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Laboratory analysis of xenoestrogen exposed breast cancer cell lines

Final Report

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Amarbayasgalan DAVAATSEREN

Bachelor programme

„Medical and Pharmaceutical Biotechnology“

Department: Breast Cancer Research

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List of Abbreviations

Ab	Antibody
ABC	Avidin Biotin Complex
AF488	Alexa Fluor
BSA	Bovine Serum Albumin
BPA	Bisphenol-A
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
CSS	Charcoal Stripped Fetal Bovine Serum
DAPI	4', 6-diamidino-2-phenylindole
DCIS	Ductal Carcinoma in situ
DMEM	Dulbecco's Modified Eagle Medium
DMK	Acetone
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetracetic acid
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ERE	Estogen Response element
E_2	Estrogen
ER α	Estrogen receptor alpha
ER β	Estrogen receptor beta
FA	Formaldehyde
FCS	Fetal Bovine Serum
FCM	Flow Cytometer
FITC	Fluorescein Isothiocyanate
HER2	Human Epidermal Growth Factor
H_2O_2	Hydrogen Peroxide
IDC	Invasive Ductal Carcinoma
IF	Immunofluorescence
LC	Low Carbon
MCF7	Human Breast Adenocarcinoma cell line

MDA-231-MB	Human Breast Cancer cell line
MeOH	Methanol
mTOR	Mechanistic target of Rapamycin
PA	Patient Cells
PBS	Phosphate Buffered Saline
PET	Polyethylene terephthalate
PI	Propidium iodide
PIK3	Phosphoinositide 3 kinase
RPMI	Roswell Park Memorial Institute medium
SKBR3	Human Breast Cancer cell line
TAM	Tamoxifen
Triton X-100	Octyl phenol ethoxylate
XEs	Xenoestrogens
UV	Ultraviolet
4EBP1	4E-binding protein 1
eIF4E	Eukaryotic translation initiation factor 4E

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1. Abstract

Estrogen signaling increases the sensitivity of the breast tissue. Hereby, estrogen mimicking hormones xenoestrogens (XEs) can be found commercially available common-use products including food, beverage storage containers and cosmetics. Through inhalation and ingestion, XEs from a variety of environmental sources eventually enter the blood stream, and are thus detectable in the body fluids of the vast majority of human adults and children. Among other adverse effects, persistent exposure to XEs disrupts normal mammary gland biology in animal models and likely in humans as well, thereby increasing breast cancer susceptibility. However, since the effects of such XE exposure have not yet been directly demonstrated as an underlying causal factor in human diseases, they are generally considered safe by environmental regulatory agencies.

A recent study of the Dairkee lab showed that chemicals which are terephthalic acid (TPA), polyethylene terephthalate (PET) expose the cells and increases the ratio of the Estrogen Receptor α (ERα) and Estrogen Receptor β (ERβ) in multiple HRBEC samples. This shows estrogenic effect in the cell. Moreover, PET causes and preserves DNA damage-harboring non-malignant human breast cells by flow cytometry and western blots. Furthermore, it is still remained unknown the quantification of the ration between ER α and ER β on the breast cancer cell lines after exposure to the XEs.

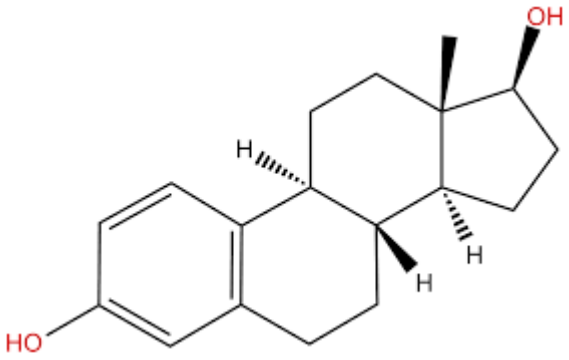
The aim of my internship was to analyse the xenoestrogen-exposed non-immortalized patient cells by optimizing the ERα and ERβ by Immunofluorescence and quantify the intensity of the nuclear signals by visualizing it under the microscope. The experimental procedures of the immunofluorescence are fixation, permeabilization, washing, and incubation of the primary and secondary antibodies. Each step has individual conditions such as storage of fixed cells, antibody dilutions, incubating temperature and duration. General IF protocol was implemented by the research group. The breast cancer cell lines which are MCF7, SKBR3 and MDA231 were used as controls.

2. Introduction

The main cause of the carcinogenesis in the breast is estrogen along with mutation of the gene further metabolism of it, and growth of the tissue is provoked. Estrogen is a primary female sex hormone that regulates the menstrual cycle and childbirth (Ryan, 1982). Even though after menopause, women still tend to produce estrogen. On the other hand, a woman who has had more menstrual cycle tend to lead higher risk of breast cancer, which means menstrual started earlier than the average age value(American Cancer Society, 2015b).

Estrogen has three kinds of steroid hormones, which are estrone, estradiol, and estriol (Burger, 2002). More in details, 17β-estradiol (E_2) stimulates the breast development at puberty and during sexual maturity, which most common hormone in breast tissue (Figure 1) (Russo & Russo, 2006).

Figure 1 Structure of 17β-estradiol

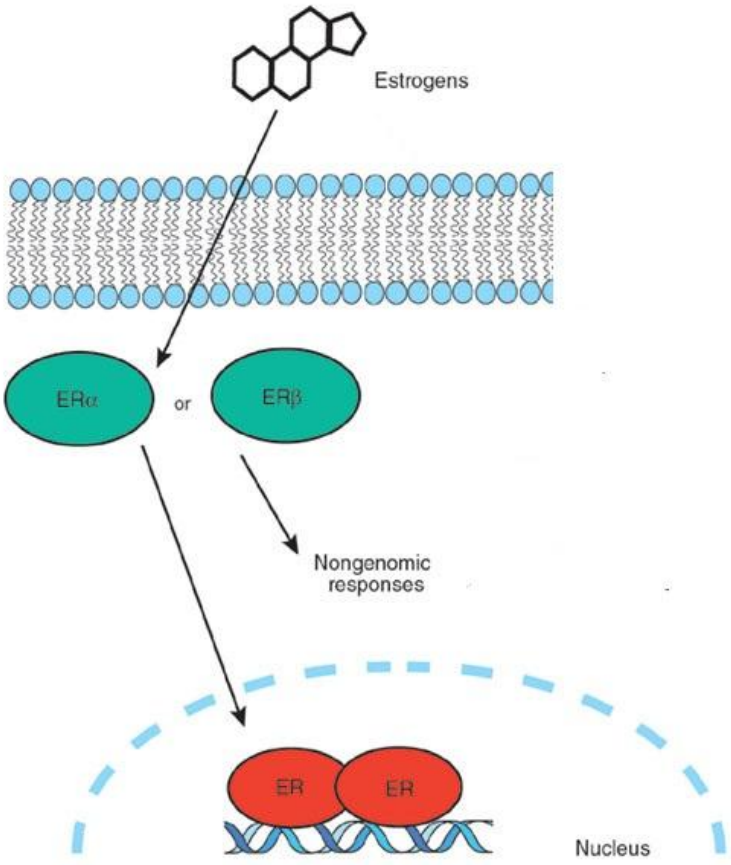


(www.wtt-pro.nist.gov; accessed 13th of March)

2.1 Breast Cancer – Estrogen signaling pathway

Estrogen receptor signaling pathway is mediated by estrogen receptors that become associated with mitochondria and the plasma membrane. Furthermore, when estrogen directly binds to the nuclear estrogen receptor which then stimulates the gene alteration by binding as a dimer to estrogen-response genes and causes the translocation of the nucleus (Yue, Yager, Wang, Jupe, & Santen, 2013). The estrogen receptor has two isomers that are ER β and ER α, while ER-β downregulates the breast cancer (Figure 2) (Alayev et al., 2015).

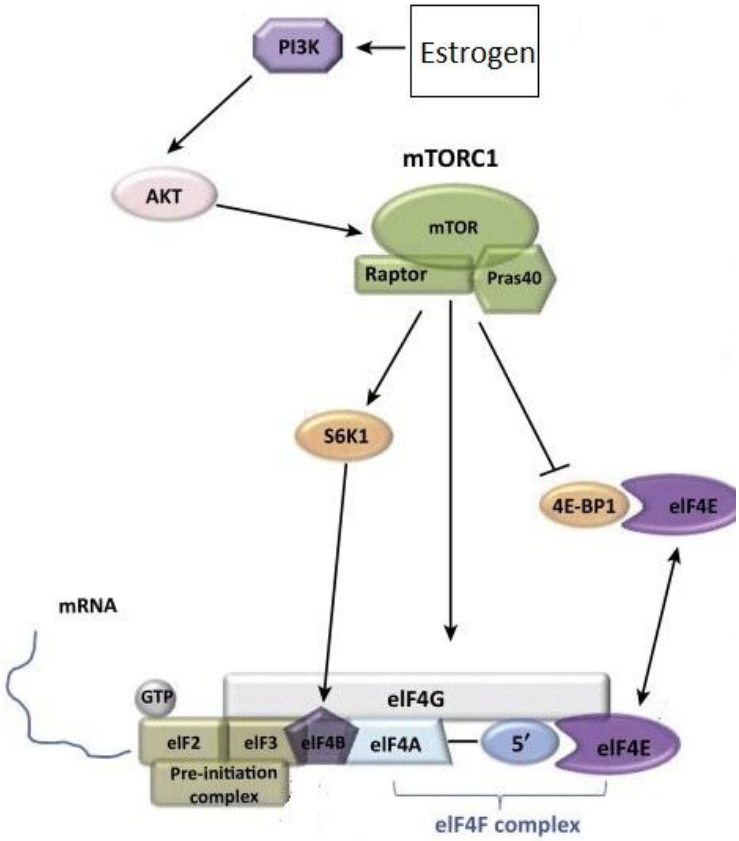
Figure 2 Estrogen Receptor signaling pathway



(www.nature.com; accessed 26th of February)

Hereby, mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that has two different complexes, mTORC1 and mTORC2 .Moreover, mTOR pathway is associated with activation of the phosphoinositide 3 kinase (PI3K) by binding estrogen, which leads to breast cancer cell growth and tumor proliferation (Paplomata & O'Regan, 2014). Once mTOR is activated it phoshorylates 4EBP1 then releases its target eIF4E(Goodson et al., 2011). The target binds to the estrogen-response elements (EREs) which is transcription factor on DNA and alters the gene. Once altered gene expression will lead to growth of breast cancer cell and cell proliferation (Figure 3) (Palmieri et al., 2002).

Figure 3 mTOR pathway after Estrogen binding to Estrogen Receptor alpha



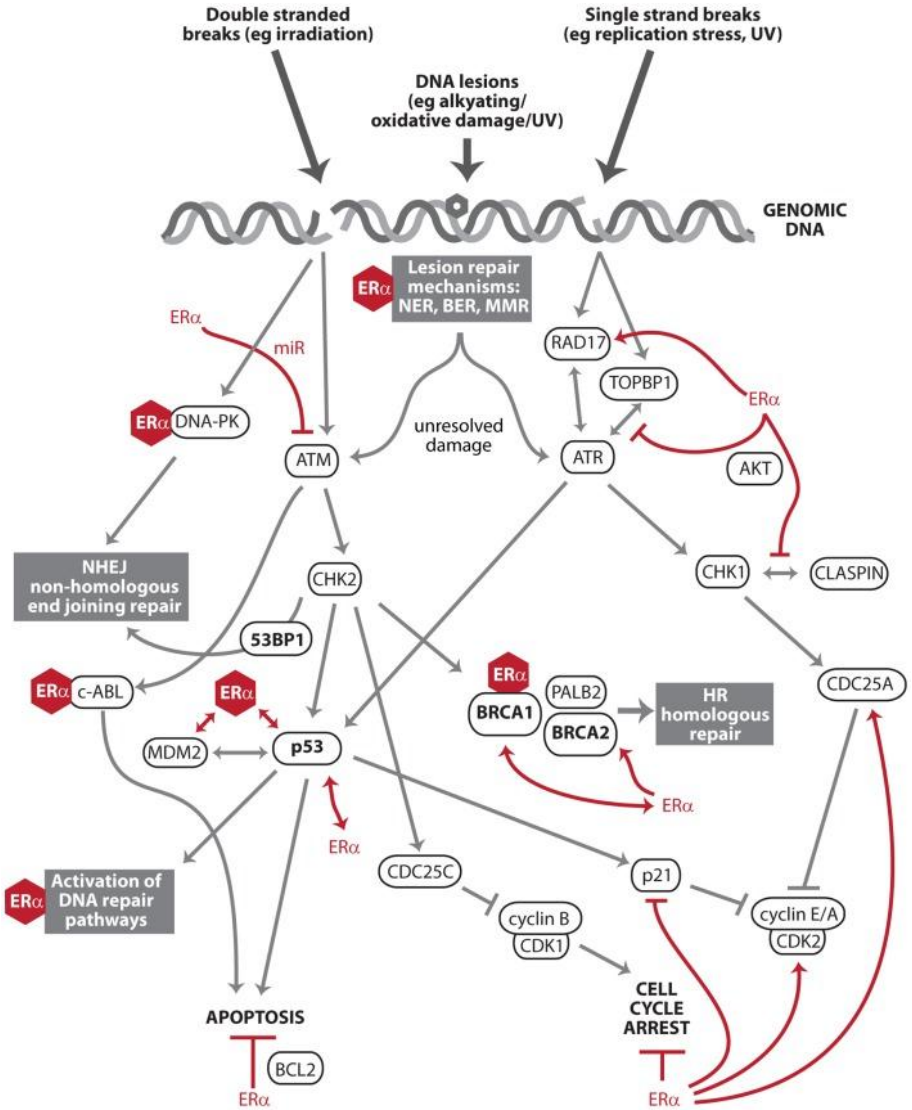
(www.cell.com; accessed 27th of February)

