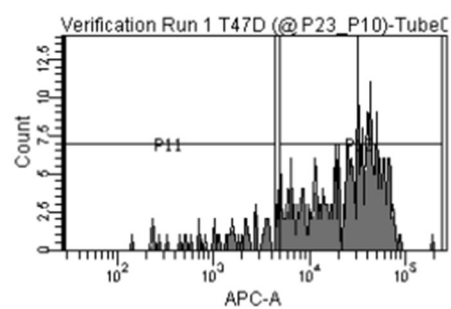
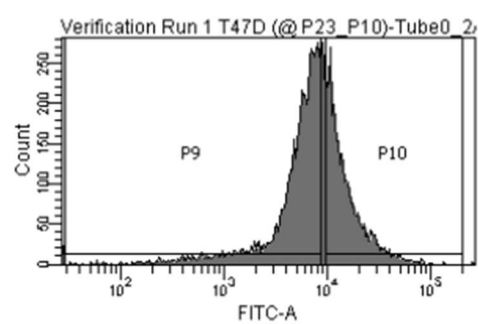
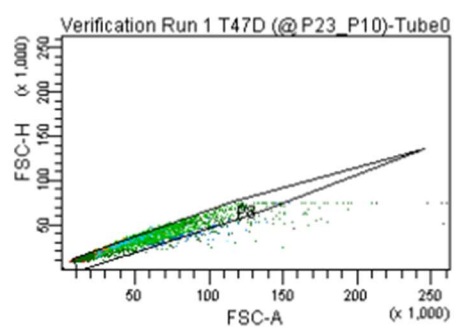
Figure E2: Representing scatter plots of different detachment media for MCF7 and T47D cell lines. For each detachments media there were no significant variations in scatter properties and thus not altering cell size or granularity. We therefore don't suspect that detachment media will influence autofluorescence between each other and thus we chose to use trypsin for experiments.

F: Compensation controls to correct spectral overlap in the flow cytometer

F1: MCF cells

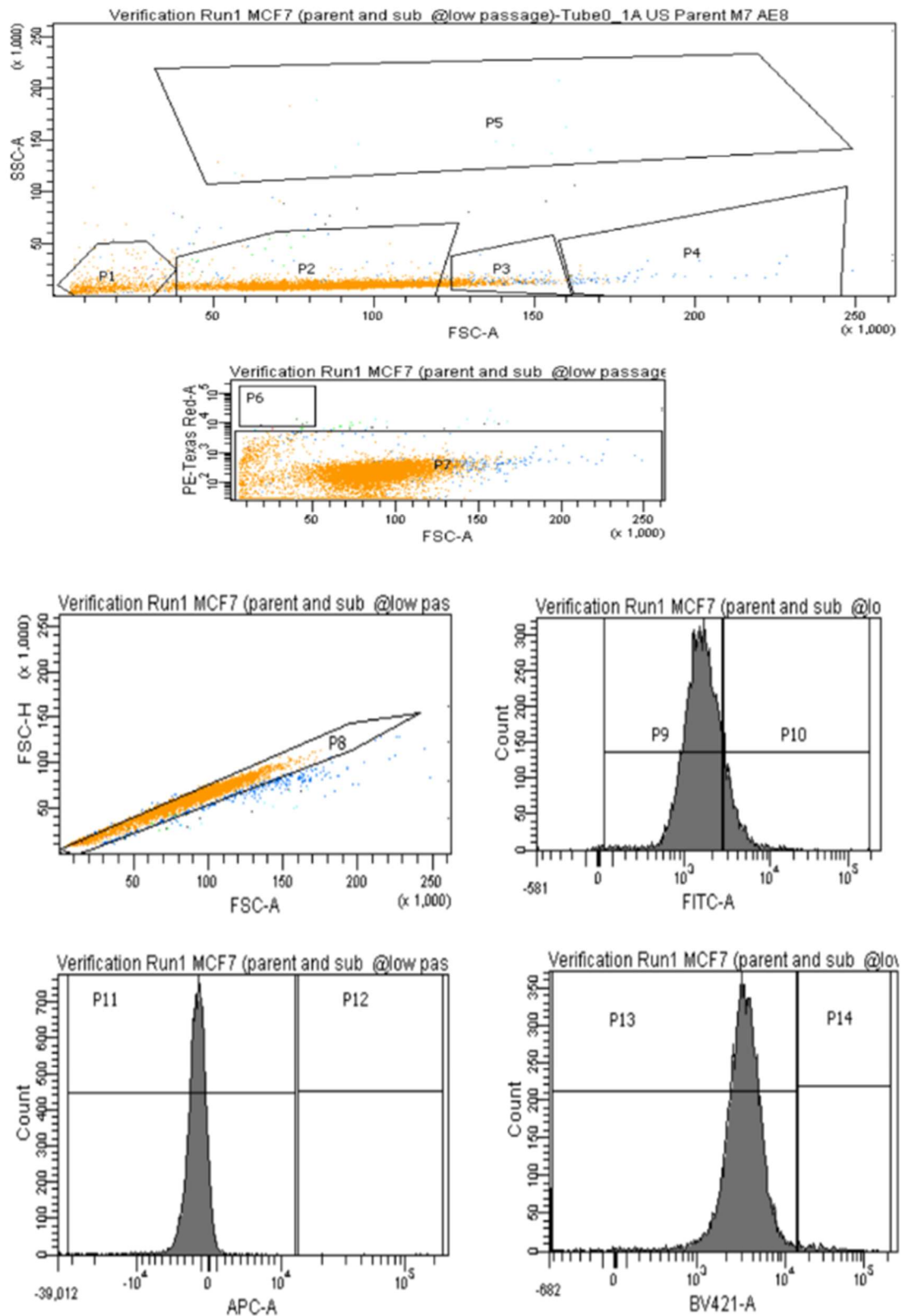


F2: T47D cells



G: Verification gating strategy

G1.1: Gating strategy

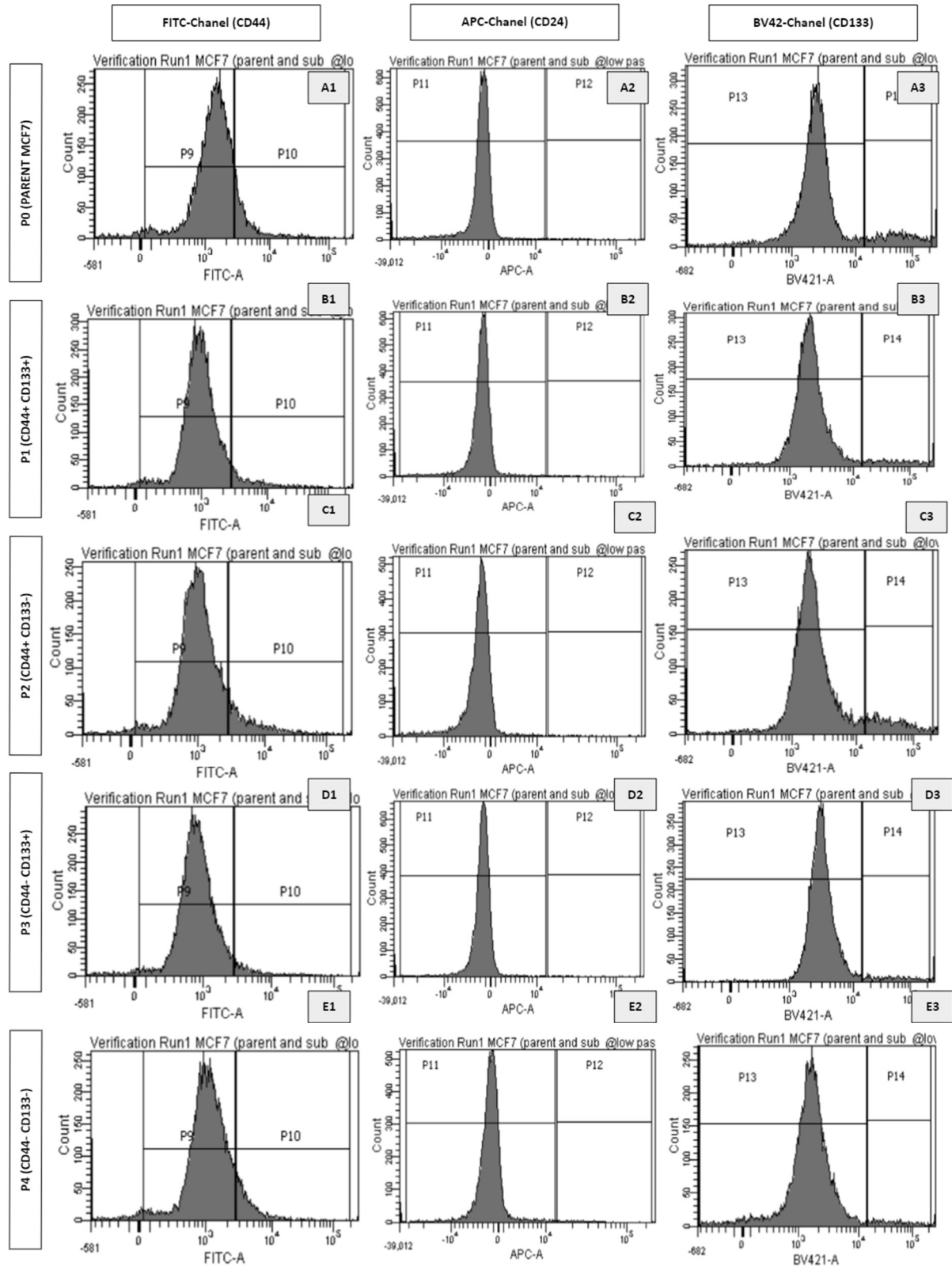


G1.1: Population hierarchy

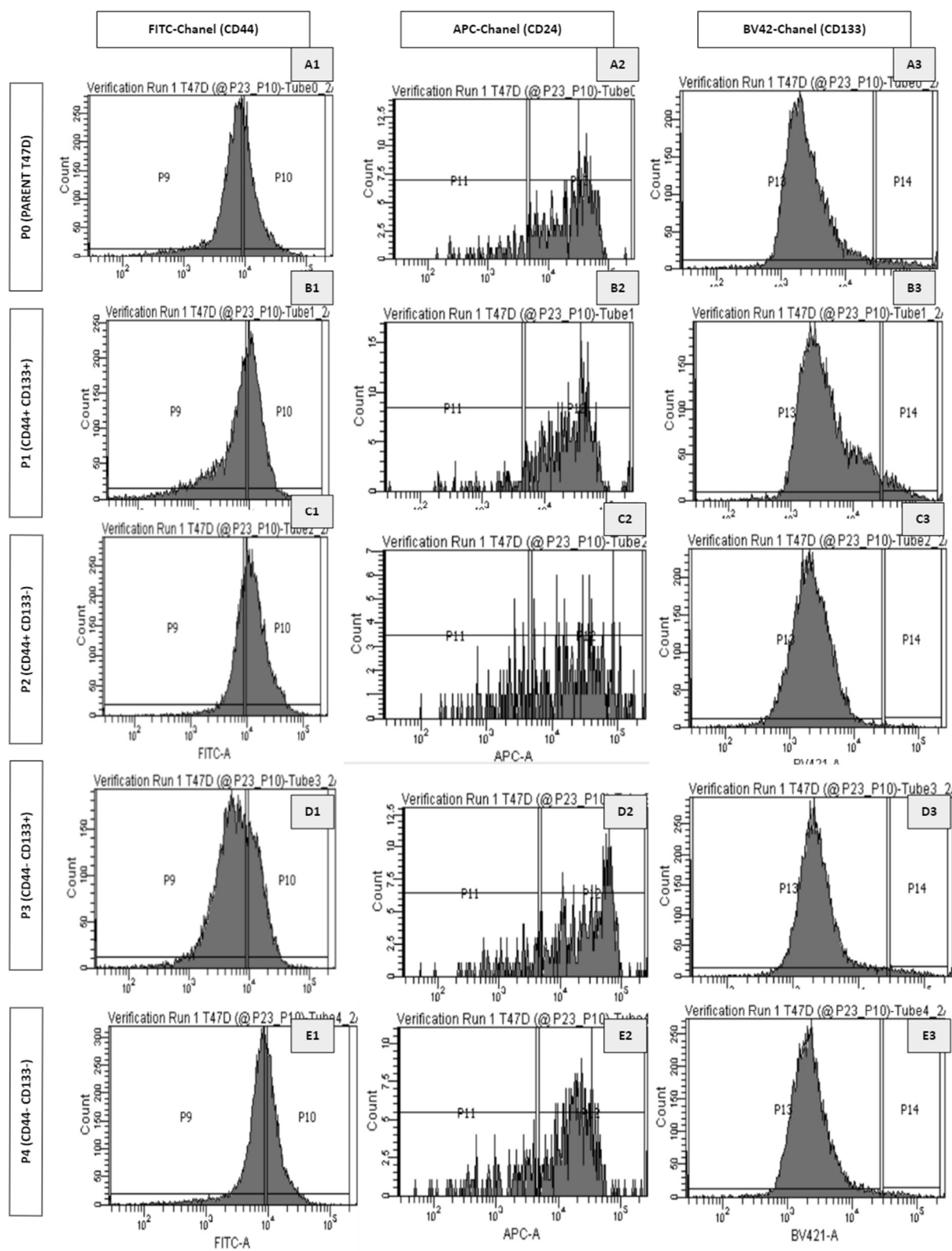
Tube: Tube0_1A US Parent T47 AE8			
Population	#Events	%Parent	%Total
■ All Events	10,000	####	100.0
■ P1	835	8.4	8.4
■ P2	6,778	67.8	67.8
■ P3	750	7.5	7.5
■ P4	486	4.9	4.9
■ P5	228	2.3	2.3
■ P6	522	5.2	5.2
■ P7	8,401	84.0	84.0
■ P8	8,283	98.6	82.8
■ P9	8,283	100.0	82.8
■ P10	0	0.0	0.0
■ Q1-3	0	####	0.0
■ Q2-3	0	####	0.0
■ Q3-3	0	####	0.0
■ Q4-3	0	####	0.0
■ P11	0	0.0	0.0
■ Q1-5	0	####	0.0
■ Q2-5	0	####	0.0
■ Q3-5	0	####	0.0
■ Q4-5	0	####	0.0
■ P12	0	0.0	0.0
■ Q1-6	0	####	0.0
■ Q2-6	0	####	0.0
■ Q3-6	0	####	0.0
■ Q4-6	0	####	0.0
■ P13	8,280	100.0	82.8
■ P14	3	0.0	0.0
■ Q1-4	0	0.0	0.0
■ Q2-4	0	0.0	0.0
■ Q3-4	3	100.0	0.0
■ Q4-4	0	0.0	0.0
■ Q1	0	0.0	0.0
■ Q2	0	0.0	0.0
■ Q3	8,283	100.0	82.8
■ Q4	0	0.0	0.0
■ Q1-1	5	0.1	0.0
■ Q2-1	0	0.0	0.0
■ Q3-1	8,278	99.9	82.8
■ Q4-1	0	0.0	0.0
■ Q1-2	0	0.0	0.0
■ Q2-2	0	0.0	0.0
■ Q3-2	8,264	99.8	82.6
■ Q4-2	19	0.2	0.2