Another fact is that, enormous amount of estrogen binding to its receptors leads to induction of DNA damage, but also oxidative DNA damage (Musgrove & Sutherland, 2009; Yager & Davidson, 2006). DNA damage response (DDR) recognizes the DNA damage. The role of DDR is to repair the DNA damage, arrest the cell cycle, or if the damage is not repairable, apoptosis. ATM, ATR, and DNA-PK are the main effector kinases of the DDR. The main role of ATM and DNA-PK identify double stranded breaks of DNA and initiates the phosphorylation of certain proteins p53, mdm2, BRCA-1, Chk2 and Nbs1, whereas ATR notices interrupt of single stranded region. Furthermore, ATR decreases the G2/M checkpoint response

Downstream of ATM/ATR activates tumor suppressor protein p53. Nuclear phosphoprotein that activates G1 cell cycle arrest is encoded by p53. Furthermore, it activates apoptotic response to DNA damage (Green & Lin, 1979).

ER α suppresses ATR/CHK1 by transactivated AKT phosphorylation of TOBP1. This action prevents interaction with ATR at sites of DNA damage (Pedram, Razandi, Evinger, Lee, & Levin, 2009). Moreover, this complex leads to reduction of Cyclin Dependent Kinases (CDKs), which arrests and slows the cell cycle at G1-S, intra S and G2-M heck points. This allows to repair the damaged DNA in the meantime. Cell death is triggered by DDR signaling if the DNA is not able to repaired. Eventually it leads to apoptotis or cellular senescence (Figure 4) (Green & Lin, 1979).

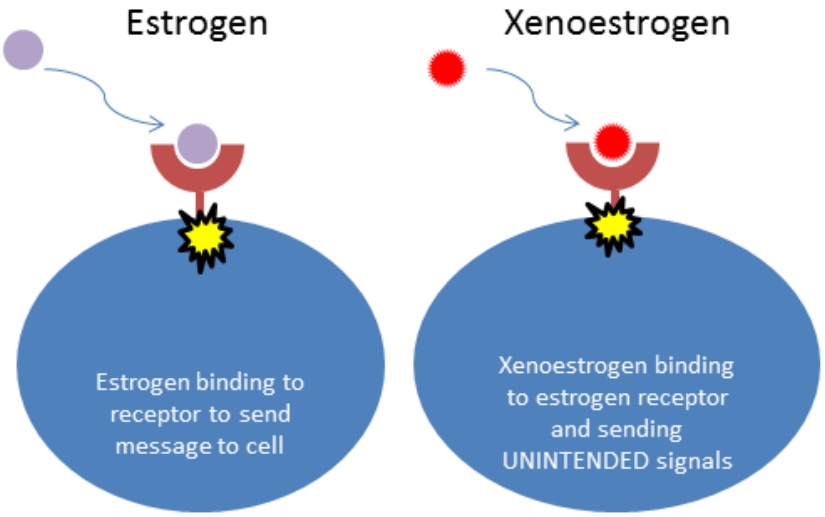
Figure 4 Oxidative DNA damage signaling pathway



(http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4030134/; accessed 8th February)

Nowadays, not only the woman is developing breast cancer, but also men have been facing with the same issue. Even though men have less of the female sex hormone, still developing breast cancer by additional sources of estrogen mimicking chemicals, which is a xenoestrogen (XE), such as bisphenol-A (BPA) and PET(Goodson et al., 2011). Furthermore, XEs bind to ERs and stimulates the breast cancer growth (Figure 5) (Fernandez & Russo, 2010).

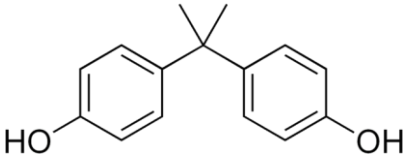
Figure 5 Comparison between Estrogen and Xenoestrogen binding to ERs



(www.weightlossforwomenwithpremenopause.com; accessed 24th February)

For instance, BPA is a chemical substance that can be found from surrounding items which are plastic, epoxy resins and thermal paper etc. (Figure 6) (Pivnenko, Pedersen, Eriksson, & Astrup, 2015). It is involved in several oncogenic pathways, STAT3, MAPK, and PI3K/AKT (Gao et al., 2015). In addition to this, the cell survival is regulated by multiple checkpoints, but BPA exposure induces abnormal expression of these checkpoints. (Dairkee, Luciani-Torres, Moore, & Goodson, 2013).

Figure 6 Structure of BPA

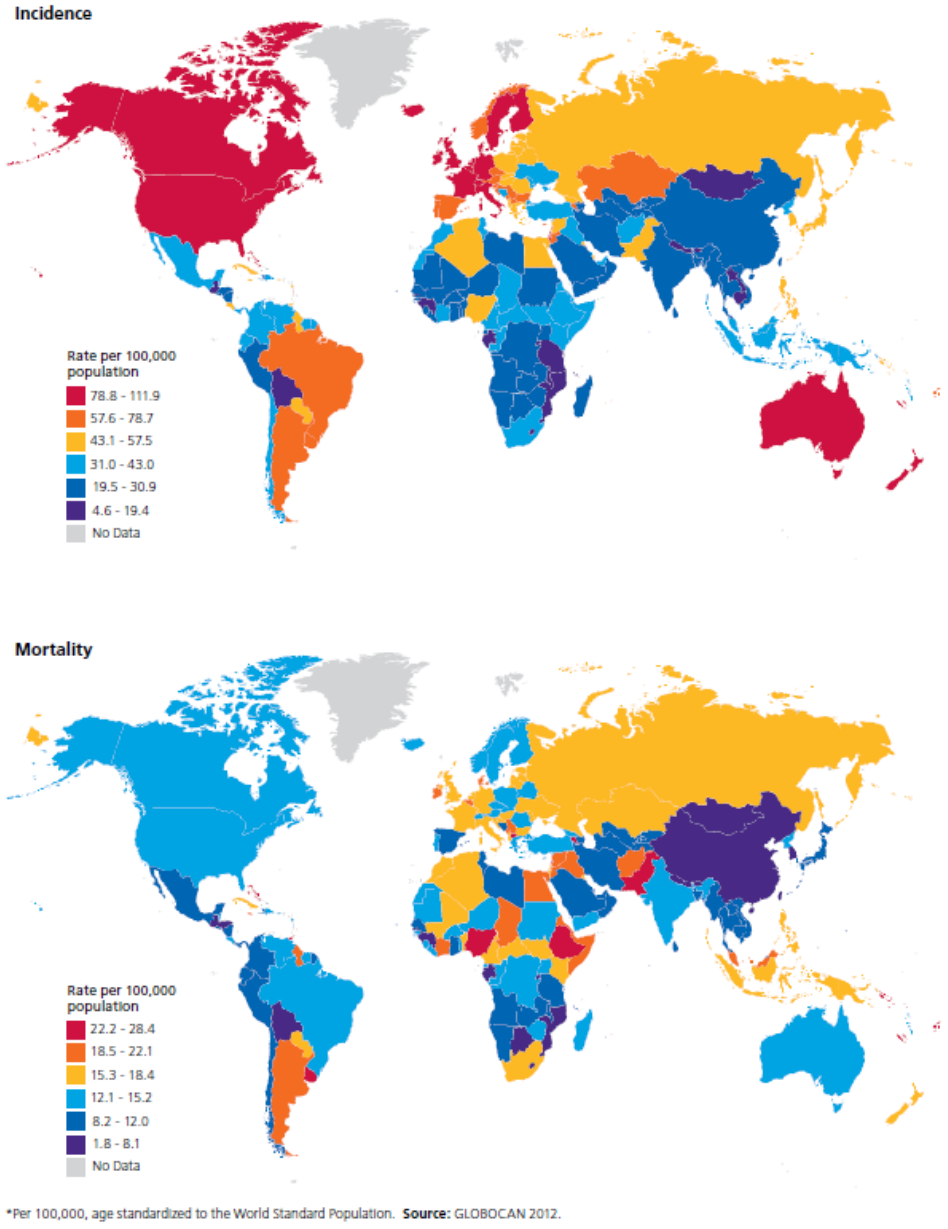


(www.chemistry.about.com; accessed 1st of March)

2.2 Breast Cancer – Statistics

The incident of the breast cancer is highly increasing in the last few years. In 2012 worldwide 1.7 million cases were diagnosed in woman. As a matter of fact, the incidence is high in more developed countries, the mortality rates differ only in the countries that have improved health care systems or economic backgrounds (Figure 7) (American Cancer Society, 2015b).

Figure 7 Breast cancer statistics of incidence and mortality of 2012 all over world



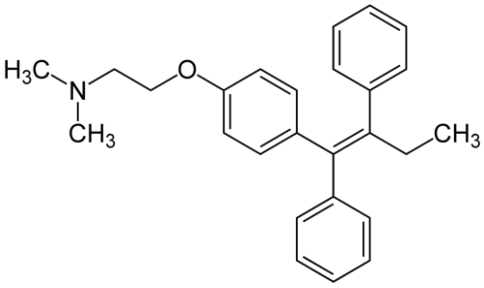
(www.cancer.gov; accessed 26th of February)

Recent statistics showed that there will be more than hundred thousands of new cases of female breast cancer are expected to be diagnosed in 2015(American Cancer Society, 2015a).

2.3 Breast Cancer – Therapy

A recent study showed that estrogen treatment leads to the development of mammary tumors (Yager & Davidson, 2006). Hereby anti-estrogen therapy or hormone therapy is recommended for the estrogen receptor alpha positive breast cancer patients. Hormone therapy reduces or stops the growth of hormone-sensitve tumors by blocking its action. The most used drugs for the breast cancers are tamoxifen and trastuzumab (Herceptin) (Leong & Zhuang, 2011). These are the main inhibitors of the estrogen receptor alpha and have less toxic effect than chemotherapeutic substances. Addition to this, tamoxifen is estrogen antagonist and tumors, which contain receptors respond more often to this (Figure 8) (Heel, Brogden, Speight, & Avery, 1978).

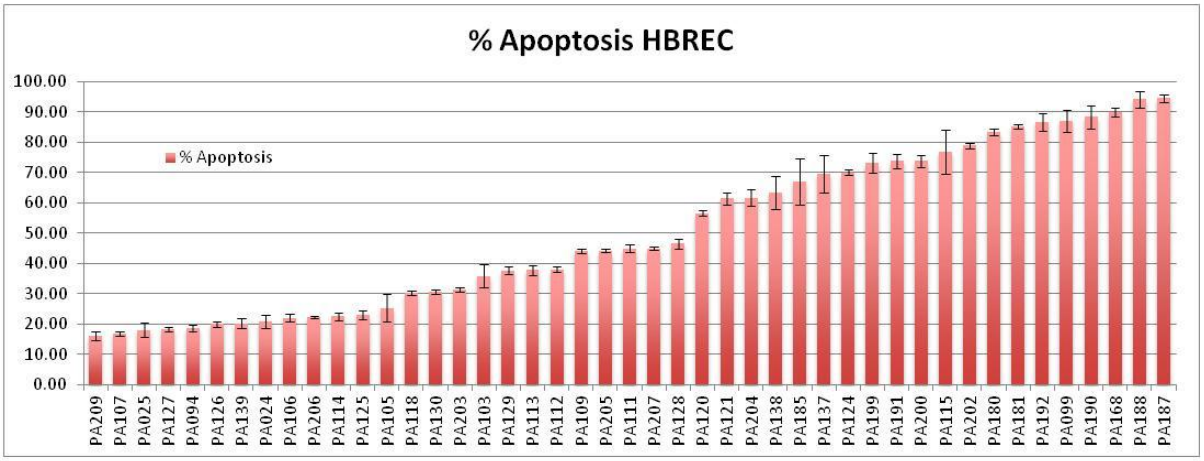
Figure 8 Structure of Tamoxifen



(www.commons.wikimedia.org; accessed 13th of March)

Tamoxifen responds individually to the patients as the reason of number of estrogen receptors. The percentage of the apoptosis shows the effect of the tamoxifen to each sample (Figure 9). The risk of the ER-positive breast cancer can be reduced by implementing tamoxifen (Cuzick et al., 2003).