G2: Flow graphs (data)

Verification of stemness markers on all subpopulations in MCF7



Verification of stemness markers on all subpopulations in T47D



Appendix VI: Immunocytochemistry to localise proteins

A: cell preparation, drug preparation and buffers

A1: Coating coverslips with Poly-d-lysine:

- Glass coverslips were placed inside 24-well plates
- 50 µl of poly-d-lysine was added to each coverslip and incubated for 5 mins at room temperature (RT)
- Glass coverslips were rinsed three times with distilled water and
- Coverslips were left to dry for at least 2 hrs at RT

A2: Drug preparation

Tamoxifen:

Need 2µM Tamoxifen treatment solution.

Recommended dilution: 1mg/1ml DMSO= 0.01g/0.001l

Molecular weight: 371.51g/mol

Dissolve 0.001g of Tam powder into 1ml of DMSO

$C_1 = m/M_w \times v$

$C_1 = (0.001g) / (371.51g/mol) (0.001l)$

$C_1 = 0.0027M = 2700\mu M$

Volume needed per run= 2000µl

- $C_1V_1 = C_2V_2$
- $V_1 = \frac{C_2V_2}{C_1}$
- $= \frac{(2\mu M)(2000\mu l)}{2700\mu M}$
- $= 1.48\mu l$

Anastrozole:

Stock 1: 1mg/1ml of Anastrozole dissolved in 100% DMSO

Stock 2: $C_1V_1 = C_2V_2$

$(1mg/ml)(V_1) = (0.1mg/ml)(5ml)$

$V_1 = 500\mu l$

500µl in 3500µl of media

Stock 3: $C_1V_1 = C_2V_2$

$$(0.1\text{mg/ml})(V_1) = (0.01\text{mg/ml})(5\text{ml})$$

$$V_1 = 500\mu\text{l}$$

500 μl in 3500 μl of media

Stock 4: $C_1V_1 = C_2V_2$

$$(0.01\text{mg/ml})(V_1) = (0.001\text{mg/ml})(5\text{ml})$$

$$V_1 = 500\mu\text{l}$$

500 μl in 3500 μl of media

(working stock) Stock 5: $C_1V_1 = C_2V_2$

$$(0.001\text{mg/ml})(V_1) = (0.0001\text{mg/ml})(4000\mu\text{l})$$

$$V_1 = 400\mu\text{l}$$

400 μl in 3600 μl of media

A3: 4% Paraformaldehyde (Fixative)

4% Paraformaldehyde is measured and dissolved in 96% distilled water.

- Dissolve 4g of paraformaldehyde in 50 ml of distilled water on a heated plate
- Once fully dissolved top up with 46 ml of distilled water

A4: 0.2% triton x-100 in PBS (Permeabilisation buffer)

- 100 μl is added to 49.59 ml of PBS

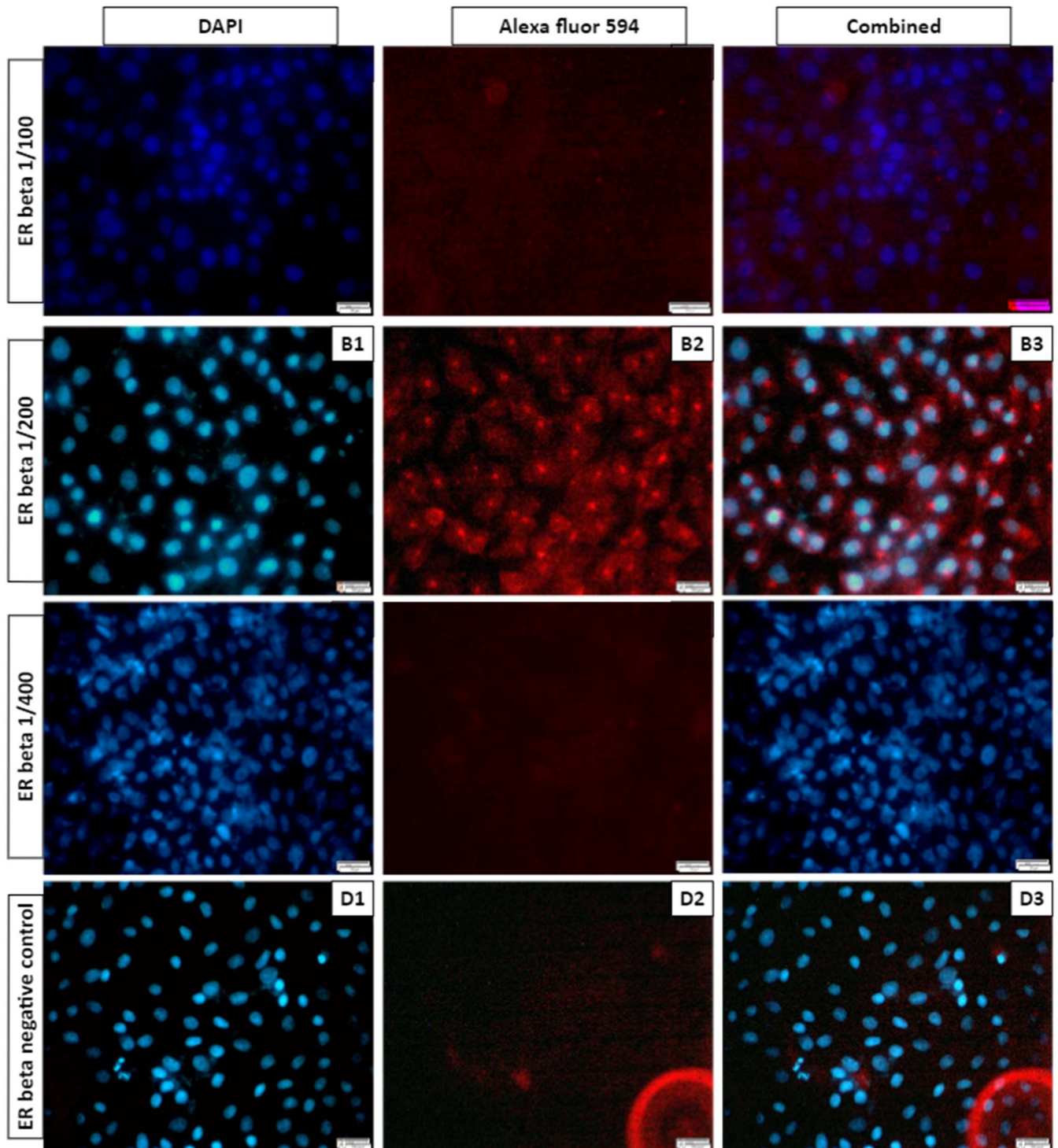
A5: 1% BSA in 0.1% tween-20/PBS (Blocking buffer)

- 50 μl of tween-20 is added to 45.90ml of PBS
- 0.5g is dissolved in the tween-20/PBS

B: Titration of ICC antibodies

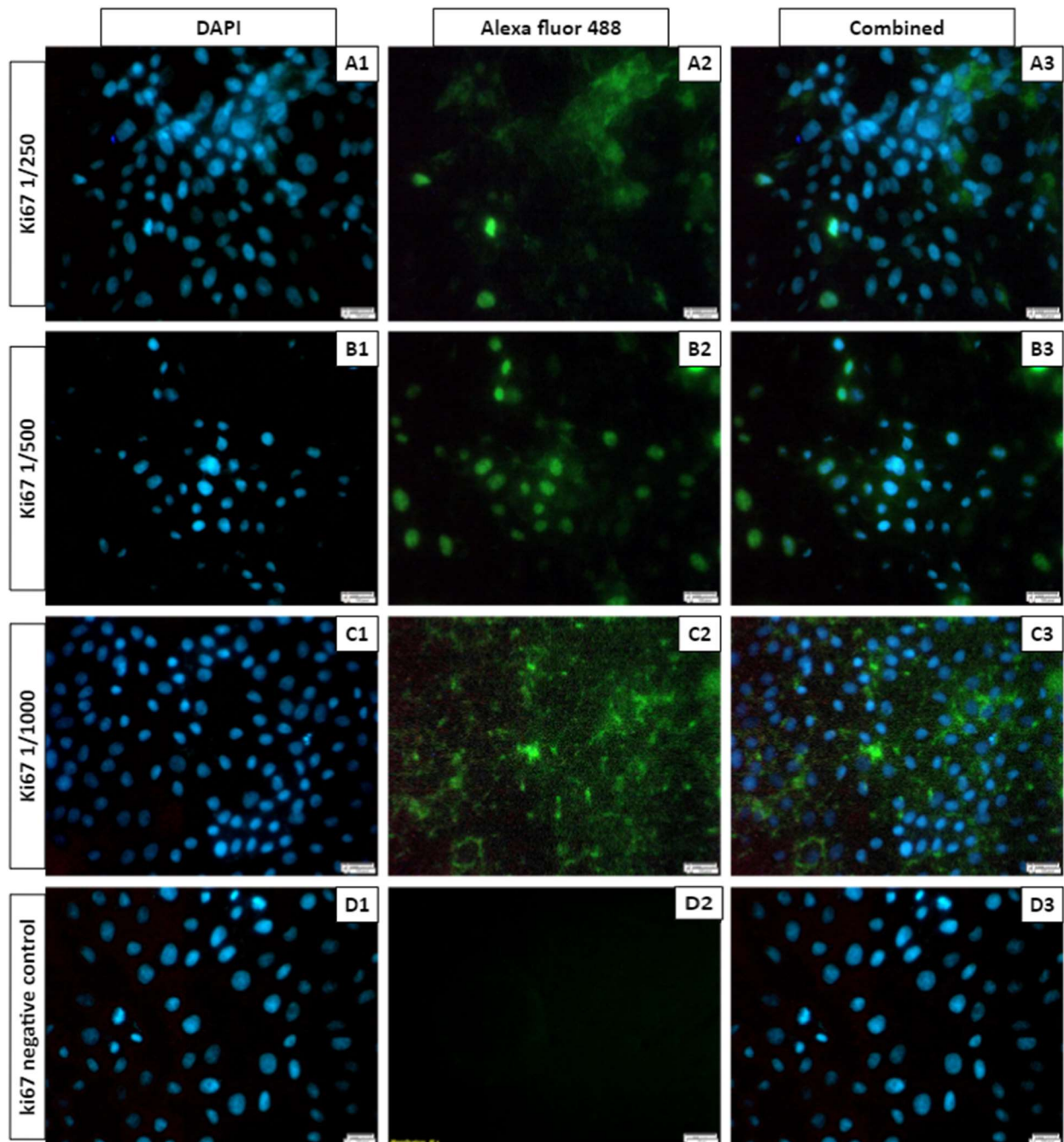
Based upon expression and specificity of those expression in the cell optimal concentrations were chosen. An excess of concentration might result in background staining which could lead to false positives.