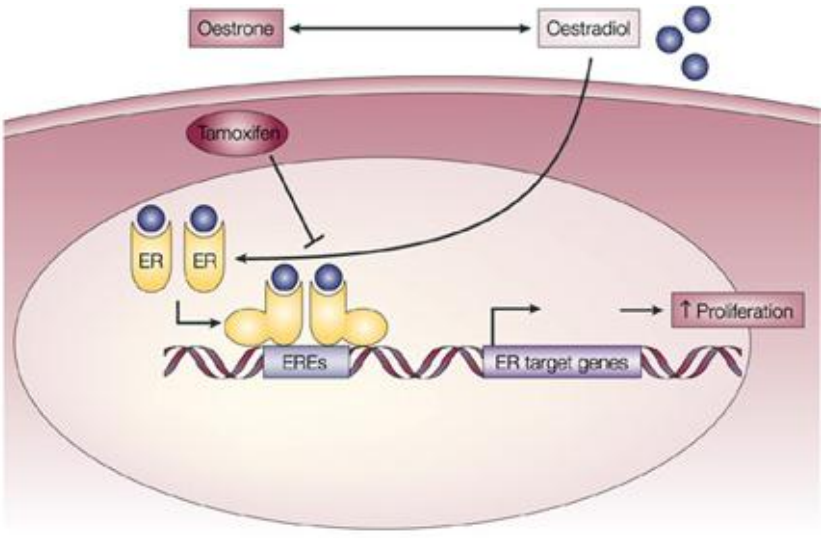
Figure 9 Cell response of the patient samples after treatment with Tamoxifen



(Personal document; accessed 20th of February)

The tamoxifen is metabolized in the liver and produces its metabolites such as 40hydroxytamoxifen and N-desmethyl-4-hydroxytamoxifen. The active metabolites inhibit the estrogen to binding the estrogen receptors. 4-hydroxytamoxifen is an antagonist of estrogen receptor that results main inhibitor of the transcription of estrogen response elements (Figure 10) (Wang, Fulthorpe, Liss, & Edwards, 2004). Concerning on the influence of tamoxifen in the cell cycle, it prevents the breast cancer cell division by persisting the cells in the phases of G_0 and G_1 (Liu et al., 2013).

Figure 10 Tamoxifen inhibits the Estrogen signaling pathway



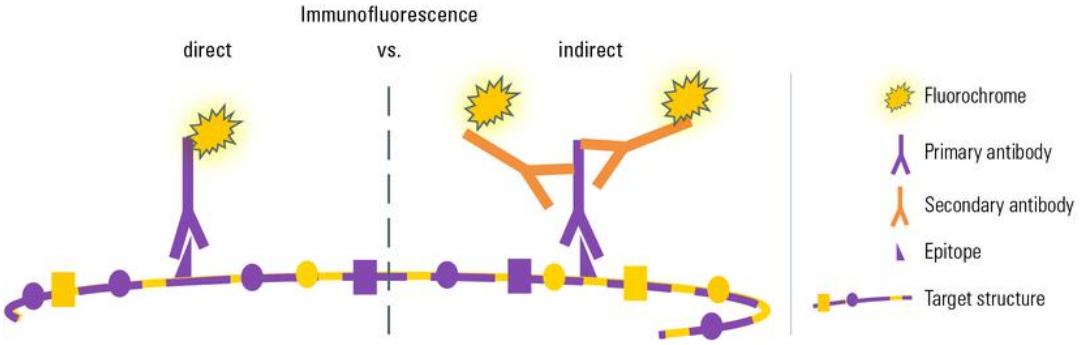
(flipper.diff.org; accessed 1st of March)

Unfortunately this medication has several crucial side effects including small increased risk of uterine cancer, stroke, eyesight problem, and pulmonary embolism. Besides, endocrine therapy cannot be implemented in the patients with triple-negative tumors or therapies that targeted to HER2 as negative breast cancers have lack expression of ER (Foulkes, Smith, & Reis-Filho, 2010; Kalimutho et al., 2015).

3. Experimental Procedure (Materials and Methods)

Immunofluorescence (IF) is the technique that based on the microscope which visualizes the target by fluorophore-labeled antibodies. This technique has two methods which are direct and indirect. The primary antibody is labeled with a fluorescent dye to detect the target for Direct Immunofluorescence (DIF). Although, the indirect method requers two steps of incubation. Hereby primary antibody localizes the target by epitope and secondary antibody specifically binds to primary antibody and fluorophore itself. This method is more sensitive than DIF because numerous secondary antibodies can bind to each primary antibody (Figure 11).

Figure 11 Direct and indirect immunofluorescence



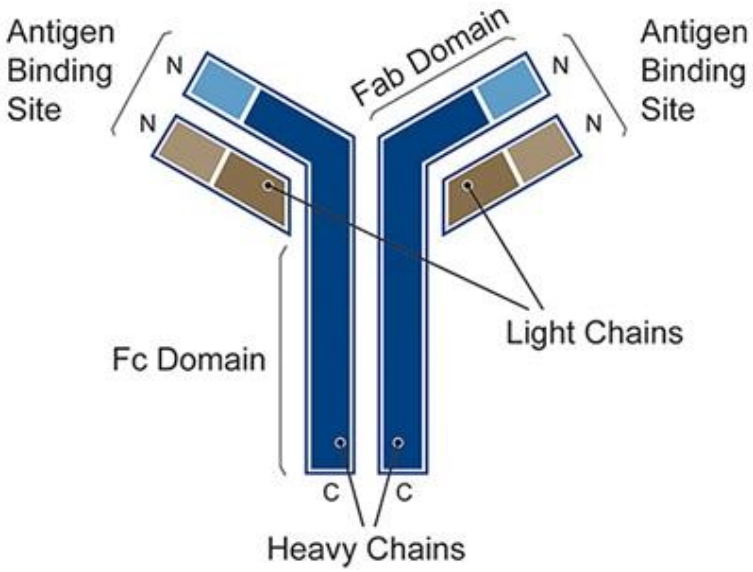
(www.leica-microsystems.com; accessed 25th of February)

Furthermore amplification of the signal can be implemented by strong affinity of Avidin binding to biotin which is called Avidin-Biotin complex (ABC). The Avidin is biotin-binding protein which is produced from egg white. Hereby secondary antibody is biotin-labeled thus tetrameric or dimeric fluorophore labeled Avidin

binds to it. The indirect method requires blocking step to prevent the non-specific binding or non-specific staining of secondary antibody.

The fluorescence can be detected by using a flow cytometer or visualizing at the confocal microscopy. One of the colossal disadvantages of the DIF is that high background can be observed by endogenous immunoglobulin further it can be counted as a positive signal. Fluorescent dye is absorbed and emitted in different wavelength as a result of fluorescence has a lower energy than the absorbed light. Antibody is Y-shaped molecule and two arms of the Y end in regions that vary between different antibody molecules, the V regions and it is produced by activated B-cells or B lymphocytes(Jr, York, & Science, 2001). Herewith, Immunoglobulin consists of two identical heavy chains and light chains. Each chain is bonded with disulfide bonds. Primary antibody has two domains, which are Fc and Fab, therefore secondary antibody binds to the Fc domain of the primary antibody further Fab domain binds to an antigen (Odell & Cook, 2013).

Figure 12 Structure of primary antibody



(www.windowssearch-exp.com; accessed 12th of March)

There are five classes of the antibodies which are IgG, IgM, IgA,IgE, and IgD. Each subtypes of the immunoglobulin have different heavy and light chains, thus bind to different antigens and functions individually. Hereby, IF mostly works with IgG, which has the binding sites of Fc and Fab (Figure 12) (Woof & Burton, 2004)

3.1 Materials

General reagents	Company
10x PBS (Mg^{++}, Ca^{++} free)	UCSF cell culture facility
37% Formaldehyde	Fisher Scientific
BSA	Santa Cruz
FCS	Corning
Mounting medium with DAPI	Vector
RPMI	Sigma Aldrich
DMEM	Sigma Aldrich
Trypsin-EDTA	UCSF cell culture facility
ER α G20	Santa Cruz Biotechnology
ER α HC20	Santa Cruz Biotechnology
ER β [14C8]	Genetex
AF488	Life tech
Biotinylated anti-mouse Ab	Vector labs
Biotinylated anti-rabbit Ab	Vector labs
AF594	Life tech
Avidin FITC	Vector labs
Streptavidin AF594	Vector labs
Tamoxifen	Sigma Aldrich
Annexin kit	MBL international
6, 8 peaks bead	BD Accuri Cytometer
Antibiotic/Antimycotic 100x	UCSF cell culture facility

General materials	
Cover slips	Thomas® Red label® Micro cover glasses
Tissue culture plates	100 mm; SantaCruz Biotechnology
25 cm^2 flask	Santa Cruz Biotechnology
75 cm^2 flask	Santa Cruz Biotechnology
6 well-plate	Ultra Cruz™

General equipments

Incubator	Sanyo
Hood	The baker company
Flow Cytometer	BD Accuri™ C6

3.2 Immunofluorescence

3.2.1 Preparation of the cover slips

Before starting the experiment, cover slips (No.2 11x22mm; 6663-Q10) were prepared so that breast cancer cell lines could seeded on it. The cover slips were placed in a big petri dish and washed with autoclaved water thoroughly. Afterwards, the cover slips were washed with PBS 1x + 0.5% Triton X-100 for 15 minutes with agitating the plate and then washed with autoclaved water till bubbles were disappeared. Triton X-100 is a detergent that normally used in the laboratory. The breast cancer cell lines and PA cells are very sensitive, thus everything should be sterilized very well. Therefore the cover slips were dried in the hood overnight with ultraviolet light. After turning off the UV light the edges of the cover slips were cut and generally 9 cover slips were transferred to each 100x 15mm tissue culture plate.

3.2.2 Cell preparation

The breast cancer cell lines (SKBR3, BT474M1 and MDA231) were incubated in the medium of RPMI + 10% FCS and DMEM + 10% FCS was used for MCF7. To conserve the media, all breast cancer cell lines that were growing in the 75 cm^2 flasks were passaged to 25 cm^2 flasks on the other hand PA115 was still growing in 75 cm^2 flask. All cancer cell lines and PAs were split from tissue culture flasks by adding Trypsin-EDTA. When the cells were started detaching from the flask, it was collected in the 15 ml in appropriate amount of medium. Cover glasses were placed in 100x 15 mm tissue culture dish and appropriate amount of $0.5x10^6$ PA115 cells and $0.5x10^6$ of breast cancer cell lines (MCF7; SKBR3; MDA231) were amounted on it. The cells were incubated for 2-4 days depending on the confluency. The PA115 grows well with 0.5% of carbon dioxide at 37°C in LC2% medium, whereas the cancer cell lines were incubated with 5.0% of carbon dioxide at 37°C.

3.2.3 Treatment of the cancer cell lines

After incubation of the breast cancer cell lines and PA115 the media were changed in according to their cell types, so that cells can be treated with estrogen and the mixture of xenoestrogens. The medium of the MCF7 was changed to 2% CSS (white medium) + DMEM and PA115 medium were changed into 0.2% LC from 2%LC.

The PA115 was treated with different concentrations of estrogen (estrodial-β) and mixture of the xenoestrogens which are BPA, MD and PFOA. The treatment took a place of 6 days in the incubator. After the treatment cover slips were ready to be fixed.