B1: Titration of antibodies for ER β



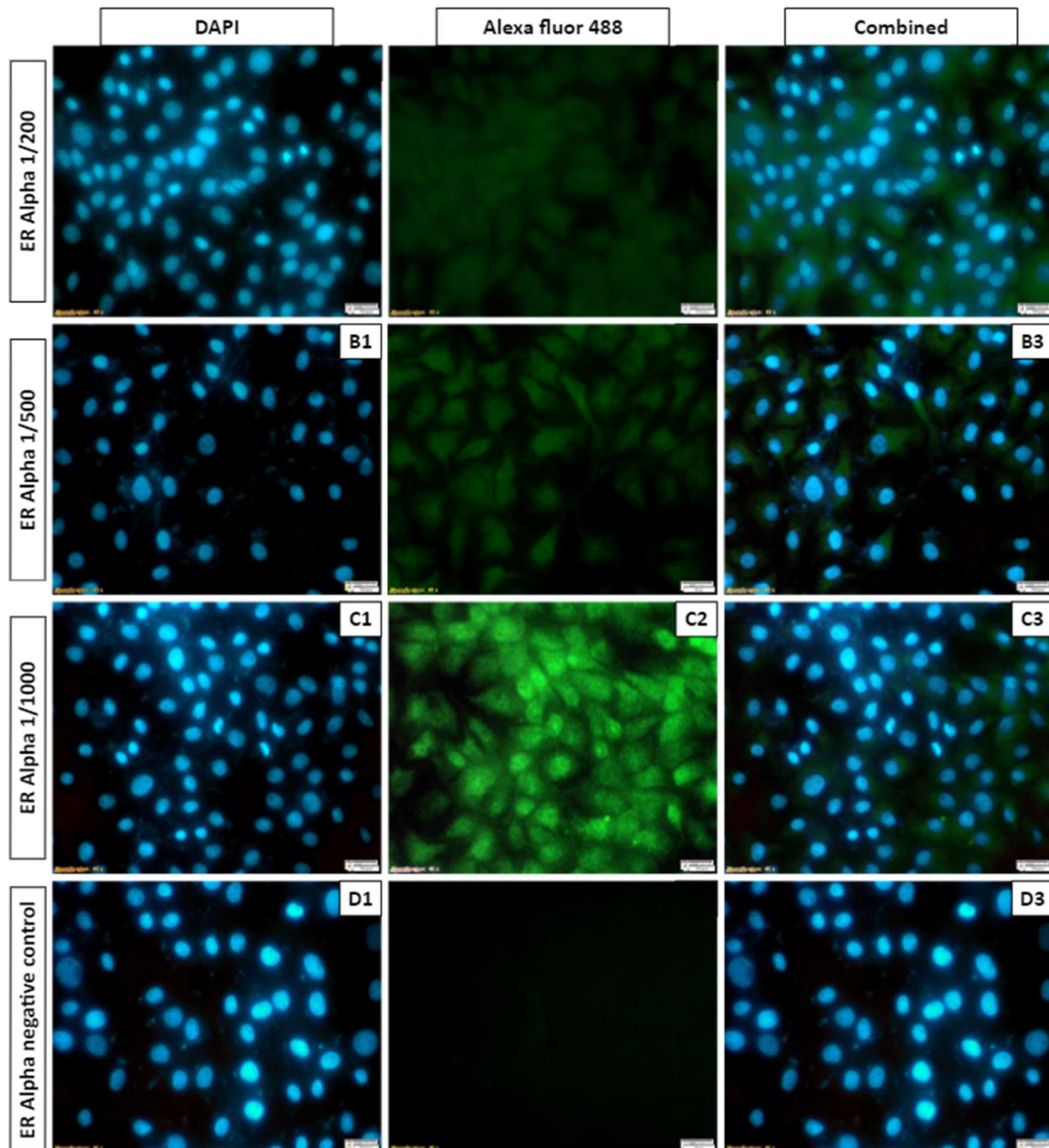
Dilutions run 1/100, 1/200, 1/400 and negative control Estrogen receptor beta (ER β) in MCF7 cell lines. 1-DAPI fluorescence, 2- Alexa fluor 594 fluorescence, 3- combined images of 1 and 2. The suitable dilution chosen is 1/200.

B2: Titration of antibodies for Ki67



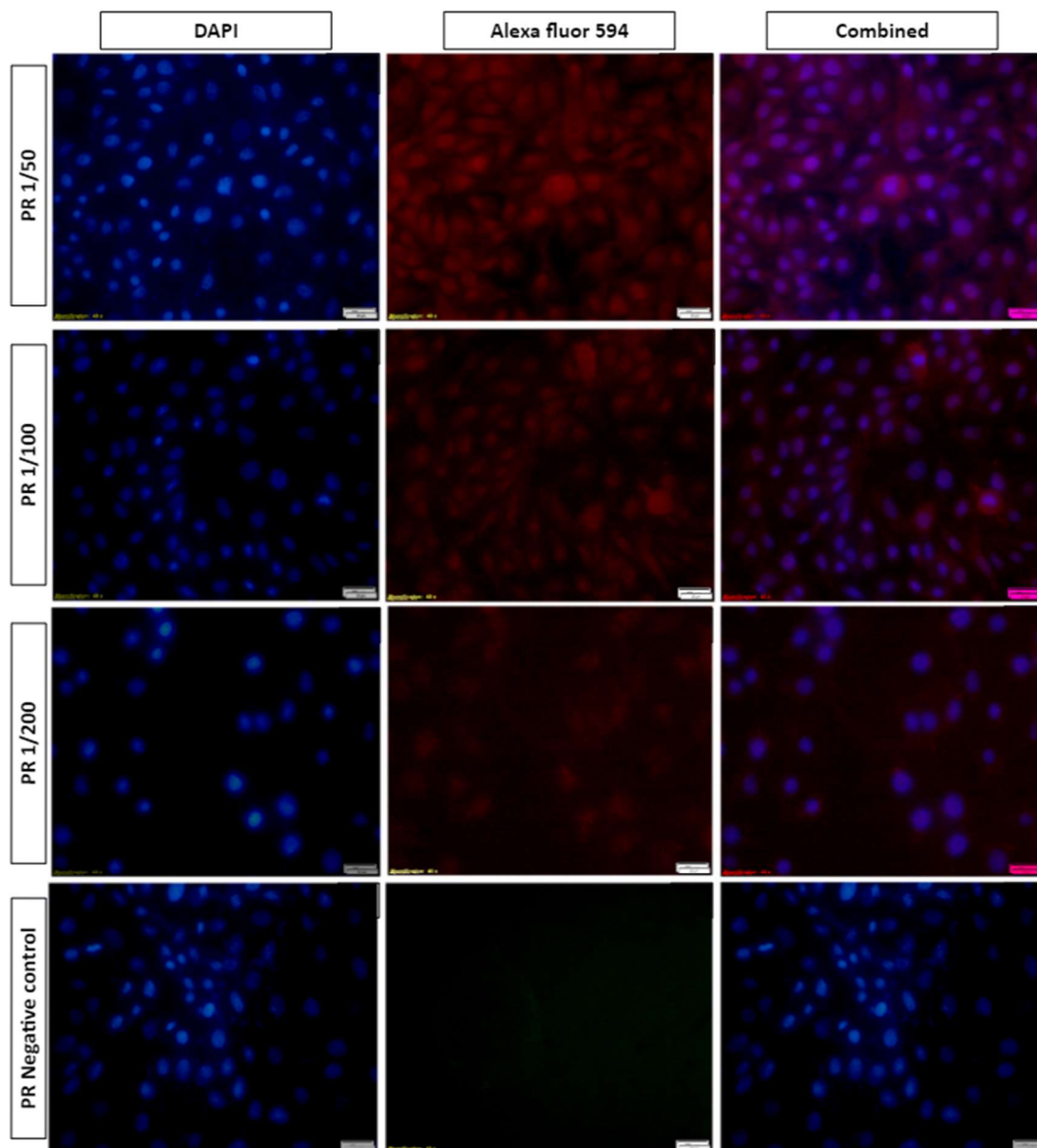
Dilutions run 1/250, 1/500, 1/1000 and negative control for Ki67 in MCF7 cell lines. 1-DAPI fluorescence, 2- Alexa fluor 488 fluorescence, 3- combined images of 1 and 2. The suitable dilution chosen is 1/1000

B3: Titration of antibodies for ER α



Dilutions run 1/200, 1/500, 1/1000 and negative control for Oestrogen receptor Alpha (ER α) in MCF7 cell lines. 1-DAPI fluorescence, 2- Alexa fluor 488 fluorescence, 3- combined images of 1 and 2. The suitable dilution chosen is 1/500.