3.2.4 Fixation

The main idea of this step is to arrest the movement of the cells. There are two types of fixations can be performed, which is acetone (DMK) or methanol (MeOH) fixation for organic components, and formaldehyde (FA) fixation for crosslinking reagents. The MeOH or DMK fixation is harsh to tissue culture cell and a patient's cancer cell, thus 4% FA is recommended. The cover slips were remained still in the tissue culture plates and medium should be discarded. After discarding the media the cover slips were washed one time with PBS 1x. After washing step the cover slips were treated with freshly made 4% FA + 1x PBS solution for 15 minutes at room temperature.

3.2.5 Storage

The cover slips were washed once with PBS 1x before it was stored. The cells can be stored as long as in PBS 1x at 4°C. This is the appropriate storage of cover slips. To prevent the fungal growth in the tissue culture plates in cold room, the plates were wrapped with parafilm.

3.2.6 Permeabilization

After cover slips were transferred in 6-well plate, the plate should be labeled according to the conditions. After the cover slips were washed, 2ml of PBS 1x + 0.2% Triton was added to the each well that has cover slips and incubated for 10 minutes at room temperature while agitating. After the permeabilization, the cover slips were washed two times with PBS 1x + 0.5 % BSA + 0.05% Triton X-100 and one time with PBS1x for 5 minutes with rocking.

3.2.7 Blocking

Blocking is very important part of the IF. Antibodies tend to bind non-specifically to the antigens or a target, to prevent this issue blocking is required. The solution that used for blocking is freshly made PBS + 10% FCS. 100% FCS was diluted with PBS 1x in the exact amount that needed. The blocking procedure took 1 hour of incubation at room temperature with agitating. After blocking the cover slips were washed with PBS 1x. In general, BSA is used for the blocking for IF. Nevertheless, FCS has a very low amount of antibodies, thus we used it as blocking solution.

3.2.8 Primary antibody incubation

The primary antibody was diluted in PBS + 1% BSA solution. There were three kinds of primary antibodies used that were polyclonal rabbit ER α HC20 (sc-543), polyclonal rabbit ER α G20 (sc-544) and monoclonal mouse ER β (14C8). Final concentrations of ER α HC20 and ER α G20 are 1:250 and ER β is 1:50. After washing step, 50 µl of the primary antibody solution was added on the cover slips, although 50 µl of PBS+1% BSA were added to the cover slips for no antibody control. The 6-well plate was incubated in the incubator at 37°C for 2 hours. After the incubation the cover slips were washed two times with PBS 1x for 5 minutes.

3.2.9 Secondary antibody incubation

Secondary antibody was diluted in PBS +1% BSA solution as well. After washing the cover slips, 50 µl of the secondary antibody solution was added to each well. The secondary antibodies were Alexa Fluor 488 (anti-rabbit), Biotinylated anti-

rabbit secondary antibody(BA-1000), and anti-mouse AF594. AF488 and AF594 have fluorescent dye thus it should be kept in a dark place. The secondary antibody incubation took 45 minutes at room temperature with agitating and the 6-well plate was covered with aluminum foil to avoid emission of the fluorescent dye. After incubation the cover slips were washed 3 times with 1x PBS thoroughly.

3.2.10 Avidin Reaction

Biotin labeled secondary antibodies were detected by fluorescently labeled Avidin diluted in 1x PBS + 1% BSA. 100 µl of the solution was added on the cover slips and was incubated for 30 min at room temperature with agitating. After incubation, cells were washed 3 times with 1x PBS while rocking.

3.2.11 Mounting

The cover slips were transferred on the microscope slides (Cytochrome™ Microscope slides; Shandoz; Order number. 9991004) by tweezers. The cells were mounted with mounting medium containing DAPI as nuclear staining dye (Vector Labs). The purpose of the mounting medium is to prevent the fluorescent dye to bleach by the light or laser from the microscope.

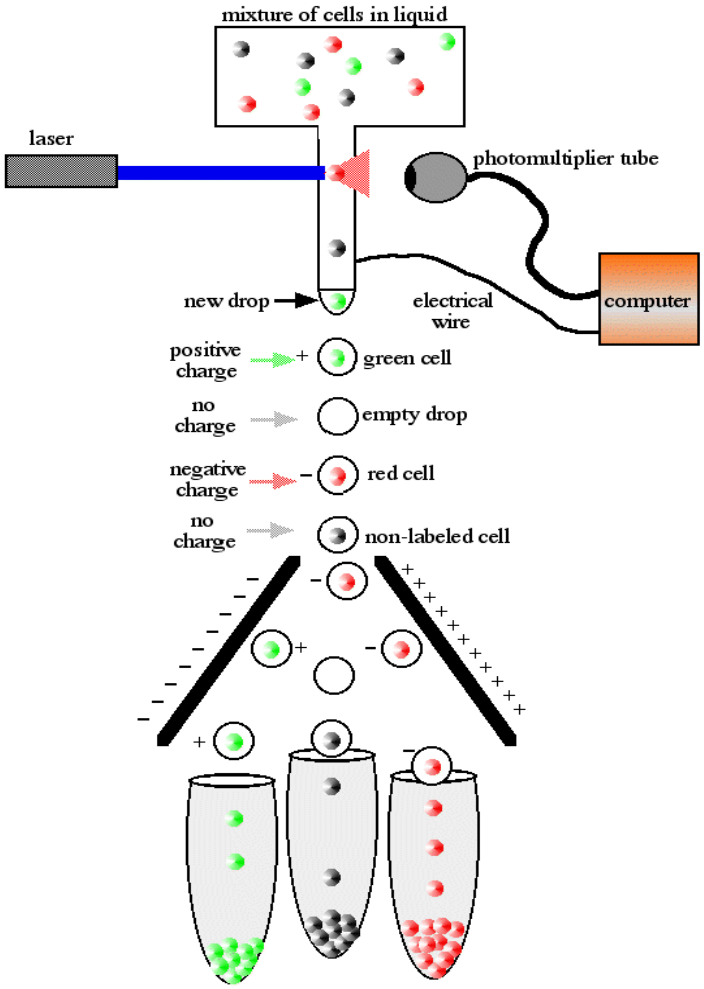
3.2.12 Microscope

All IF pictures were taken with Zeiss AX10 Observer Z.1 equipped with Axiocam 506 mono, 503 color and HXP120C as light source. Zen pro was used as software. Furthermore the set exposure was done by the software.

3.3 FACS – flow cytometry

Flow cytometry is used for quantitative and qualitative analysis of cells (Pospichalova et al., 2015). Hereby fluorescence-activated cell sorting (FACS) is one of the specialized type of flow cytometry (Julius, Masuda, & Herzenberg, 1972). To analyse the the samples, the cells are stained with fluorescence labeled antibodies that binds to the intracellular molecules and to the cell surface. At the certain point of the wavelength the fluorescence emits when cells are passed through the laser beams (Aghaeepour et al., 2013). The cells are sorted according to their electrical charges. Thereafter, the cells were determined and analyzed at the computer (Figure 13).

Figure 13 Principle of the flow cytometry

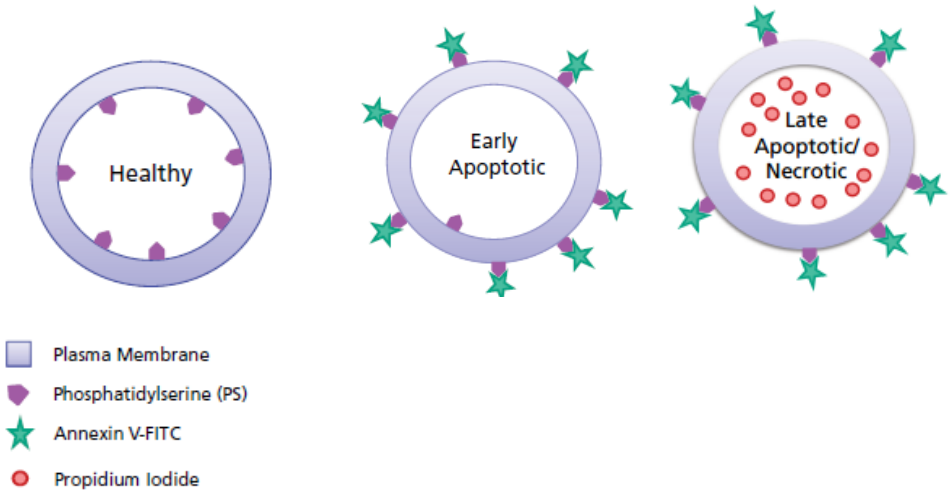


(www.bio.davidson.edu; accessed 26th February)

FACS performence with Annexin V

Quantification of the apoptotic cells in the samples, Annexin A5 affinity assay is used (Van Engeland, Nieland, Ramaekers, Schutte, & Reutelingsperger, 1998). Annexin binds to the phosphatidylserine (PS) to detect the cells underlying in the early stages of apoptosis, moreover it is family of calcium-dependent phospholipid binding protein (Figure 14). The assay is implemented together with propidium iodide (PI) and it identifies dead cells (Moore, Donahue, Bauer, & Mather, 1998; Schutte, Nuydens, Geerts, & Ramaekers, 1998).

Figure 14 Annexin V and PI binding to the target



(www.bdbiosciences.com; accessed 12th of March)

3.3.1 Cell preparation

PA115 and MCF7 were seeded in the 6-well plate individually and appropriate amount of cells which were 250.000 cells/well. The MCF7 plate was incubated for 24 hours at 37°C applying 5.0% CO_2 on the other hand plate that seeded with PA115 was incubated at 37°C applying 0.5% CO_2.

3.3.2 Cell treatment

After the incubation, first lower row of the both 6-well plates were treated with tamoxifen (TAM) for 24 hours with an appropriate incubation conditions. After 24

hours of treatment, upper rows of the cells were treated with 0.5mM, 1mM of hydrogen peroxide (H_2O_2). The treatment with H_2O_2 took 2 hours of incubation. The one well of the cells were kept as a control.

3.3.3 Further procedure

The supernatants were collected in the 15 ml tubes and the tubes were labeled according to the conditions and treatments. The plates were washed one time with PBS 1x and the supernanats were collected also into the according tubes. 150 µl Trypsin + EDTA was added to each well and incubated for 5 minutes in the incubator. After the incubation, 2 ml of 10% FCS + PBS was added to each well and the supernatants were collected in to the according tubes. The tubes were then spun down at 1500 rpm for 3 min at 14°C. The supernatant was discarded as dry as possible. The pellets were vortexed with remaining supernatant in the tube. 108 µl of Annexin V solution (Ex: 488 nm; Em: 350 nm) was added into the each pellet. The 108 µl of the Annexin V solution was consisted of 100 µl of Annexin binding buffer, 4 µl of Annexin V, and 4 µl of PI. Annexin V is the indicator of the apoptosis, whereas PI identifies the dead cells. The tubes were then vortexed and incubated for 20 minutes in a dark place. After the incubation, 200~250 µl of Annexin binding buffer was added in to the tubes.

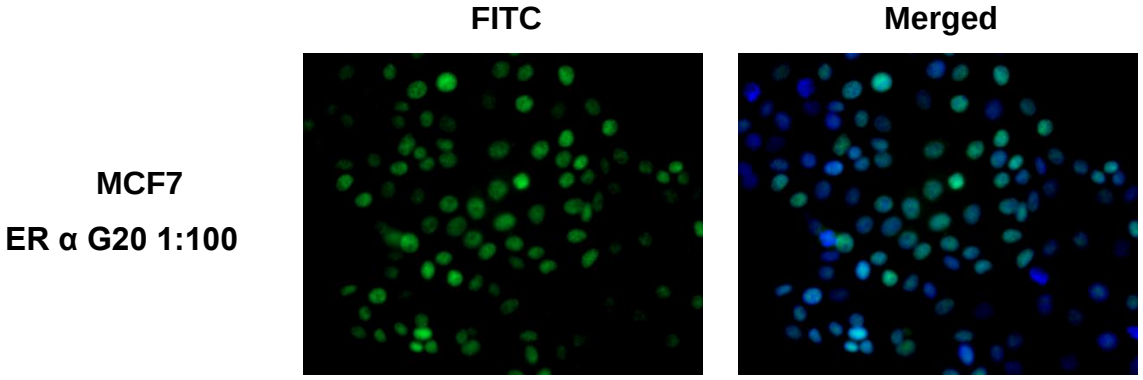
3.3.4 Performance at flow cytometer

The flow cytometer (BD Accuri™ C6) had to be turned on at least 15 min before analyzing the samples. To check laser performance of FACS machine the 6 peaks and 8 peaks bead tests were done before performing the experiment. There were 2 tubes of 6 peaks and 1 tube of 8 peaks bead were provided by the company of BD Accuri™ C6 and to perform the bead test, 2 drops from each tube were added in 500 µl water in separate glass tubes. After calibration of bead test, the samples were transferred to the glass tubes so that it could be read at the BD Accuri™ C6. The all collected data were analyzed by BD Accuri C6 software which was provided by the company. The washing step had to be done before turning off the machine. Hereby, the machine was washed with 500 µl of decontamination solution and 500 µl for 5 min.