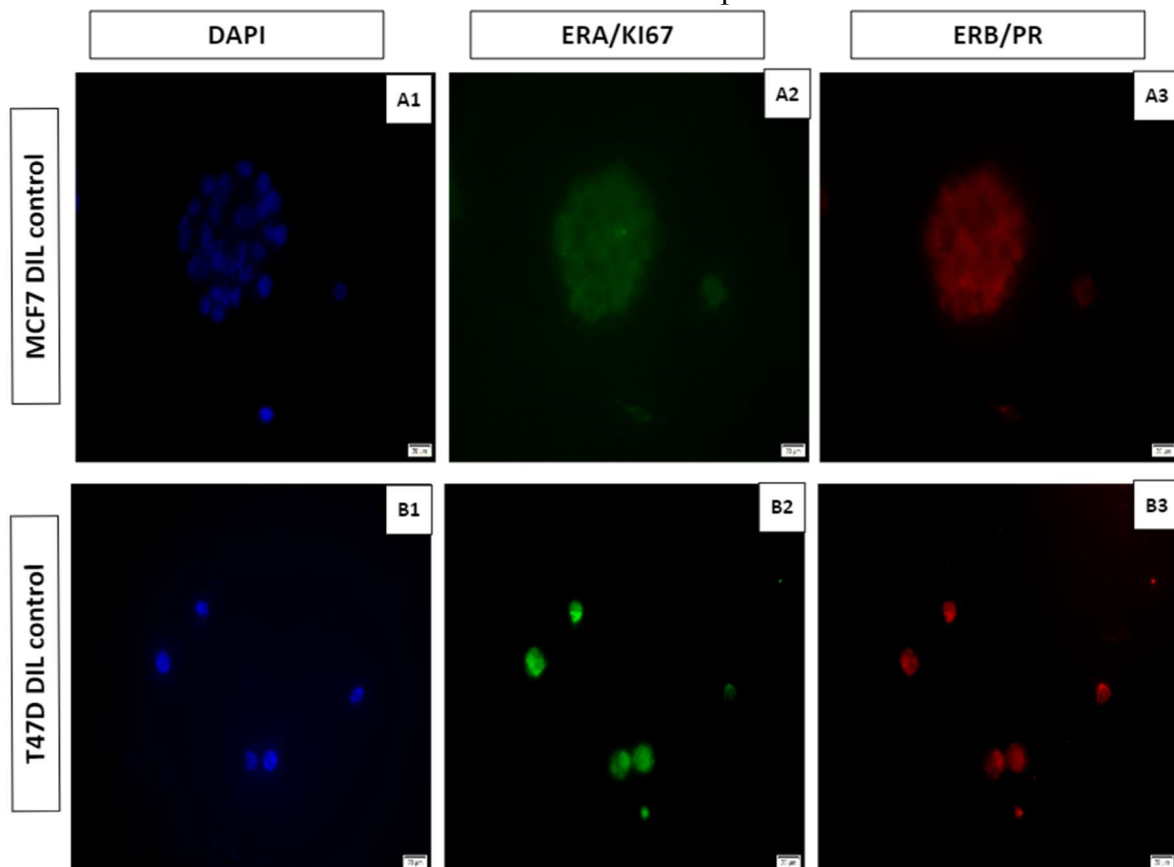
B4: Titration of antibodies for PR



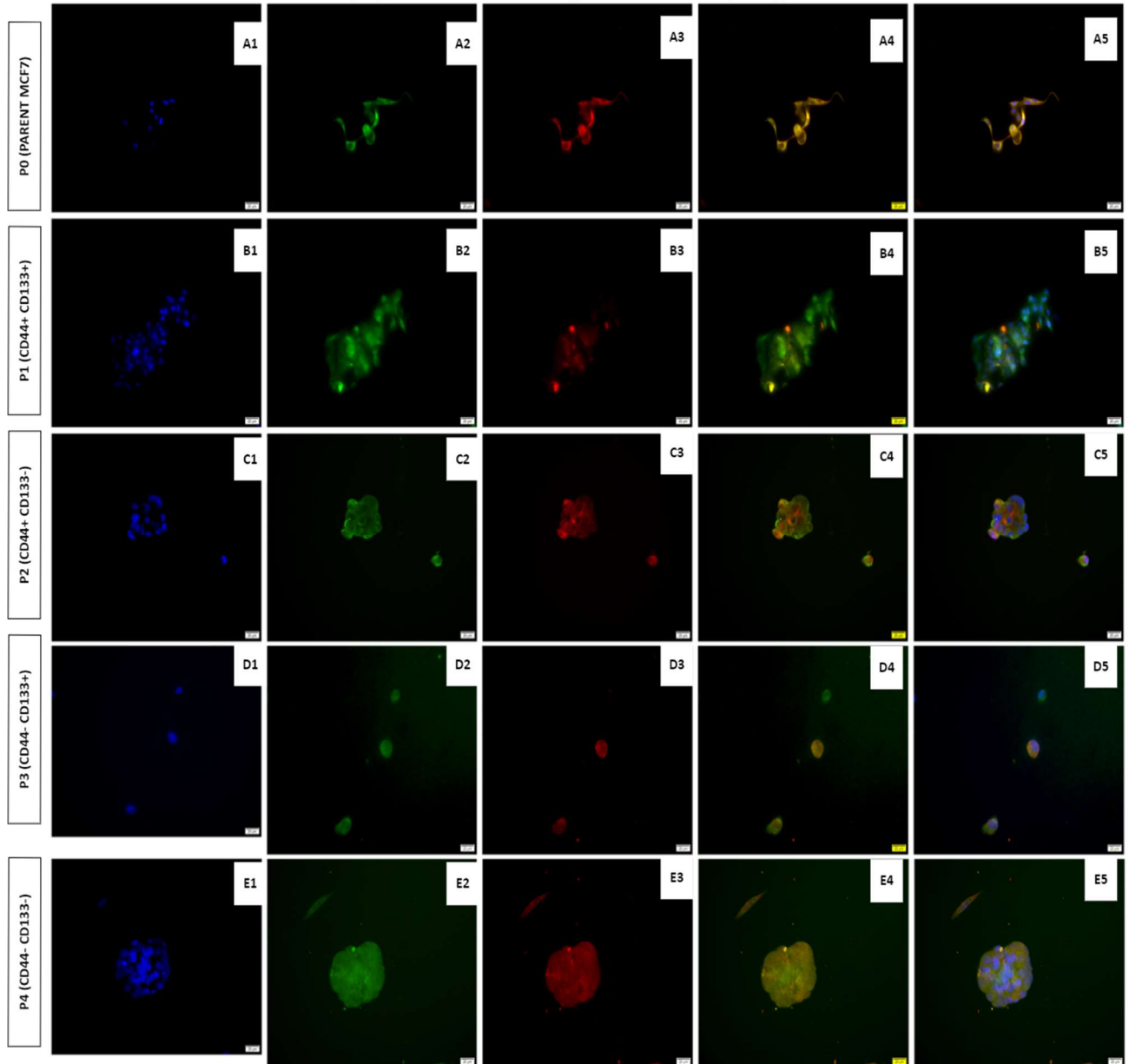
Dilution runs 1/50, 1/100, 1/200 and negative control for Progesterone (PR) in MCF7 cell lines. 1- DAPI fluorescence, 2-Alexafluor 594 fluorescence, 3- Combined images of 1 and 2. The suitable dilution chosen is 1/100

B5: All Biomarker expression of breast cancer cell lines and subpopulations

B5.1: Diluent control of MCF7 and T47D Biomarker expression

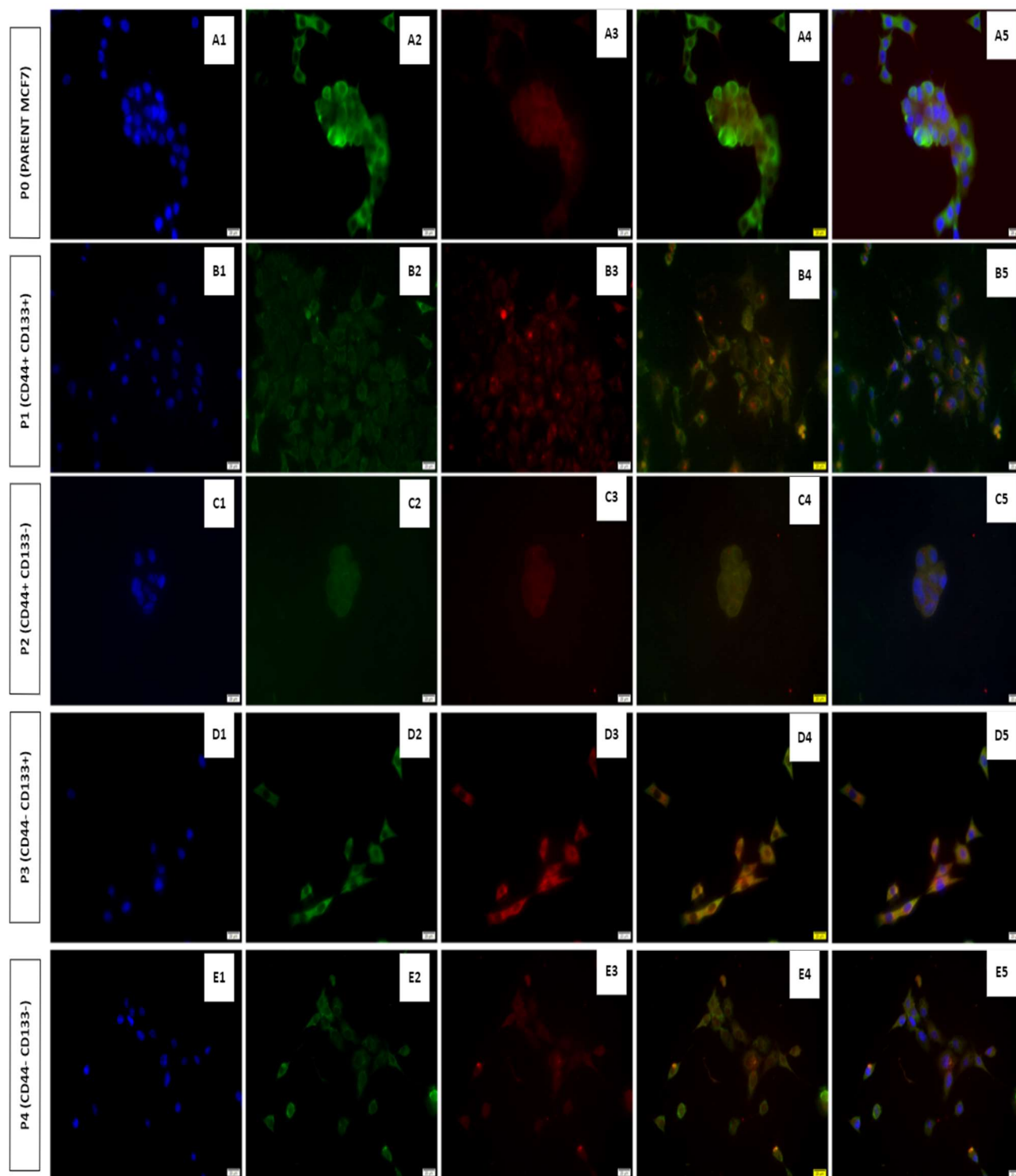


B5.2: Untreated MCF7 populations showing ER α and ER β expression



Untreated MCF7 populations showing ER α and ER β expression. Magnification; 20 μ m. 1: DAPI (blue), 2: ER α (green), 3: ER β (red), 4: Overlay of ER α and ER β , 5: Overlay of DAPI, ER α and ER β .

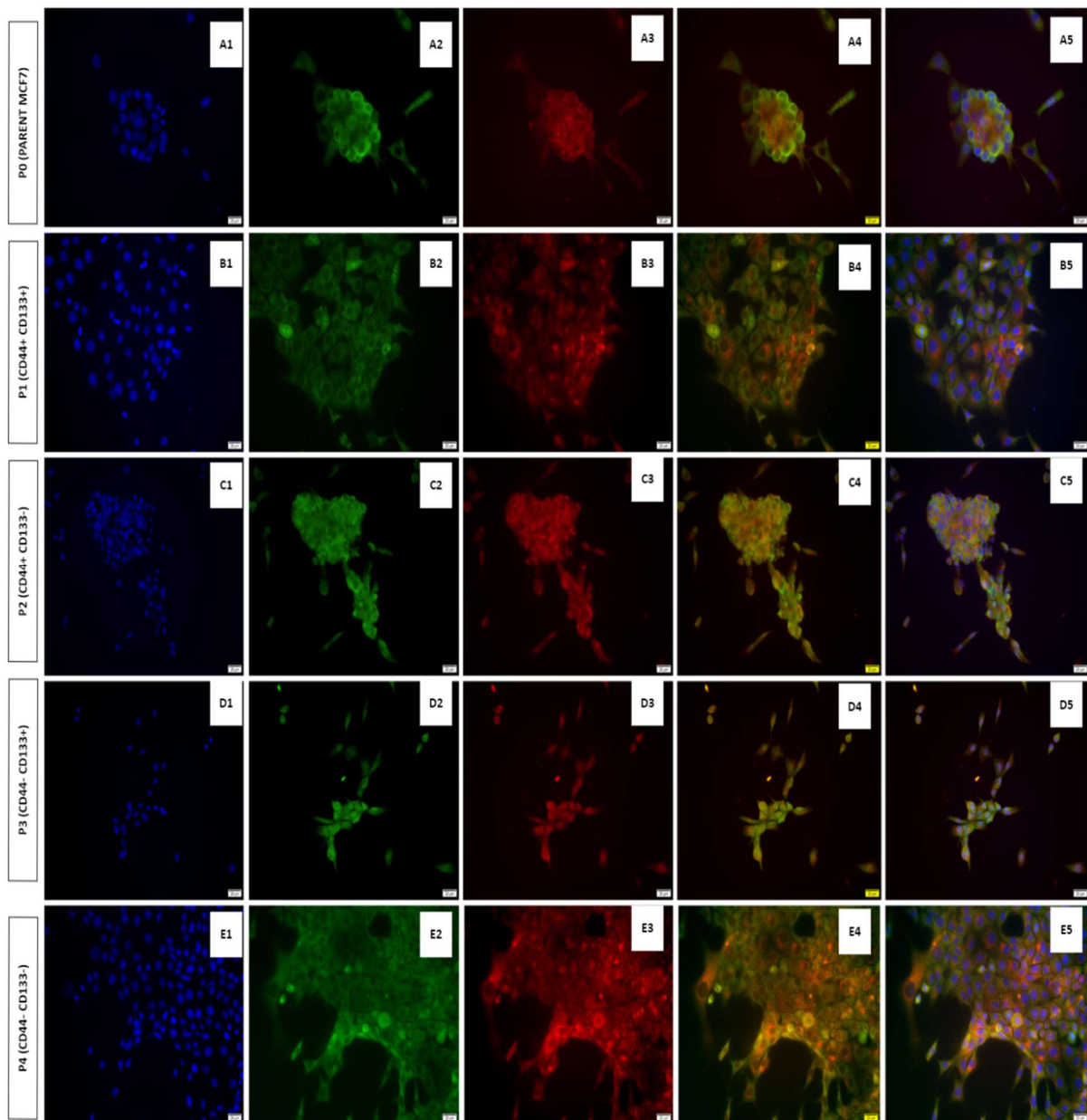
B5.3: Anastrozole treated MCF7 populations showing ER α and ER β .