4. Results

4.1 Primary antibody test

Before starting the experiment, the newly arrived polyclonal rabbit primary antibody ER α G20 was tested on the MCF7. In order to determine the appropriate concentration of the primary antibody, several dilutions were made, which were 1:100, 1:250, 1:500, and 1:1000. The MCF7 in the amount of 500000 cells/plate was firstly seeded on the cover slips and incubated the cover slips till the cells were confluent enough to be fixed. The supporting medium is DMEM + 10%FCS. After 2 days of incubation, the cover slips were fixed with 4% FA + PBS1x for 15 min at the room temperature. After fixation the cover slips were permeabilized with PBS 1x+ 0.2% Triton for 10 min, further it was blocked with 10%FCS + PBS 1x for 1h at room temperature with agitating. Since the cells were blocked, four serial dilutions of primary Ab were added on the each cover slips. The cover slips were then incubated in the incubator for 2 hours. After incubation with primary antibody, fluorescene labeled secondary anti-rabbit antibody AF488 in the dilution of 1:100 was added on the cover slips. The cover slips were incubated in the dark place to prevent from the bleaching. After secondary antibody incubation, the cover slips were incubated with mounting medium that has DAPI for overnight. The upcoming day the photos were taken at the microscopy. It can be seen from figure 15 that the intensity of nuclear signals was decreasing by in each dilution starting from the highest to lowest. The nuclear signal from ER α is indicated as green fluorescence.



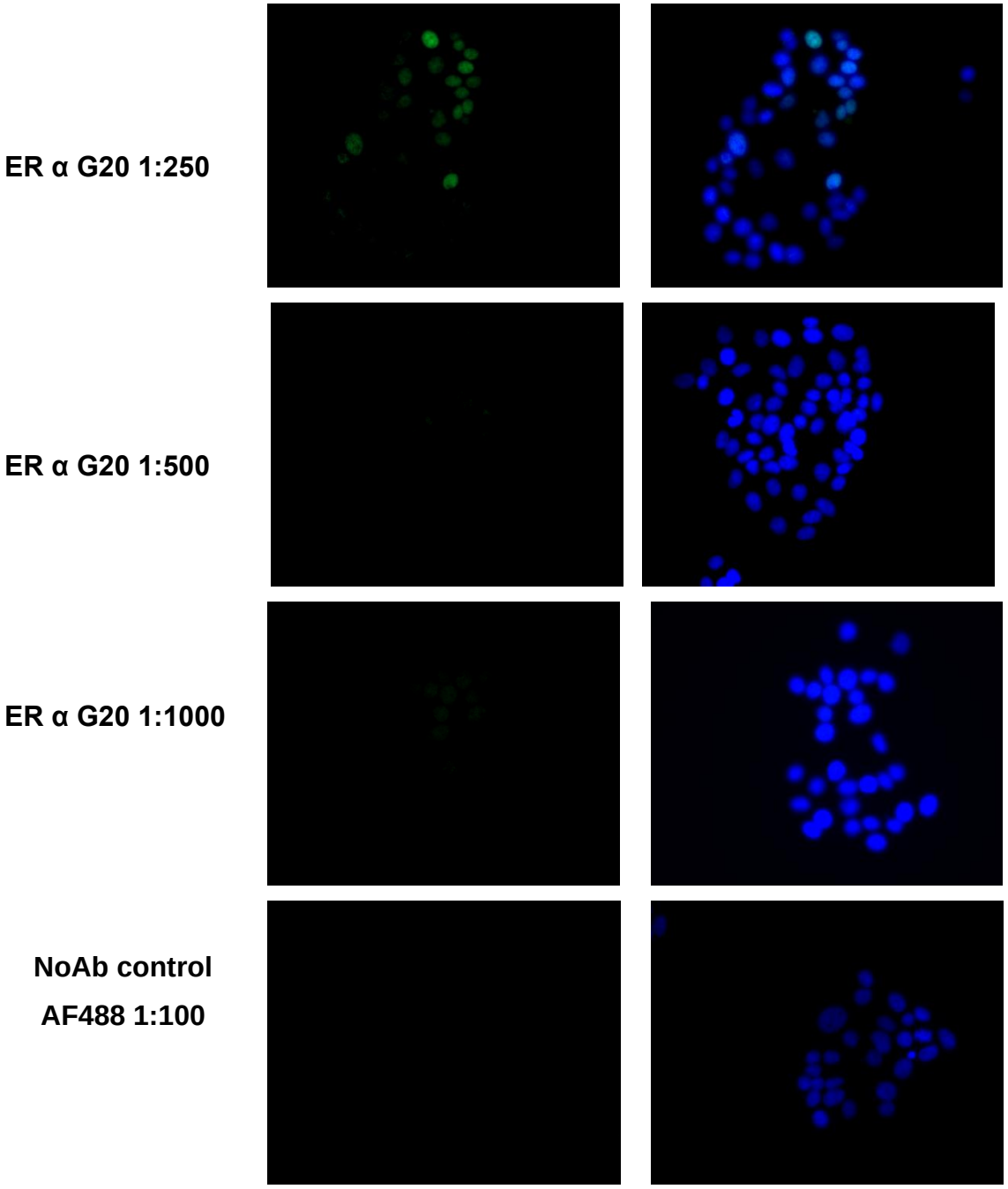


Figure 15 Testing the appropriate concentration of polyclonal rabbit primary antibody ER α G20 on MCF7. Four types of dilutions were made for the primary Ab, which are 1:100, 1:250, 1:500, and 1:1000. To see the nuclear signal, the cells were incubated with DAPI. All photos were taken at 40x

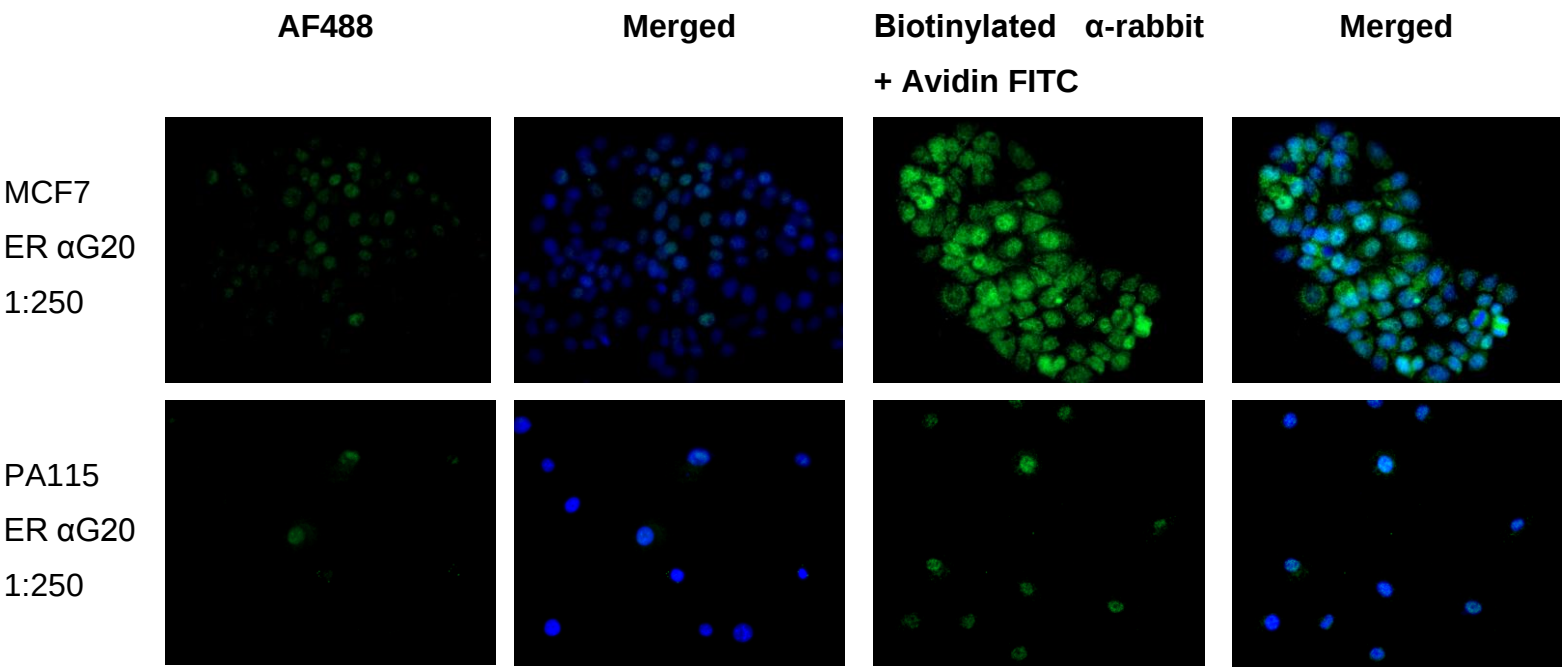
DAPI localizes the nucleus thus it was able to determine the nuclear signal by merging the pictures. The background signal of secondary antibody was

determined by NoAb control. Hereby secondary Ab worked and background signal was not observed (Figure 15).

4.2 Testing two different secondary antibodies

The aim of the experiment was to compare the intensity of two different secondary antibodies on the MCF7 and PA115 under the same condition. For primary antibody incubation ER α G20 in the dilution of 1:250 was used. The two secondary antibodies were anti-rabbit AF448 in the dilution of 1:100 and anti-rabbit Biotinylated antibody in the dilution of 1:100 combination of Avidin (FITC) in the dilution of 1:200. The incubation time of the Avidin was 30 minutes at the room temperature in dark place.

It can be seen from the figure 16, the Biotinylated anti-rabbit secondary antibody gave stronger nuclear signal on both MCF7 and PA115. The AF488 is gave dimmer nuclear signal than ABC (Biotin Avidin complex). There was background signal observed from the NoAb control on MCF7 by ABC. However PA115 didn't display any background signal by both secondary antibodies.



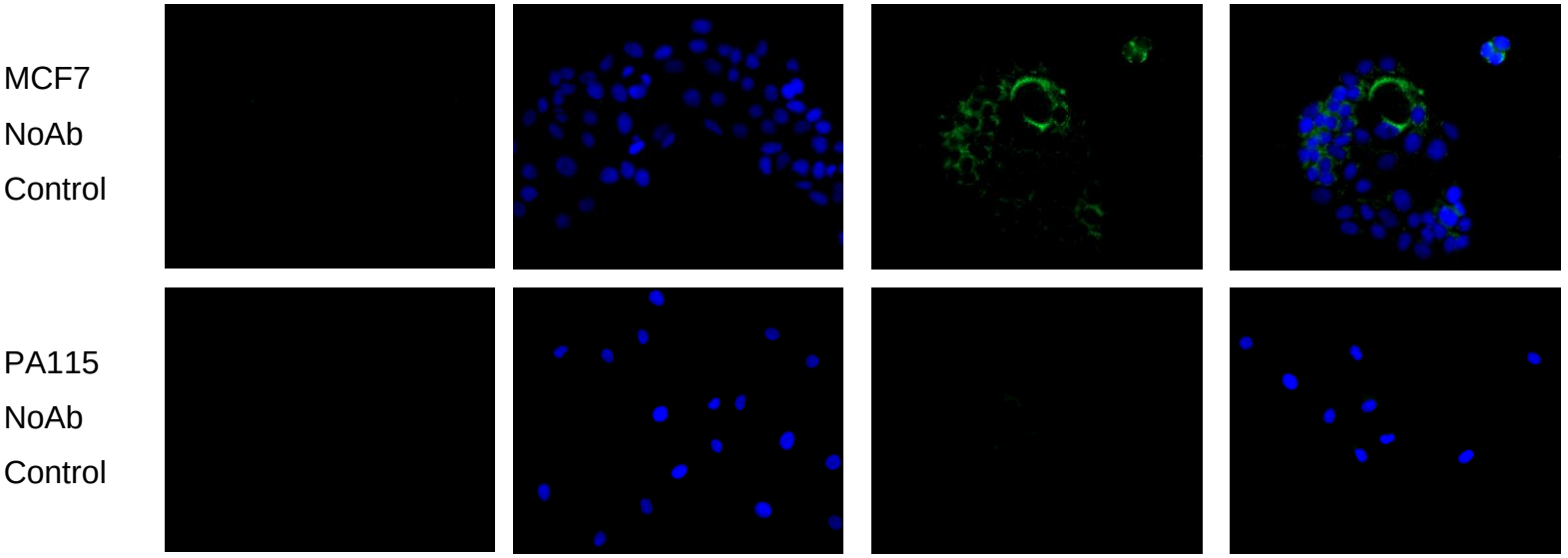


Figure 16 Testing anti-rabbit AF488 and Biotinylated anti-rabbit secondary antibody combination with Avidin (FITC) on the MCF7 and PA115. The primary antibody is ER alpha G20. The photos were taken at the magnification of 40x

4.3 Reproducibility of experiments

The aim of the experiment was to see reproducibility of the experiments which was 2 days of experiments. Hereby MCF7 and PA115 were used for the IF performance under the same conditions which primary antibody was ER α G20 in the dilution of 1:250 and anti-rabbit biotinylated secondary antibody in the dilution of 1:200 with the combination of Avidin (FITC) in the dilution of 1:100. All photos were taken at the same set exposure. On the first day (1st experiment) one IF experiment was performed and upcoming two days the photos were taken (DAY1, DAY2). On the second day (2nd experiment) also one IF experiment was performed and upcoming two days the photos were taken (DAY1, DAY2). Slightly bolbs were visible in the center of the photos on photos (Figure 17).

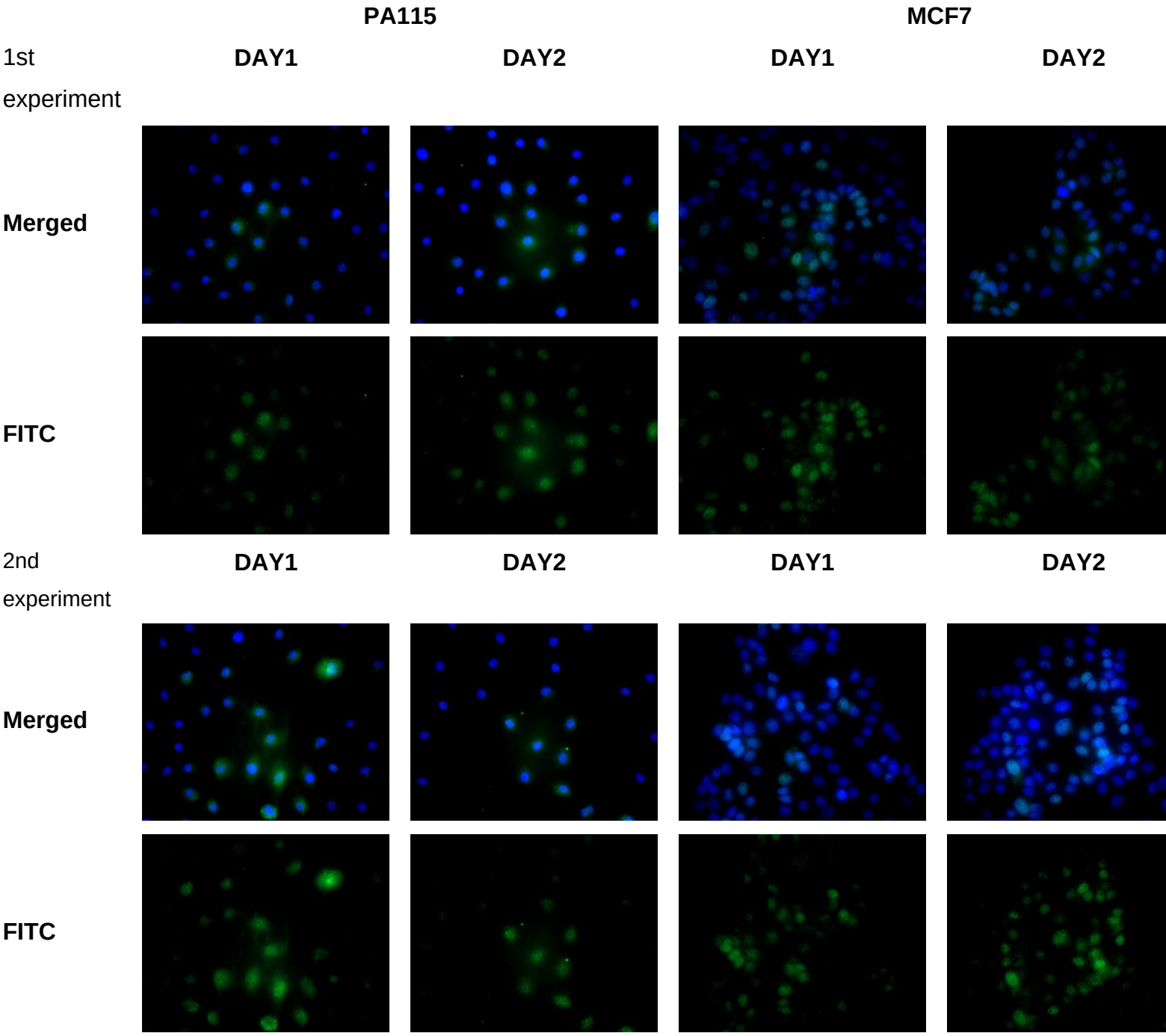
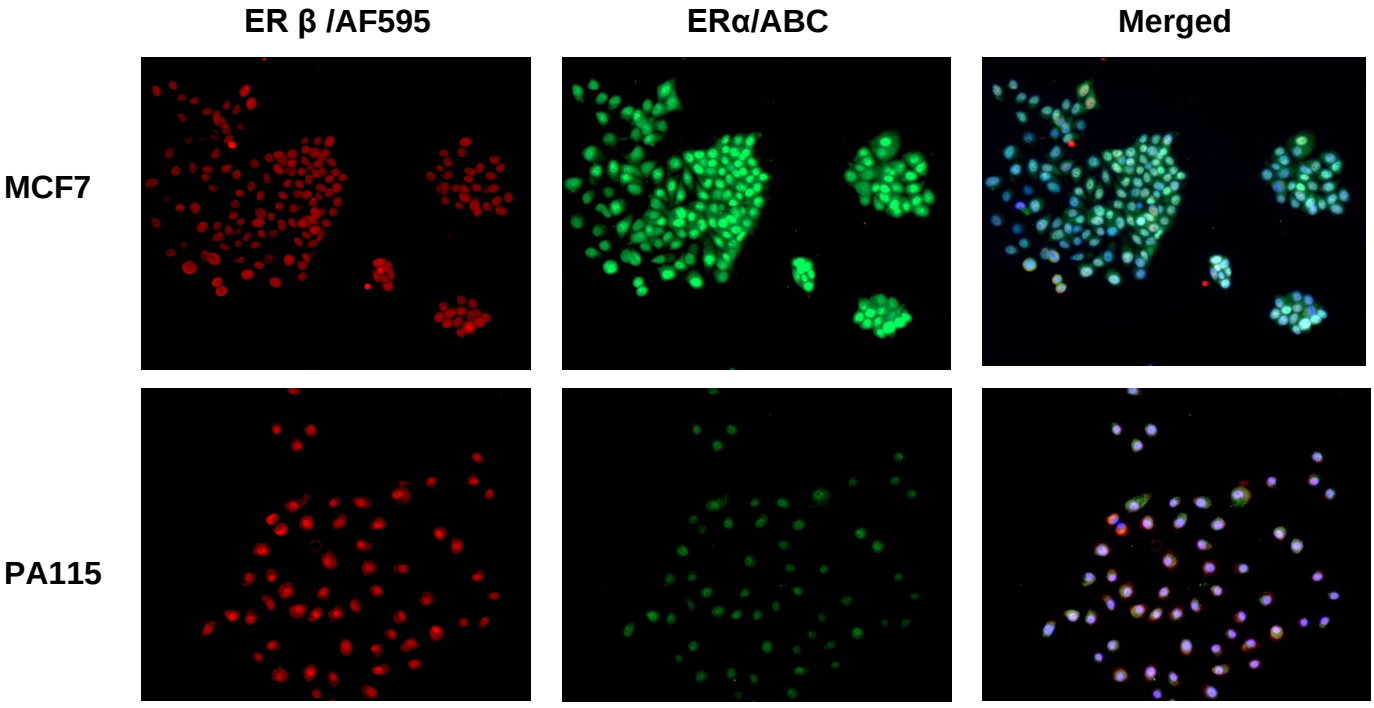


Figure 17 Reproducibility of the experiments on MCF7 and PA115 with ER α G20 in the dilution of 1:250 and ABC complex (1:200/1:100). The photos were taken at 20x

4.4 ER α/ERβ double staining

Two different types of primary and secondary antibodies combinations were used to determine the intensity ration of ER α and ER β of MCF7 and PA115. Dilution of the rabbit ER α G20 was 1:250 which was detected by anti-rabbit biotinylated secondary antibody combination with fluorescence labeled Avidin (FITC). On the other hand, the dilution of the monoclonal mouse ER β 14C8 was 1:50 and was detected by fluorescence labeled goat anti-mouse AF594. Only secondary antibodies were implemented on the no antibody control (NoAb control) to check the background signal. The dilutions of the secondary antibodies were 1:100 for AF594, whereas 1:200 for Biotinylated secondary Ab and 1:100 for Avidin.

It can be seen from the figure 10 that stronger nuclear signals were observed from MCF7 comparing to result of PA115. There were no background fluorescence signals optimized on both MCF7 and PA115 (Figure 18). The merged photos describe the positive nuclear signal of both ERs and were showing the combination of AF594, Avidin (FITC), and DAPI.