Anastrozole treated MCF7 populations showing ER α and ER β . Magnification; 20 μ m. 1:

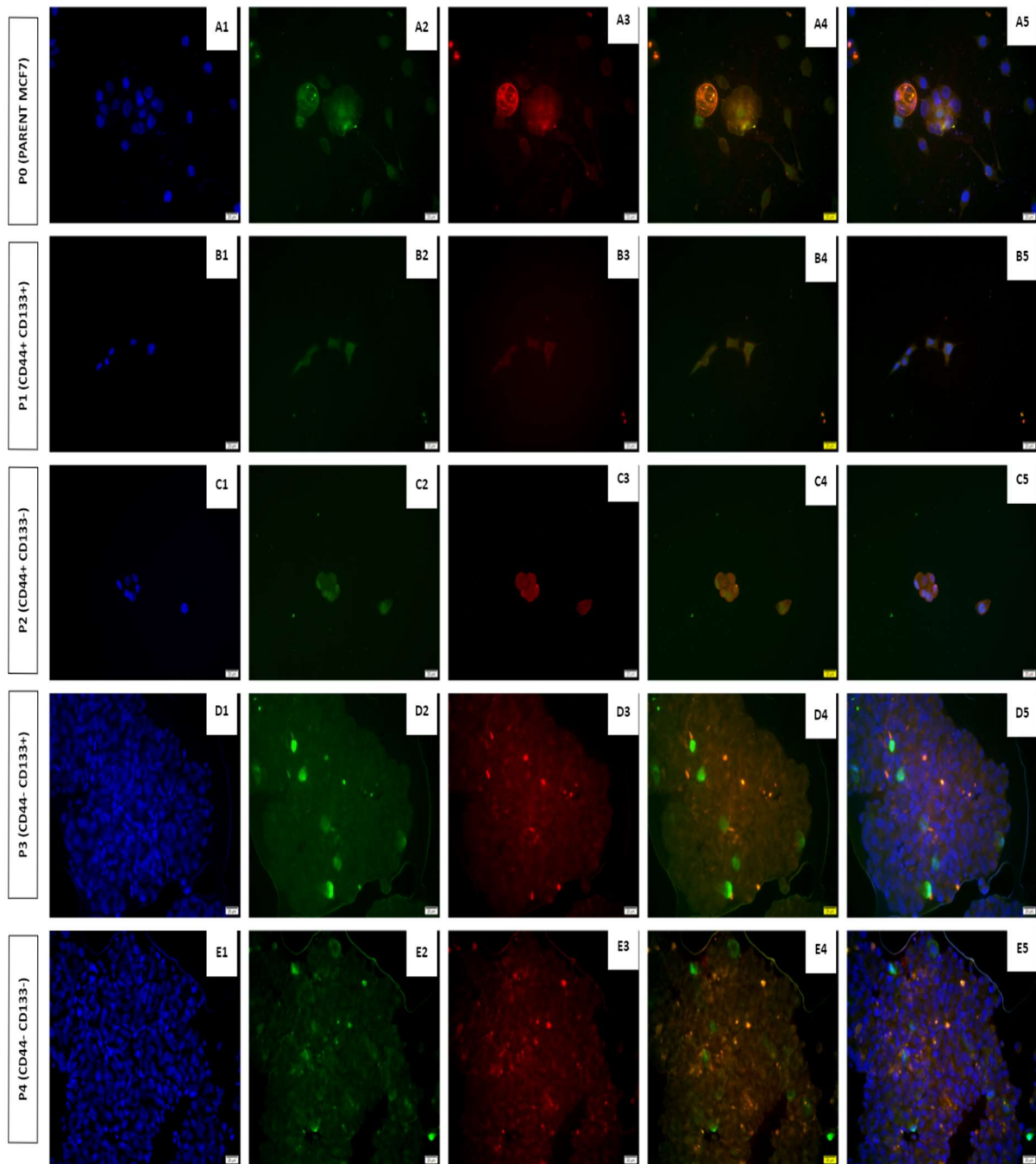
DAPI, 2: ER α , 3: ER β , 4;Overlay of ER α and ER β , 5: Overlay of DAPI, ER α and ER β .

B5.4: MCF7 populations treated with Tamoxifen and ER α and ER β expression.



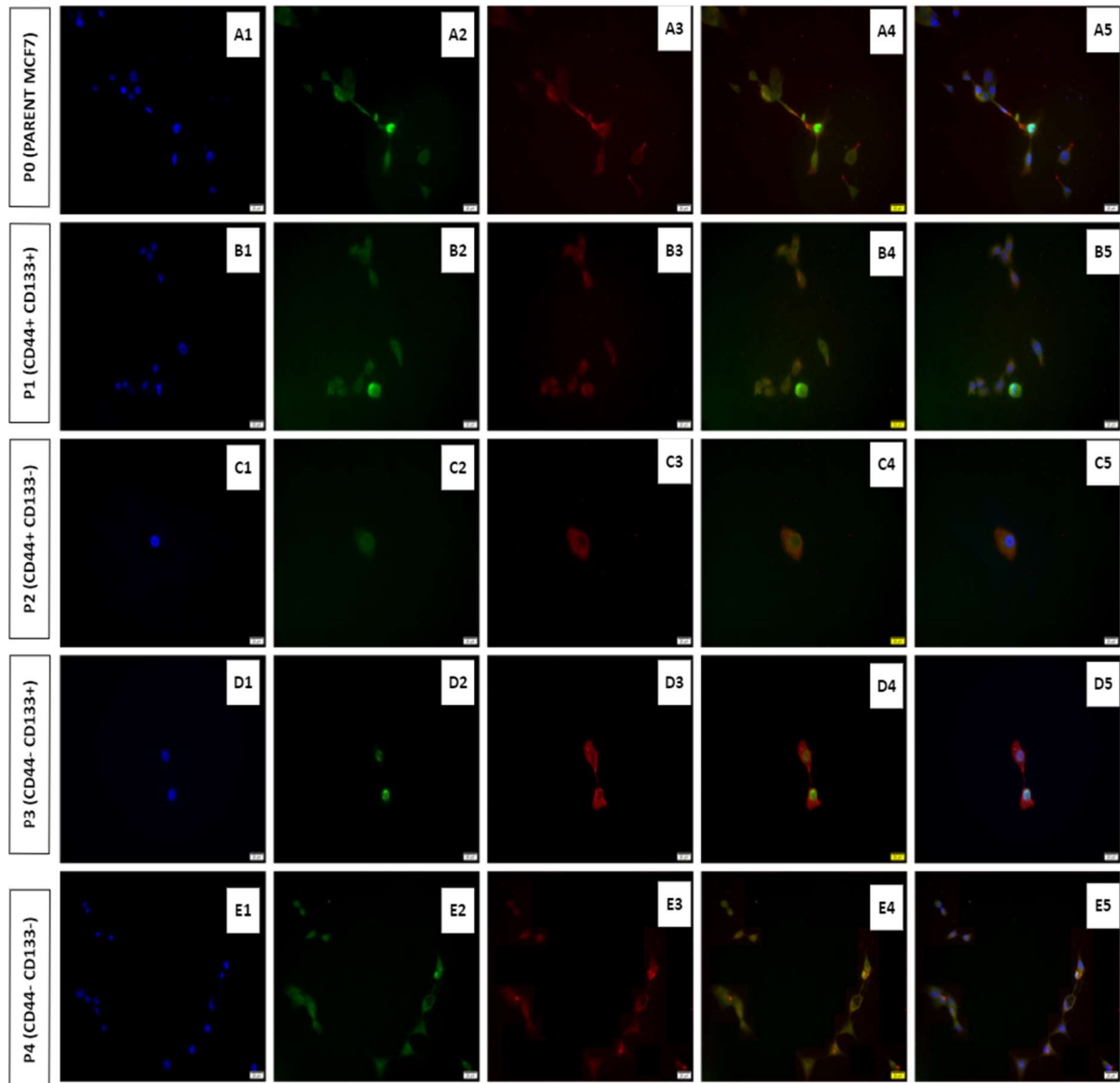
MCF7 populations treated with Tamoxifen and ER α and ER β expression. Magnification; 20 μ m. 1: DAPI, 2: ER α , 3: ER β , 4: Overlay of ER α and ER β , 5: Overlay of DAPI, ER α and ER β .

B5.5: MCF7 populations untreated with Ki67 and PR expression.



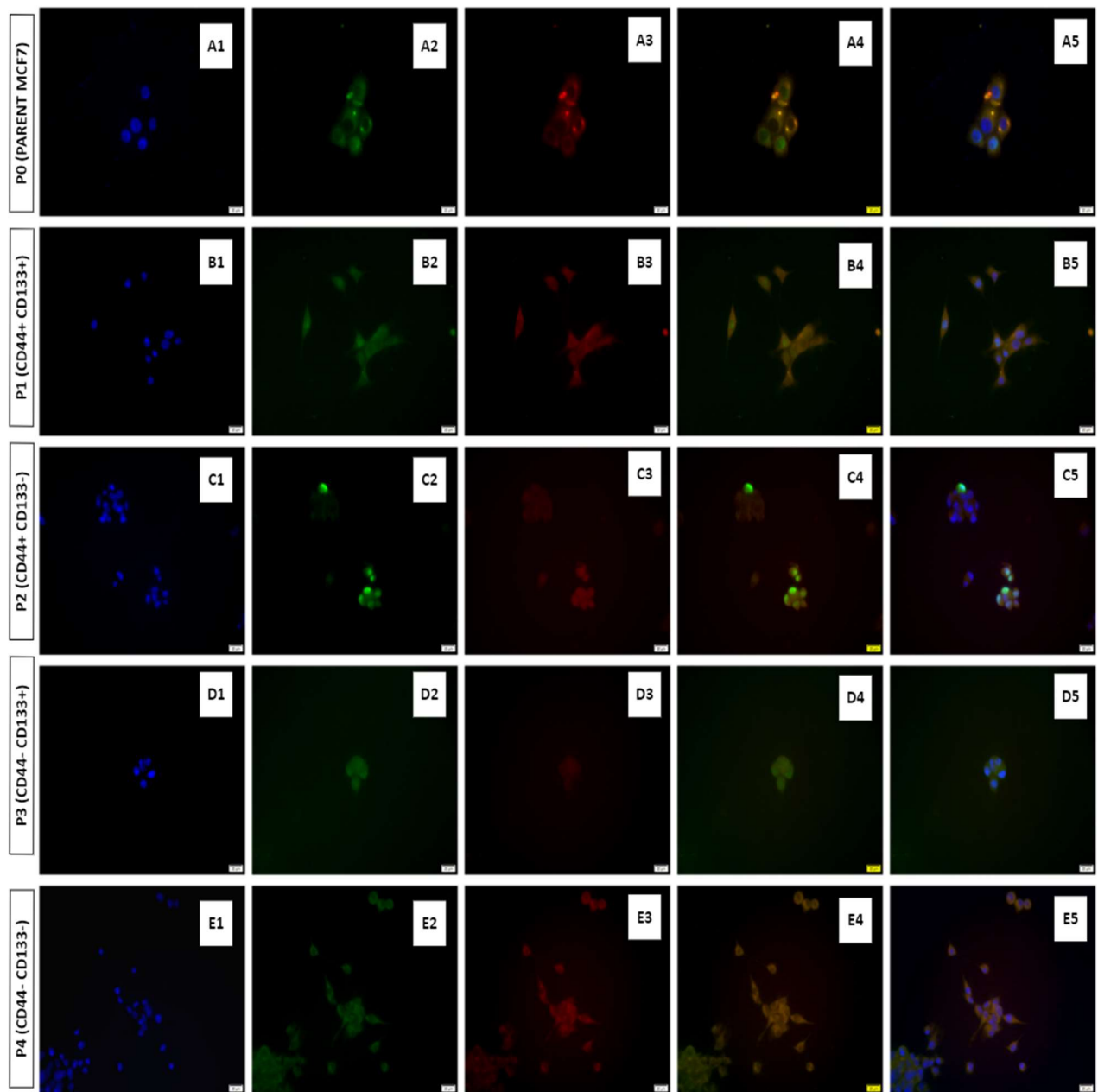
MCF7 populations untreated with Ki67 and PR expression. Magnification; 20μm. 1: DAPI, 2: Ki67, 3: PR, 4;Overlay of Ki67 and PR, 5: Overlay of DAPI, Ki67 and PR.

B5.6: MCF7 populations untreated with Anastrozole Ki67 and PR expression



MCF7 populations untreated with Anastrozole Ki67 and PR expression. Magnification; 20µm. 1: DAPI, 2: Ki67, 3: PR, 4;Overlay of Ki67 and PR, 5: Overlay of DAPI, Ki67 and PR. UT: Untreated, Nuclear expression (*), Cytoplasmic expression (white arrow).

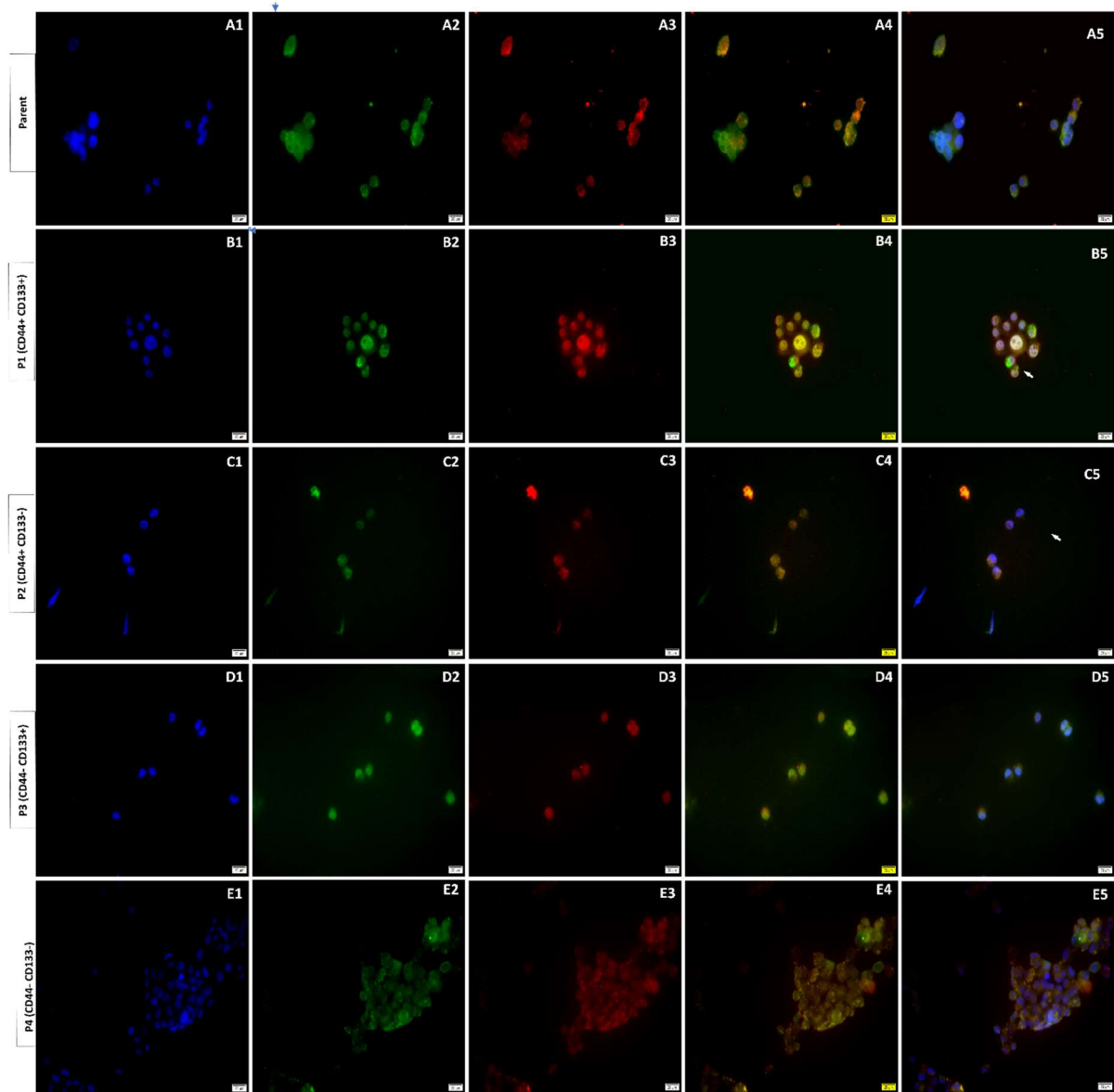
B5.7: MCF7 populations treated with Tamoxifen Ki67 and PR expression.