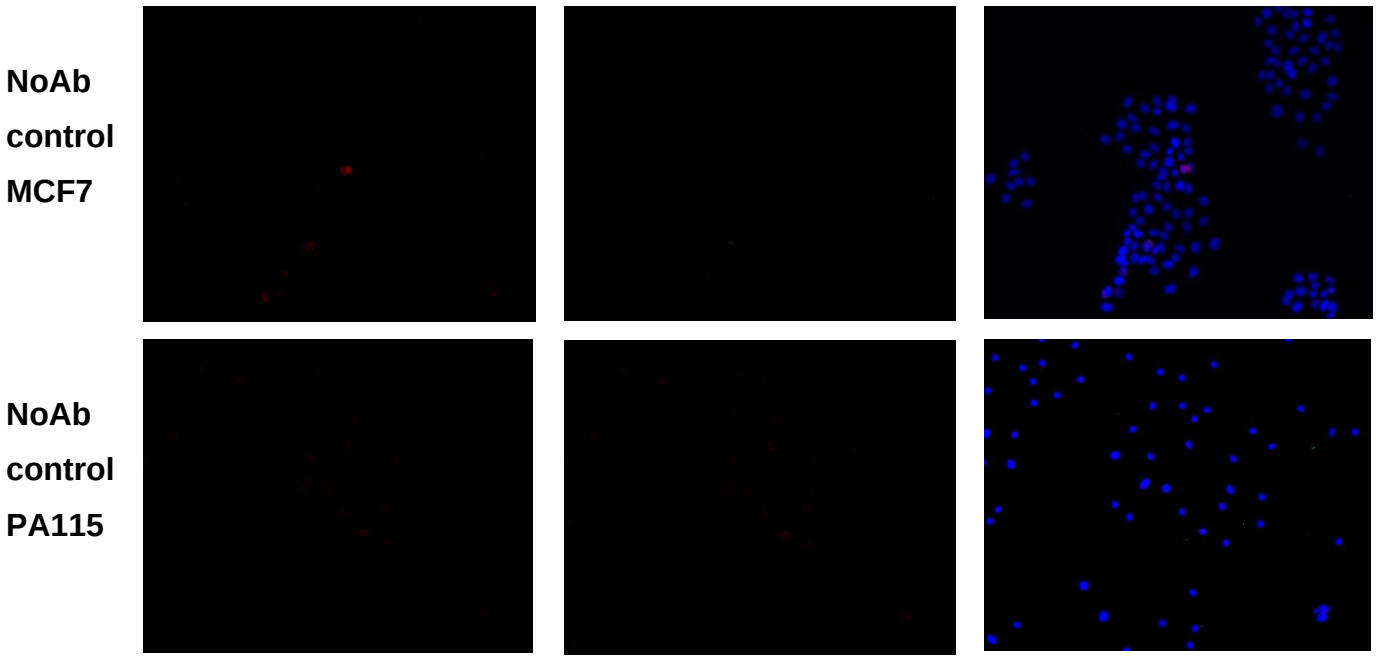


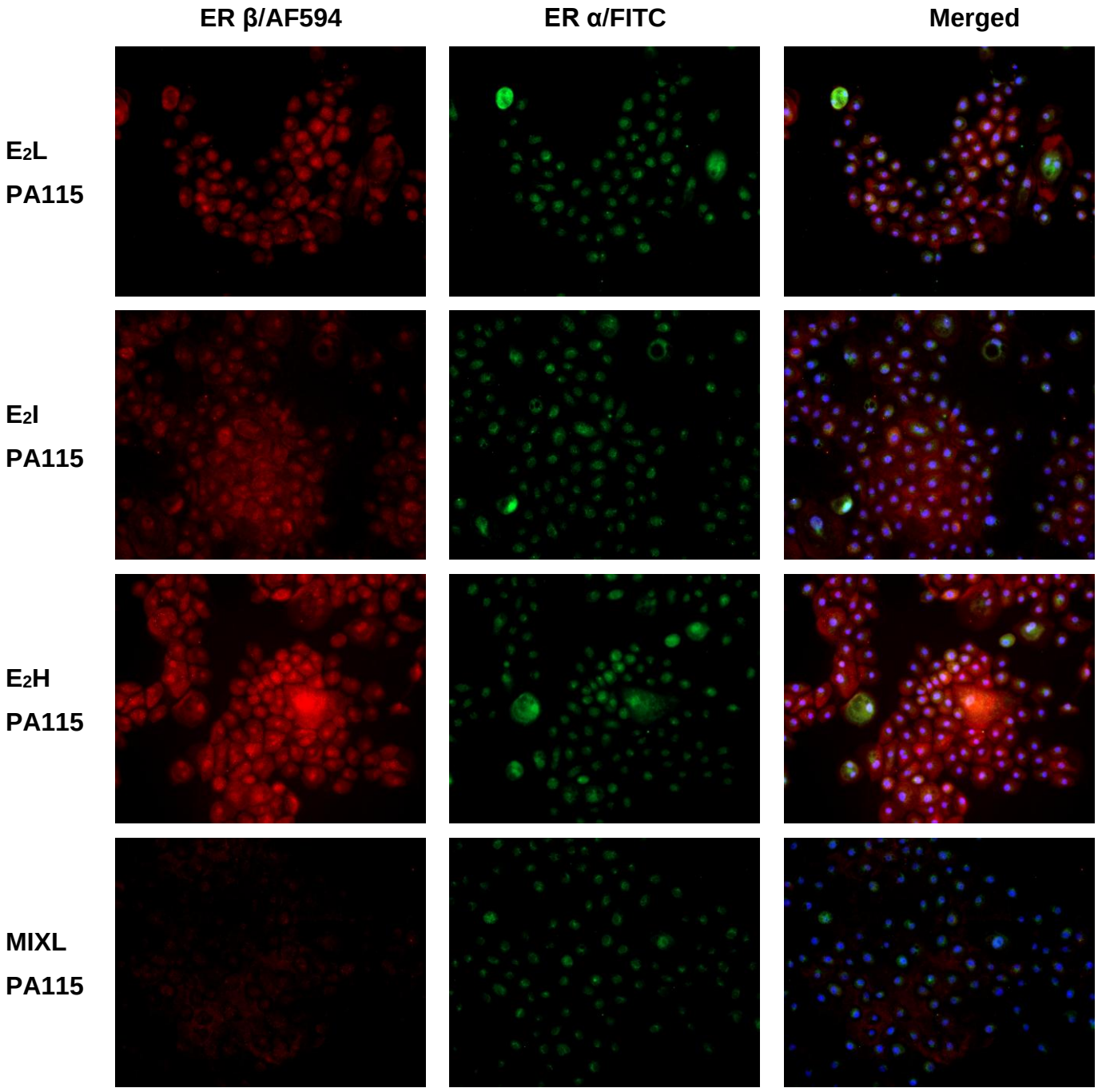
Figure 18 Double staining performance on MCF7 and PA115. MCF7 was used as positive control to test the primary antibodies which were ER α G20 and ER β 14C8. Furthermore, two different types of secondary antibodies were used to detect primary antibodies. The photos were taken at 20x

4.5 Exposure to Estrogen and Xenoestrogen on PA115

The goal of the experiment is to determine the ration of the nuclear signal of ER α and ER β after exposing to estrogen and mixture of xenoestrogens on the PA115. The treatment took 6 days of incubation in the supporting medium of LC 0.2%. In the dilution of ER α G20 polyclonal rabbit antibody was used to localize the ER α further the antibody was detected with in the dilution of 1:200 Biotinylated anti-rabbit secondary antibody combination with Avidin (FITC) in the dilution of 1:100. On the other hand, ER β was localized by mouse antibody ER β 14C8 in the dilution of 1:50 and fluorescence labeled anti-mouse AF594 was used as secondary antibody in the dilution of 1:100.

It can be seen from the figure 19 that the lowest intensity of nuclear signals of ER α and ER β showed on treatment with E_2L (Estrogen low concentration) and MIXL (mixture of xenoestrogens low concentration). As the concentration of Estrogen and mixture of Xenoestrogens were increasing the intensity of the nuclear signals were became stronger and brighter. To check the background signal of the

secondary antibodies, NoAb controls were done. There was no background signal observed from the NoAb control. PA115 control (PA115 LC 2%) was performed to compare differences of the exposure to XEs and Estrogens on the PA115.



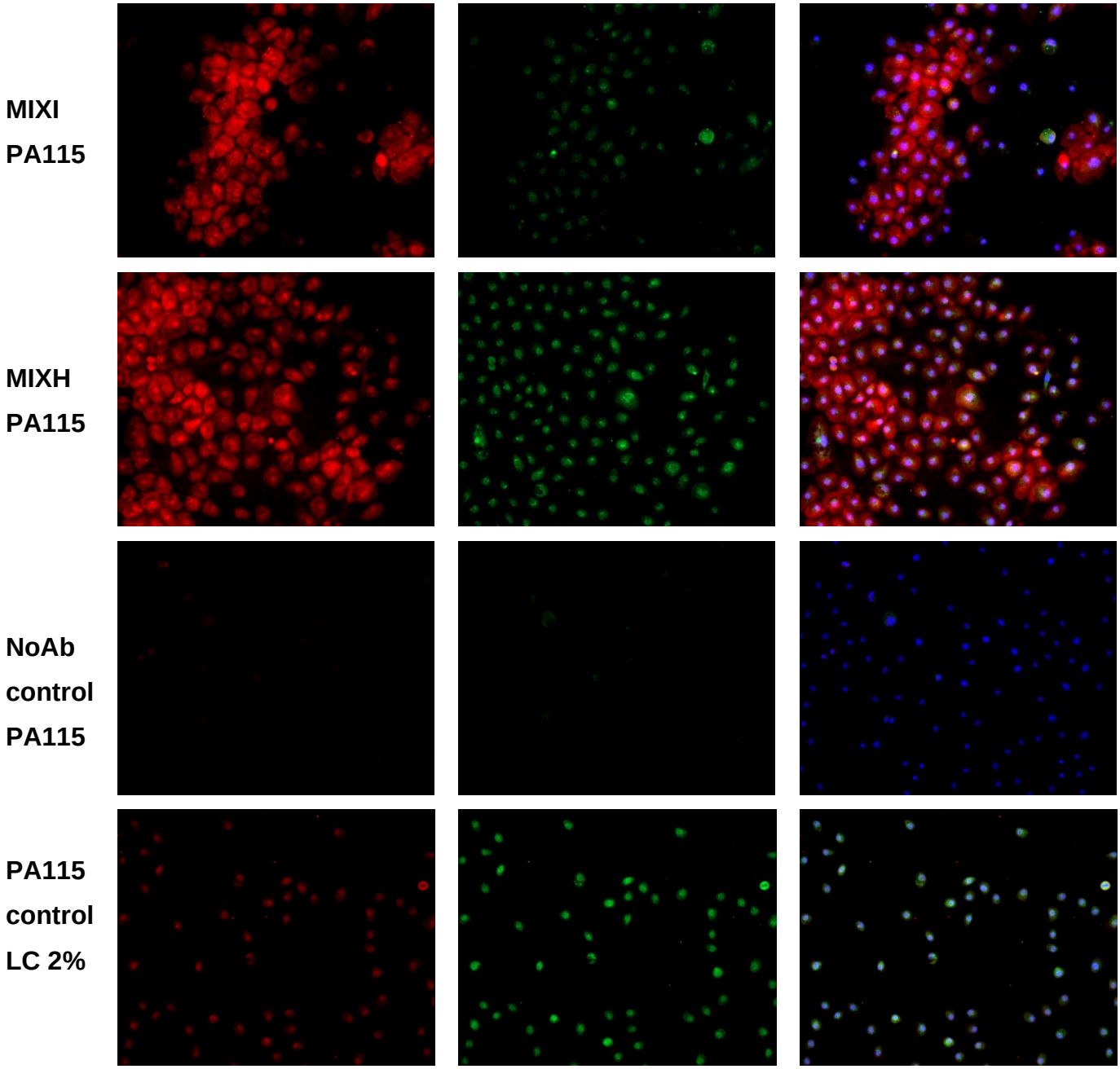


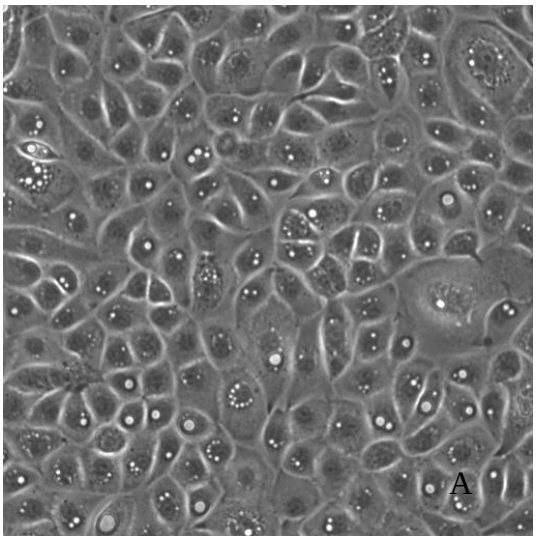
Figure 19 Double staining Immunofluorescence. Three concentrations of Estrogen and mixture of xenoestrogens treatments (E2L, E2I, E2H, MIXL,MIXI,MIXH) on the PA115. The concentrations of the treatments were E_2L 5*10^{-6}M, E_2I 5*10^{-5}M, $E_2$$10^{-4}$M, MIXL BPA 10^{-6} + MP 10^{-5} + PFOA 10^{-6}, MIXI BPA 10^{-5} + MP 10^{-4} + PFOA 10^{-5}, and MIXH BPA 10^{-4} + MP 10^{-3} + PFOA 10^{-4}. The photos were taken at the 20x

4.6 Tamoxifen application on the PA115 and MCF7

4.6.1 Tamoxifen treatment on PA115

The PA115 was seeded on the 6-well plate and incubated for 2 days until the cells were confluent enough to be treated. Tamoxifen (TAM) was added directly and the treatment took 1 day of incubation at the 37°C applying 0.5% CO_2. The supporting media for PA115 was LC 2%. Comparing the picture A to B, most of the PA115 cells went apoptosis after implementing TAM. The pictures were taken at the bright field microscopy (Figure 20).