MCF7 populations treated with Tamoxifen Ki67 and PR expression. Magnification; 20 μ m.

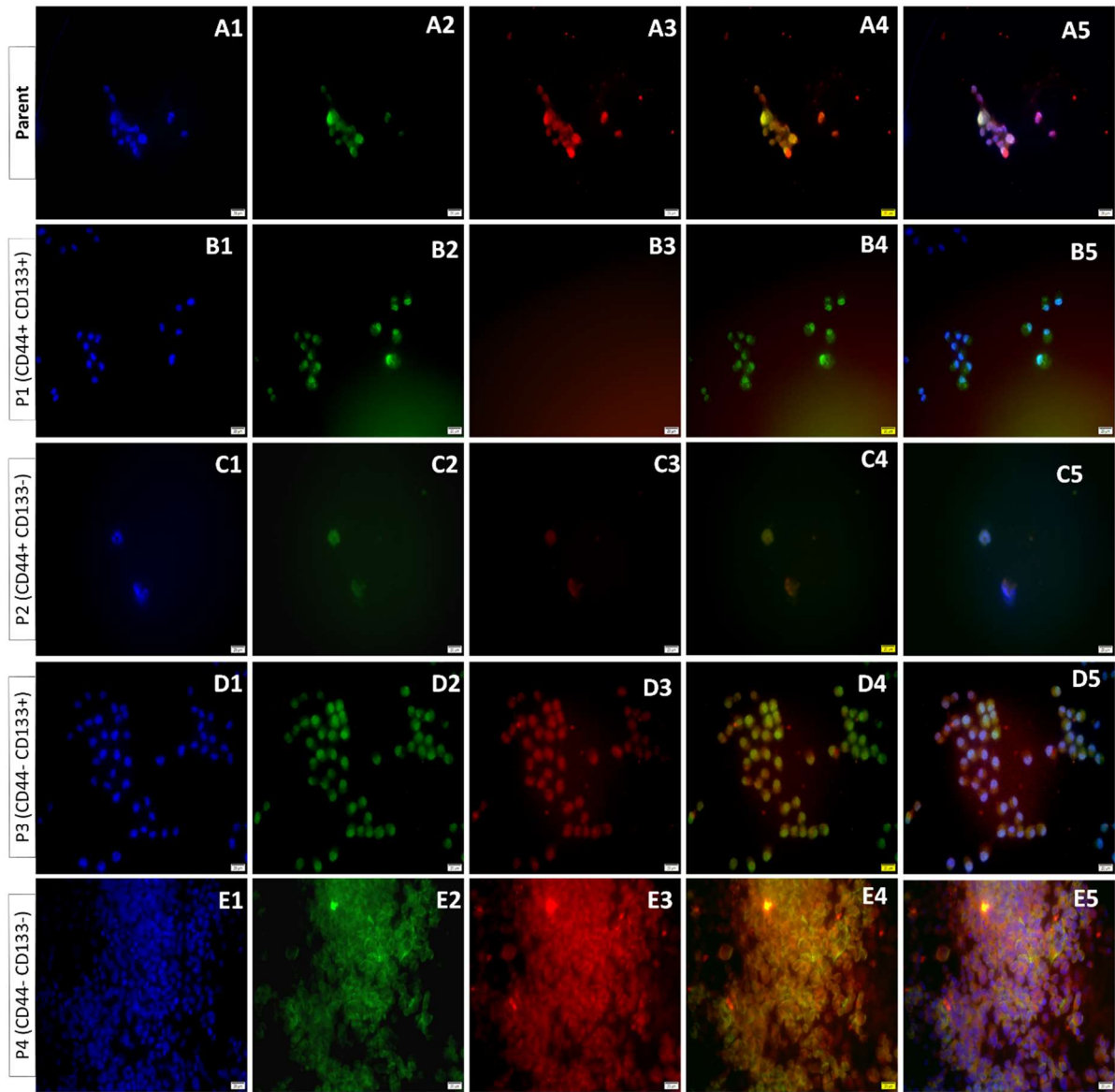
1: DAPI, 2: Ki67, 3: PR, 4;Overlay of Ki67 and PR, 5: Overlay of DAPI, Ki67 and PR.

B5.8: The effects of the untreated on all populations of T47D cells



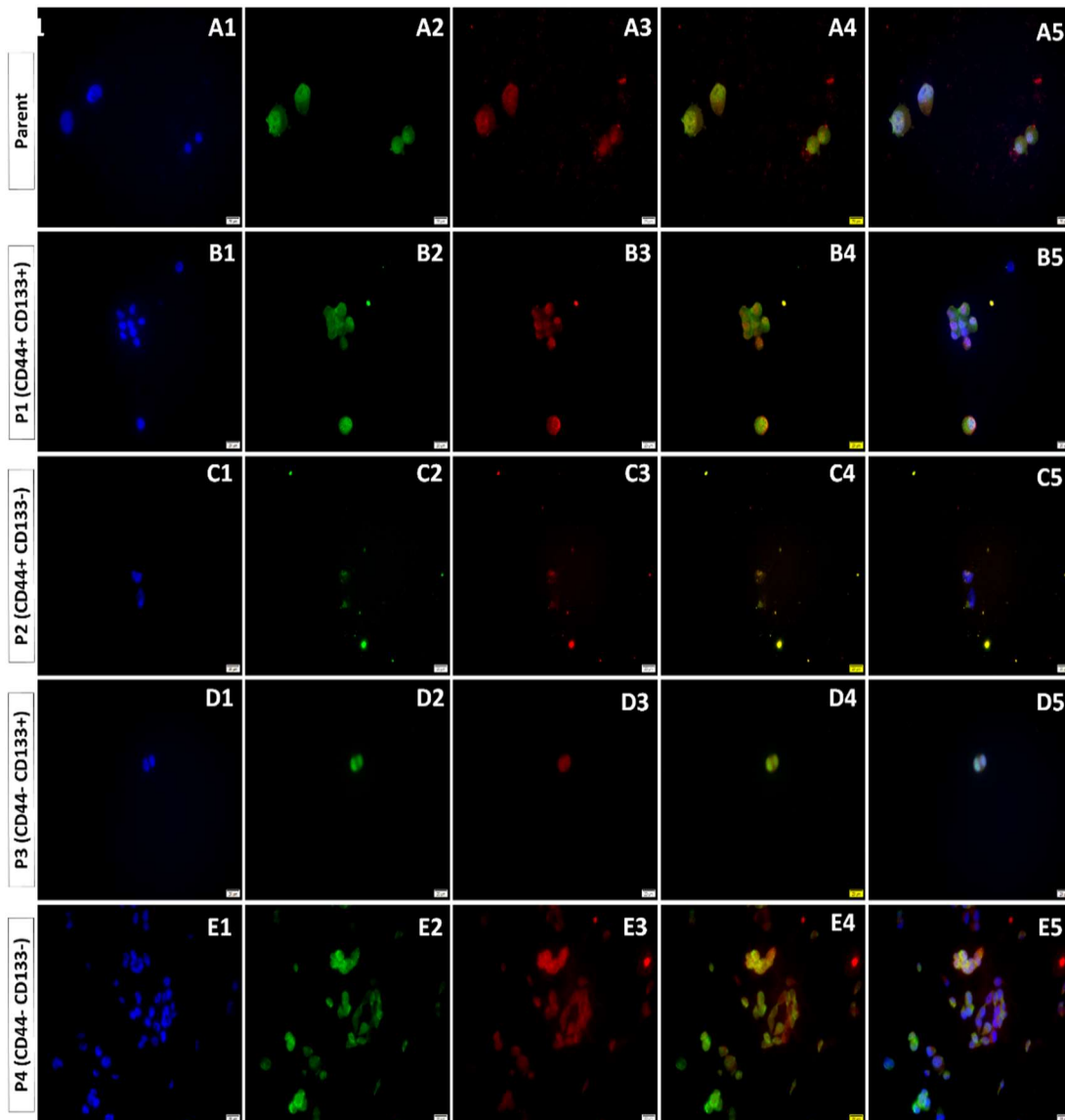
The effects of the untreated on all populations of T47D cells. Key: 1: DAPI, 2: ER α , 3: ER β , 4: Overlay of ER α and ER β , 5: Overlay of DAPI, ER α and ER β .

B5.9: The effects of the treated with Anastrozole on all populations of T47D cells



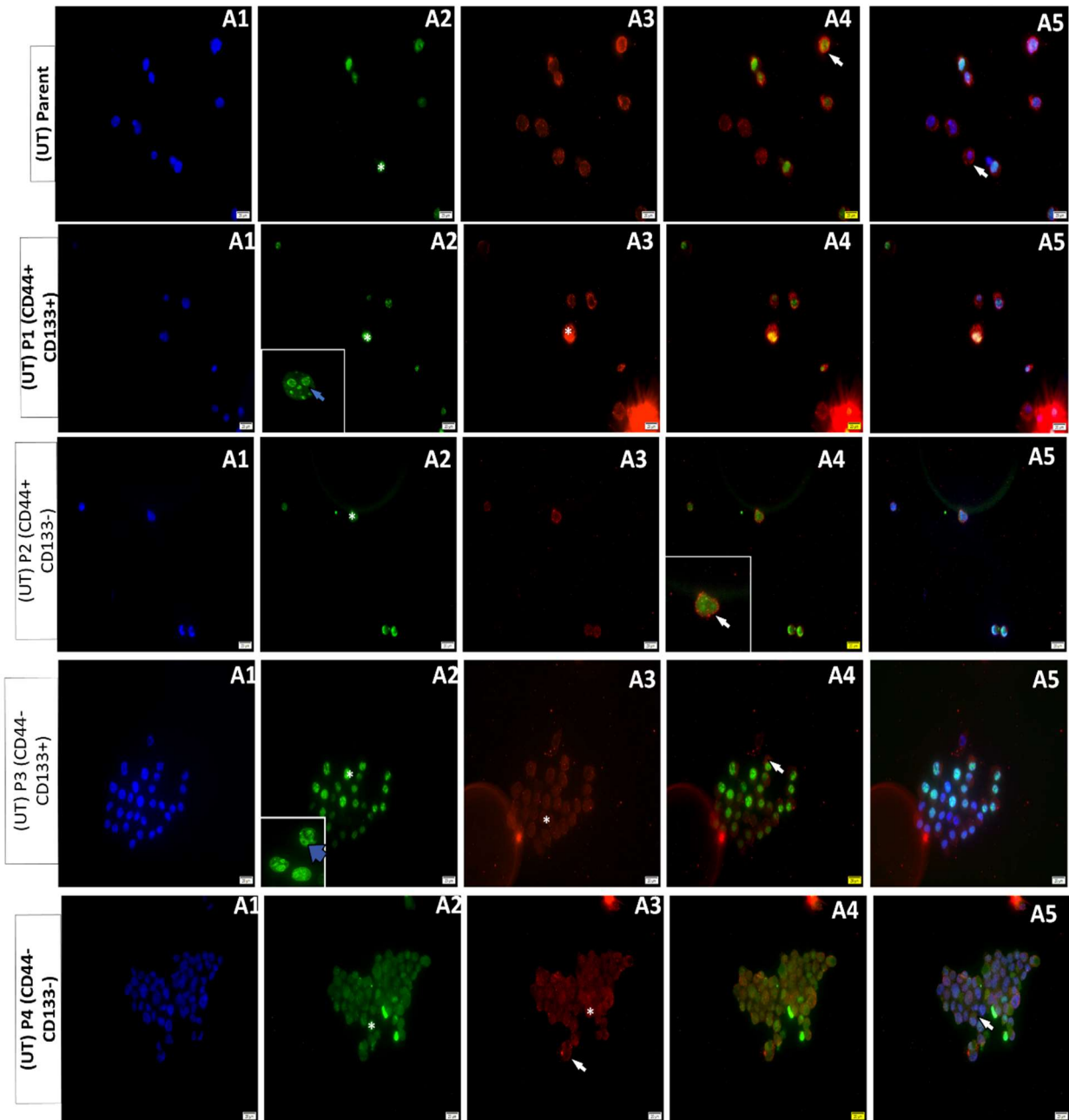
The effects of the treated with Anastrozole on all populations of T47D cells. Key: 1: DAPI, 2: ER α , 3: ER β , 4;Overlay of ER α and ER β , 5: Overlay of DAPI, ER α and ER β .

B5.10: The effects of the treated with Tamoxifen on all populations of T47D cells



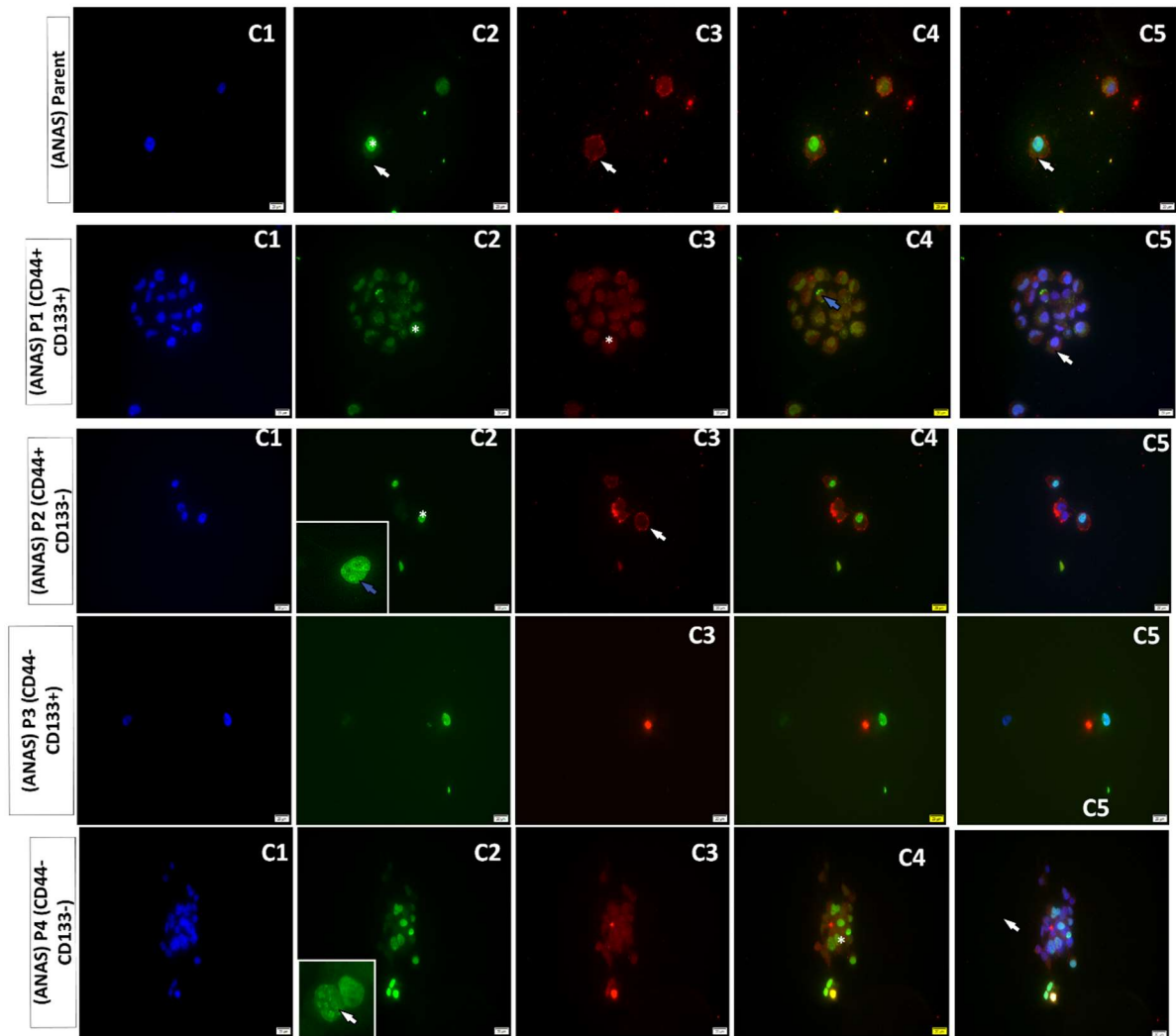
The effects of the treated with Tamoxifen on all populations of T47D cells. Key: 1: DAPI, 2: ER α , 3: ER β , 4; Overlay of ER α and ER β , 5: Overlay of DAPI, ER α and ER β .

B5.11: MCF7 populations untreated with Ki67 and PR expression



MCF7 populations untreated with Ki67 and PR expression. Magnification; 20 μ m. 1: DAPI, 2: Ki67, 3: PR, 4; Overlay of Ki67 and PR, 5: Overlay of DAPI, Ki67 and PR. Nuclear expression (Blue arrow), Cytoplasmic expression (white arrow).

B5.12: T47D populations treated with Anastrozole Ki67 and PR expression.

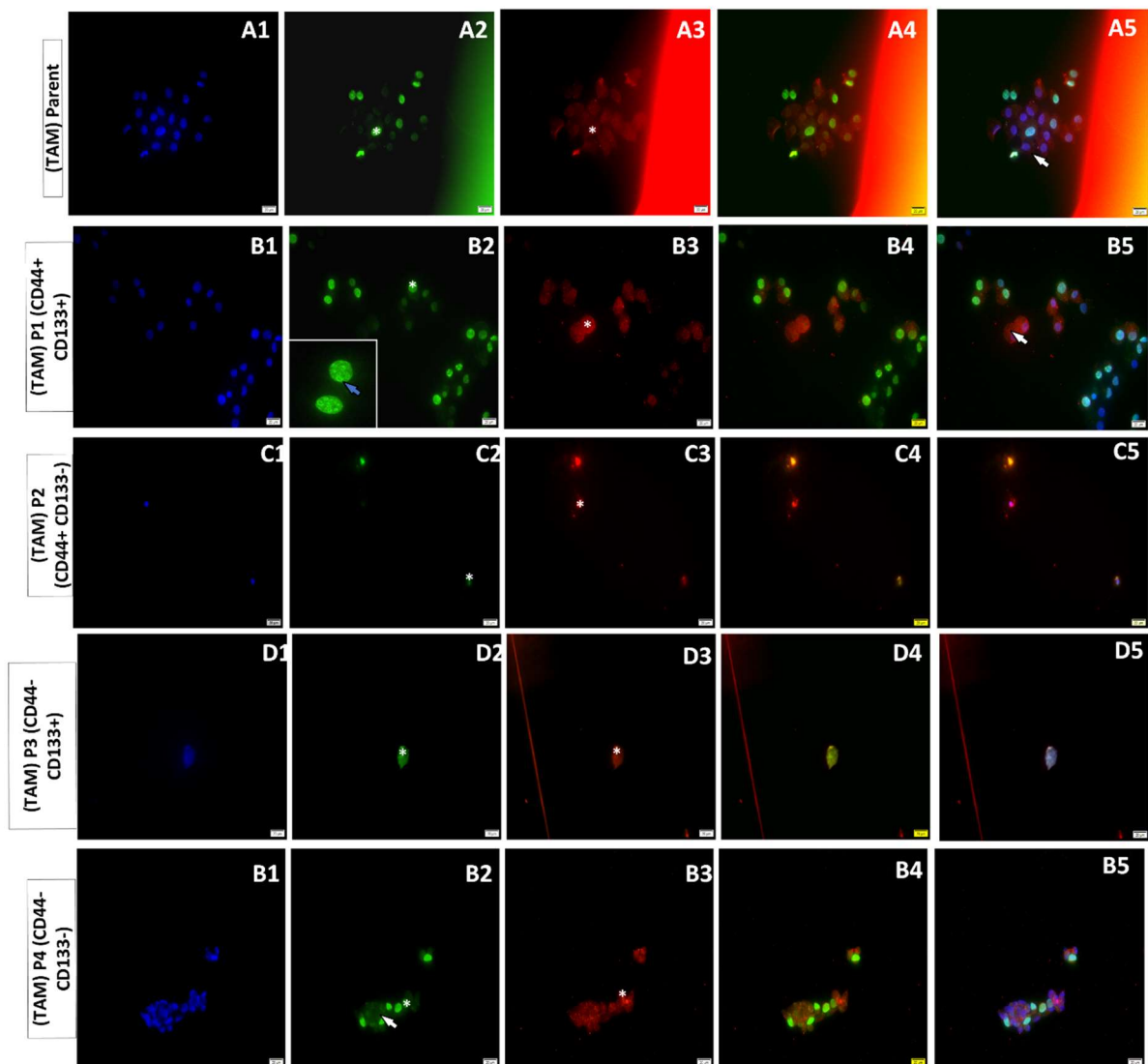


T47D populations treated with Anastrozole Ki67 and PR expression. Magnification; 20µm.

1: DAPI, 2: Ki67, 3: PR, 4;Overlay of Ki67 and PR, 5: Overlay of DAPI, Ki67 and PR.

Nuclear expression (Blue arrow), Cytoplasmic expression (white arrow).

B5.13: T47D populations treated with Tamoxifen Ki67 and PR expression



T47D populations treated with Tamoxifen Ki67 and PR expression. Magnification; 20µm. 1: DAPI, 2: Ki67, 3: PR, 4;Overlay of Ki67 and PR, 5: Overlay of DAPI, Ki67 and PR. Nuclear expression (Blue arrow), Cytoplasmic expression (white arrow).

C: Statistical outputs from data analysis

C1: Kruskal-Wallis test of each treatment group per cell line for each for ER α AND Ki67 population. Red indicates significant differences.

Depend.: MFI G	Multiple Comparisons z' values; MFI G					
	Independent (grouping) variable: Population+treatment+Cell line +Receptor					
	Kruskal-Wallis test: H (62, N= 1089) =292,9028 p =0,000					
	P2utM+Ki67PR R:747,03	P4utM+Ki67PR R:216,20	P4utT+EraErb R:349,50	P2TamM+Ki67PR R:275,85	P4utM+EraErb R:783,66	P0utM+EraErb R:862,90
P4utM+EraErb	1,849798	1,465468	4,308942	0,052009	1,062768	2,001613
P2utM+Ki67PR	0,294508	4,941380	1,291973	4,386114	1,826412	0,887567
P2TamM+Ki67PR	4,386114	1,190461	2,424156	0,524848	2,007395	1,068549
P4utM+Ki67PR	4,941380	2,000557	1,517653	0,678557	0,876256	0,062590
P0utT+Ki67PR	3,614617	3,440171	0,174447	4,477822	2,815455	2,048891
P2utT+Ki67PR	0,607725	4,201301	0,586684	4,332497	0,067366	0,871480
P3utT+Ki67PR	0,990878	0,246879	3,033332	4,243312	0,413997	0,524848
P4utT+EraErb	1,746981	4,170635	0,556018	1,519245	4,308942	0,763142
P1AnasM+EraErb	0,808136	3,140544	0,474073	2,359006	1,741650	4,340374
P4utT+EraErb	2,809749	2,310460	1,207750	2,363326	1,607223	4,214768

C2: Kruskal Wallis test of each treatment group per cell line for each for ER α AND Ki67 population. Red indicates significant difference.

Depend.: MFI G	Multiple Comparisons p values (2-tailed); MFI G (in ICC Stats Data) Independent (grouping) variable: Population+treatment+Cell line +Receptor Kruskal-Wallis test: H (62, N= 1089) =292,9028 p =0,000				
	P0utM+EraErb R:862,90	P4utM+EraErb R:783,66	P2TamM+Ki67PR R:275,85	P4TamM+Ki67PR R:278,90	P4utM+Ki67PR R:216,20
P1AnasM+EraErb	0,027780	1,000000	1,000000	1,000000	1,000000
P4utT+EraErb	1,000000	0,032037	1,000000	1,000000	0,318873
P2utM+Ki67PR	0,480420	0,764633	0,022536	1,000000	0,001515
P4TamM+Ki67PR	1,000000	1,000000	0,025667	1,000000	1,000000
P4utM+Ki67PR	1,000000	1,000000	0,001515	1,000000	1,000000

C3: Kruskal Wallis of each treatment group per cell line for each for ER β and PR population. Red indicates significant differences.

Depend.: MFI R	Multiple Comparisons z' values; MFI R (M+T (2) in ICC Stats Data) Independent (grouping) variable: Population+treatment+Cell line +Receptor Kruskal-Wallis test: H (62, N= 1089) =320,4657 p =0,000								
	P4utM+Era Erb R:818,24	P1AnasT+Ki6 7PR R:331,75	P1TamT+Ki6 7PR R:331,80	P1AnasM+Ki6 7PR R:322,80	P0AnasM+Ki6 7PR R:328,60	P1utM+Era Erb R:331,30	P4AnasM+Ki6 7PR R:664,30	P2TamM+Ki6 7PR R:273,60	P4AnasT+Era Erb R:765,09
P4utM+EraErb	0,166409	1,901627	1,231839	1,822958	0,251025	4,832764	0,536338	2,610321	1,873166
P3AnasM+Ki6 7PR	1,301980	3,375963	2,700154	4,603078	4,555463	1,029588	0,448874	2,475173	1,742359
P4utM+Ki67P R	1,355970	3,429954	2,758471	0,785966	1,288017	0,444915	4,312907	3,196818	1,256784
P2utM+Ki67P R	0,075556	2,149539	1,375465	1,813960	3,887943	3,253156	0,508400	5,231502	1,021487
P4utT+EraErb	3,232377	1,497158	2,350796	0,902950	1,003341	0,211229	0,244664	1,829319	4,280271
P1utT+Ki67PR	1,021788	4,548074	4,547664	0,902170	2,976153	2,268311	1,264972	0,761327	0,101833

C4: Kruskal Wallis test of each treatment group per cell line for each for ER β and PR population. Red indicates significant difference.

Depend.: MFI R	Multiple Comparisons p values (2-tailed); MFI R (M+T (2) in ICC Stats Data) Independent (grouping) variable: Population+treatment+Cell line +Receptor Kruskal-Wallis test: H (62, N= 1089) =320,4657 p =0,000									
	P3AnasT+EraErb R:560,84	P1TamT+Ki67PR	P0AnasM+Ki67PR R:328,60	P4utM+Ki67PR	P1AnasT+EraErb R:364,27	P0DilM+Ki67PR R:129,20	P1AnasM+Ki67PR R:322,80	P4utM+EraErb R:818,24	P4AnasM+Ki67PR R:664,30	P2TamM+Ki67PR R:273,60
P1utM+EraErb	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,002630	1,000000	1,000000
P3AnasM+Ki67PR	1,000000	0,483260	0,010208	0,185384	1,000000	1,000000	0,008130	1,000000	1,000000	1,000000
P4utT+EraErb	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,001565	0,544533	1,000000
P4utM+Ki67PR	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,031467	1,000000
P2utM+Ki67PR	1,000000	1,000000	1,000000	0,000045	0,134623	1,000000	1,000000	1,000000	1,000000	0,000328
P1utT+Ki67PR	1,000000	0,010594	1,000000	1,000000	0,395027	1,000000	0,010573	1,000000	1,000000	0,364023

C4: Mann-Whitney U test between MCF7(M) and T47D (T) in all biomarkers per population and treatment group

variable	Population+treatment+Receptor=P0Anas+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	541,0000	279,0000	69,00000	3,530037	0,000416	3,530037	0,000416

variable	Population+treatment+Receptor=P1Anas+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	325,0000	756,0000	115,0000	-3,20190	0,001365	-3,20200	0,001365

variable	Population+treatment+Receptor=P2Anas+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	304,0000	224,0000	104,0000	0,868543	0,385098	0,868543	0,385098

variable	Population+treatment+Receptor=P3Anas+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value

variable	Population+treatment+Receptor=P3Anas+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	315,0000	465,0000	105,0000	-2,37425	0,017585	-2,37425	0,017585

variable	Population+treatment+Receptor=P4Anas+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	363,0000	340,0000	153,0000	-0,502856	0,615066	-0,502856	0,615066

variable	Population+treatment+Receptor=P0Tam+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	310,0000	320,0000	100,0000	-1,65000	0,098944	-1,65000	0,098944

variable	Population+treatment+Receptor=P1Tam+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	309,0000	394,0000	99,00000	-2,14856	0,031670	-2,14856	0,031670

variable	Population+treatment+Receptor=P2Tam+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	369,0000	261,0000	141,0000	0,283333	0,776922	0,283373	0,776891

variable	Population+treatment+Receptor=P3Tam+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	293,0000	235,0000	83,00000	-1,42076	0,155387	-1,42076	0,155387

variable	Population+treatment+Receptor=P4Tam+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	513,0000	307,0000	97,00000	2,772634	0,005561	2,772634	0,005561

variable	Population+treatment+Receptor=P0ut+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	230,0000	235,0000	25,00000	3,277563	0,001047	3,277563	0,001047

variable	Population+treatment+Receptor=P1ut+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	345,0000	475,0000	135,0000	-1,74473	0,081033	-1,74473	0,081033

variable	Population+treatment+Receptor=P2ut+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	400,0000	380,0000	190,0000	0,014049	0,988791	0,014049	0,988791

variable	Population+treatment+Receptor=P3ut+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	230,0000	235,0000	94,00000	-0,727485	0,466930	-0,727485	0,466930

variable	Population+treatment+Receptor=P4ut+Era Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	534,0000	246,0000	36,00000	4,312978	0,000016	4,312978	0,000016

variable	Population+treatment+Receptor=P0Dil+Erc Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	64,00000	107,0000	9,000000	-2,71000	0,006729	-2,71000	0,006729

variable	Population+treatment+Receptor=P0Anas+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	273,0000	288,0000	63,00000	-2,45011	0,014282	-2,45011	0,014282

variable	Population+treatment+Receptor=P1Anas+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	411,0000	409,0000	199,0000	0,013525	0,989209	0,013525	0,989209

variable	Population+treatment+Receptor=P2Anas+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	278,0000	352,0000	68,00000	-2,71667	0,006595	-2,71667	0,006595

variable	Population+treatment+Receptor=P3Anas+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	169,0000	156,0000	36,00000	2,135596	0,032713	2,135596	0,032713

variable	Population+treatment+Receptor=P4Anas+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	327,0000	303,0000	117,0000	-1,08333	0,278661	-1,08333	0,278661

variable	Population+treatment+Receptor=P0Tam+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	396,0000	345,0000	135,0000	1,300969	0,193270	1,300969	0,193270

variable	Population+treatment+Receptor=P1Tam+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	397,0000	423,0000	187,0000	-0,338126	0,735268	-0,338126	0,735268

variable	Population+treatment+Receptor=P2Tam+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	292,0000	236,0000	82,00000	-1,45969	0,144378	-1,45969	0,144378

variable	Population+treatment+Receptor=P3Tam+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	233,0000	173,0000	68,00000	1,355457	0,175273	1,355457	0,175273

variable	Population+treatment+Receptor=P4Tam+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	347,0000	473,0000	137,0000	-1,69063	0,090908	-1,69063	0,090908

variable	Population+treatment+Receptor=P0ut+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	341,0000	605,0000	131,0000	-2,39840	0,016467	-2,39840	0,016467

variable	Population+treatment+Receptor=P1ut+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	263,0000	143,0000	88,00000	0,071919	0,942666	0,071929	0,942658

variable	Population+treatment+Receptor=P2ut+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	171,0000	105,0000	51,00000	-0,548673	0,583231	-0,548673	0,583231

variable	Population+treatment+Receptor=P3ut+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	211,0000	254,0000	58,00000	-2,17629	0,029534	-2,17629	0,029534

variable	Population+treatment+Receptor=P4ut+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI G	327,0000	493,0000	117,0000	-2,23163	0,025640	-2,23163	0,025640

variable	Population+treatment+Receptor=P0Dil+Ki67 Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000							
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value	Valid N M
MFI G			0,00	0,00	1,000000	0,00	1,000000	10

variable	Population+treatment+Receptor=P0Anas+Erβ Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	405,0000	415,0000	195,0000	-0,121725	0,903117	-0,121725	0,903117

variable	Population+treatment+Receptor=P1Anas+Erβ Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	527,0000	554,0000	203,0000	1,251954	0,210587	1,251993	0,210573

variable	Population+treatment+Receptor=P2Anas+Erβ Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	302,0000	226,0000	106,0000	0,793018	0,427768	0,793018	0,427768

variable	Population+treatment+Receptor=P3Anas+Erβ Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	457,0000	323,0000	133,0000	1,587513	0,112398	1,587513	0,112398

variable	Population+treatment+Receptor=P4Anas+Erβ Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	292,0000	411,0000	82,00000	-2,66666	0,007661	-2,66666	0,007661

variable	Population+treatment+Receptor=P0Tam+Erβ Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	379,0000	251,0000	131,0000	0,616667	0,537455	0,616667	0,537455

variable	Population+treatment+Receptor=P1Tam+Erβ Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	332,0000	371,0000	122,0000	-1,44761	0,147726	-1,44761	0,147726

variable	Population+treatment+Receptor=P2Tam+Er β Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	390,0000	240,0000	120,0000	0,983333	0,325444	0,983471	0,325376

variable	Population+treatment+Receptor=P3Tam+Er β Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	342,0000	186,0000	108,0000	0,447637	0,654416	0,447637	0,654416

variable	Population+treatment+Receptor=P4Tam+Er β Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	423,0000	397,0000	187,0000	0,338126	0,735268	0,338126	0,735268

variable	Population+treatment+Receptor=P0ut+Er β Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	193,0000	272,0000	62,00000	1,649780	0,098989	1,649780	0,098989

variable	Population+treatment+Receptor=P1ut+ER β Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell lin Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	292,0000	528,0000	82,00000	-3,17839	0,001481	-3,17839	0,001481

variable	Population+treatment+Receptor=P2ut+ER β Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	386,0000	394,0000	176,0000	-0,379317	0,704453	-0,379317	0,704453

variable	Population+treatment+Receptor=P3ut+ER β Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	283,0000	182,0000	77,00000	1,434184	0,151521	1,434184	0,151521

variable	Population+treatment+Receptor=P4ut+ER β Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	560,0000	220,0000	10,00000	5,043515	0,000000	5,043515	0,000000

variable	Population+treatment+Receptor=P0Dil+ERβ Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	59,00000	112,0000	4,000000	-3,15426	0,001609	-3,15426	0,001609

variable	Population+treatment+Receptor=P0Anas+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	233,0000	328,0000	23,00000	-3,92387	0,000087	-3,92387	0,000087

variable	Population+treatment+Receptor=P1Anas+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	406,0000	414,0000	196,0000	-0,094675	0,924573	-0,094675	0,924573

variable	Population+treatment+Receptor=P2Anas+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	280,0000	350,0000	70,00000	-2,65000	0,008050	-2,65000	0,008050

variable	Population+treatment+Receptor=P3Anas+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	175,0000	150,0000	30,00000	2,468416	0,013572	2,468416	0,013572

variable	Population+treatment+Receptor=P4Anas+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	354,0000	276,0000	144,0000	-0,183333	0,854537	-0,183333	0,854537

variable	Population+treatment+Receptor=P0Tam+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	344,0000	397,0000	173,0000	-0,190029	0,849286	-0,190029	0,849286

variable	Population+treatment+Receptor=P1Tam+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	501,0000	319,0000	109,0000	2,448033	0,014364	2,448033	0,014364

variable	Population+treatment+Receptor=P2Tam+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	331,0000	197,0000	119,0000	0,019462	0,984472	0,019462	0,984472

variable	Population+treatment+Receptor=P3Tam+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	207,0000	199,0000	94,00000	0,160817	0,872238	0,160817	0,872238

variable	Population+treatment+Receptor=P4Tam+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	352,0000	468,0000	142,0000	-1,55538	0,119857	-1,55538	0,119857

variable	Population+treatment+Receptor=P0ut+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	301,0000	645,0000	91,00000	-3,37237	0,000745	-3,37237	0,000745

variable	Population+treatment+Receptor=P1ut+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	194,0000	212,0000	23,00000	-3,18843	0,001431	-3,18843	0,001431

variable	Population+treatment+Receptor=P2ut+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	200,0000	76,00000	40,00000	1,258720	0,208133	1,258720	0,208133

variable	Population+treatment+Receptor=P3ut+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	208,0000	257,0000	55,00000	-2,30185	0,021344	-2,30185	0,021344

variable	Population+treatment+Receptor=P4ut+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000						
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value
MFI R	310,0000	510,0000	100,0000	-2,69148	0,007114	-2,69148	0,007114

variable	Population+treatment+Receptor=P0Dil+PR Mann-Whitney U Test (w/ continuity correction) (Spreadsheet1 (1)) By variable Cell line Marked tests are significant at p <,05000							
	Rank Sum M	Rank Sum T	U	Z	p-value	Z adjusted	p-value	Valid N M
MFI R			0,00	0,00	1,000000	0,00	1,000000	10

Appendix VII: Turn it in report

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ORIGINALITY REPORT

12%	9%	9%	4%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

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