PA115 control



PA115 – TAM 10 µM

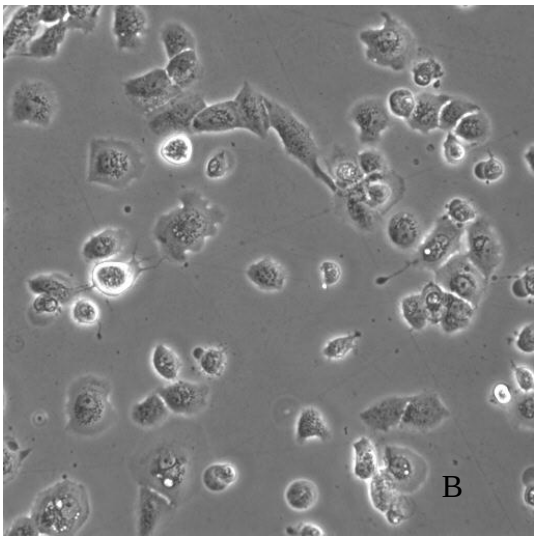


Figure 20 Picture A shows the control of PA115 (without treatment). Picture B shows the PA115 treatment with 10 µM of Tamoxifen. The photos were taken at the bright field 40x

4.6.2 Determining the apoptosis of MCF7 and PA115 by Annexin V

MCF7 and PA115 were seeded in 2x 6-well plates in amount of 250.000 cells/well. Supporting medium of MCF7 was DMEM + 10% FCS, whereas medium for PA115 was LC 2%. The 2x 6-well plates were incubated for overnight. After the incubation 10 µM of three types of TAMs were added directly on the lower three wells. The treatment required overnight incubation. After 24 hours of treatment, upper rows of the cells were treated with 0.5mM, 1mM of hydrogen peroxide (H_2O_2). The treatment with H_2O_2 took 2 hours of incubation. The one well of the cells were kept

as control. After the incubation or treatment with TAM and H_2O_2, the supernatants were collected in the 15 ml tubes according to their labels. The wells were then washed one time with PBS 1x. The supernatants were also collected in the according tubes. 150 µl of Trypsin-EDTA was added to each well and incubated for 5 min in the incubator. After detachment of the cells, 2ml of 10%FCS + PBS was added on the wells. The supernatants were collected in the according tubes. The tubes were then centrifuged at 1500 rpm for 3 min at 14°C. The supernatants were discarded as dry as possible. The pellets were then vortexed in remaining supernatants. 108 µl of Annexin V solution was added on pellets and the tubes were vortexed. Furthermore the tubes were incubated for 20 min in dark place. After the incubation 250 µl of Annexin binding buffer was added in the tubes. The samples were then transferred into glass tubes and were analyzed by FACS machine.

Most of the MCF7 underwent apoptosis after treating with TAM and it can be seen from the table 1 which was indicated by Annexin V. There was no slightly difference between 0.5 mM and 1mM of H_2O_2. The PI shows the dead cells after treating with H_2O_2 and TAM. The intensity of the TAM1 resulted over 10000,0 which is the highest intensity among all results.

	Annexin V	**PI**
MCF7 control	3158,58	975,99
MCF7 H_2O_2 0.5 mM	7 460,21	2 140,12
MCF7 H_2O_2 1mM	6 328,99	1 769,43
MCF7 TAM1	10 553,86	3 301,49
MCF7 TAM2	7 289,50	2 280,59
MCF7 TAM3	8 082,13	2 514,15

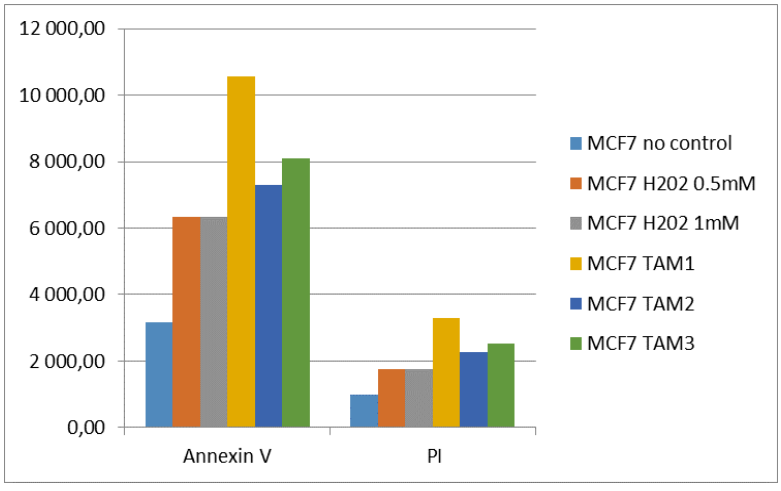
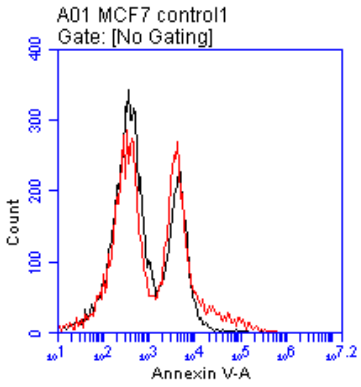


Table 1 Intensity comparison of the Annexin V and PI after MCF7 was treated with 0.5 mM, 1mM of H_2O_2 and TAM1, TAM2, TAM3.

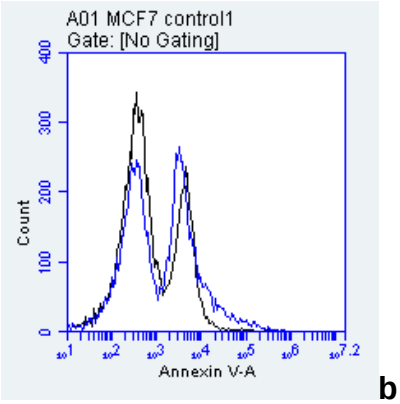
Hereby, the first peak indicates the cells undergoing apoptosis in G1 phase. Further the cell population was decreased in S phase although the population was increased in G2/M phase by MCF7 control. Only the cells of MCF7 treated with 1mM H_2O_2 populated high in the phase of G2/M and the peak is indicated as blue curve (b; table 2).

MCF7 control + MCF7 H_2O_2 0.5mM



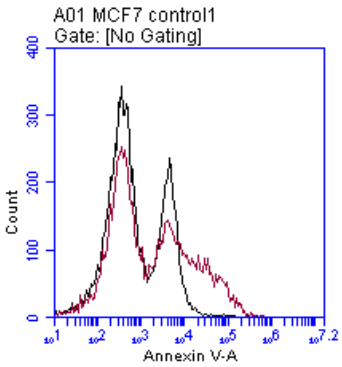
a

MCF7 control + MCF7 H_2O_2 1mM



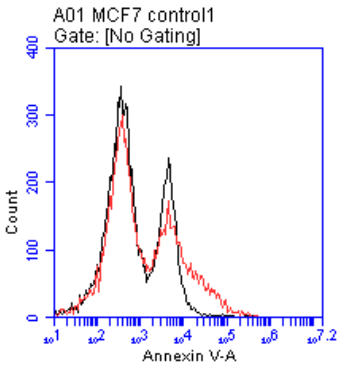
b

MCF7 control + MCF7 TAM1



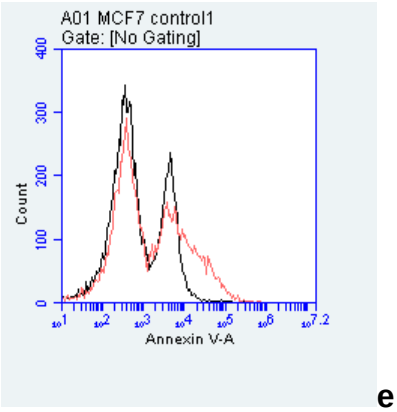
c

MCF7 control + MCF7 TAM2



d

MCF7 control + MCF7 TAM3



e

Table 2 Histogram plots of MCF7 control comparing to the MCF7 H_2O_2 0.5mM, 1mM and MCF7 TAM1, TAM2, TAM3 by Annexin V and PI. a) black curve indicates MCF7 control and red curve indicates MCF7 H_2O_2 0.5mM b) black curve indicates MCF7 control and blue curve indicates MCF7 H_2O_2 1mM c) black curve indicates MCF7 control and purple curve indicates MCF7 TAM1 d) black curve indicates MCF7 control and light red curve indicates MCF7 TAM2 e) black curve indicates MCF7 control and orange curve indicates MCF7 TAM3

The intensity of the PA115 decreased by both Annexin V and PI comparing to the control of PA115. The control of PA115 is excessively high among all the results. There were less death cells comparing to the intensity of PI to Annexin V. Furthermore, TAM1 showed highest value among all TAMs (Table 3).

	Annexin V	**PI**
PA115 control	3532,63	1172,47
PA115 H_2O_2 0.5 mM	2082,46	783,14
PA115 H_2O_2 1mM	1750,35	716,53
PA115 TAM1	1534,1	678,62
MCF7 TAM2	731,96	338,89
MCF7 TAM3	632,44	246,47

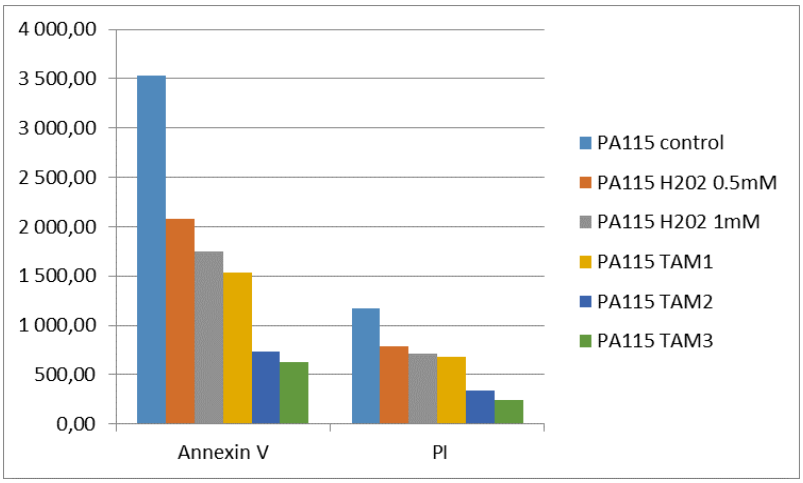
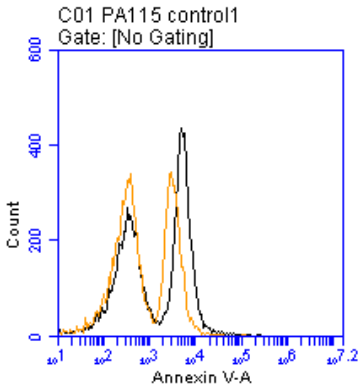


Table 3 Intensity comparison of the Annexin V and PI after PA115 was treated with 0.5mM, 1mM of H_2O_2 and TAM1, TAM2, TAM3

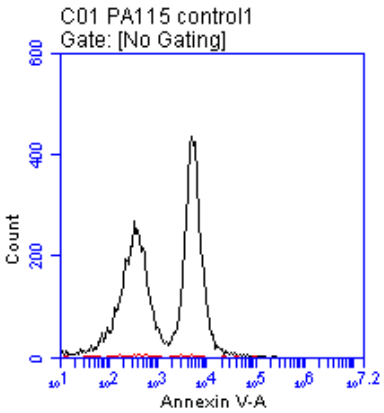
The first peak indicates the cells undergoing apoptosis in G1 phase and the second rising peak indicates the cell population in G2/M phase (table 4). Suprisingly, the cell population was decreased in G1 phase although the population was increased in G2/M phase by PA115 control and it is indicated as black curve on the histogram (a,b,c,d,e; table 4). Only PA115 that treated with 0.5 mM of H_2O_2 resulted sufficiently (a; table 4). Unusually outcome of the rest of the samples were deficient comparing.

PA115 control + PA115 0.5mM H_2O_2



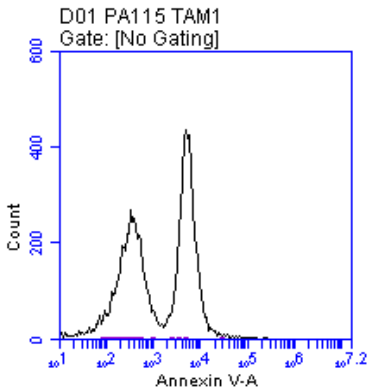
a

PA115 control + PA115 1mM H_2O_2



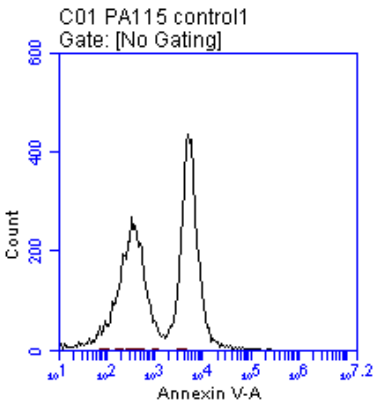
b

PA115 control + PA115 TAM1



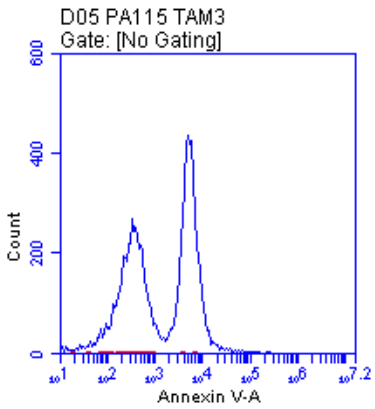
c

PA115 control + PA115 TAM2



d

PA115 control + PA115 TAM3



e

Table 4 Histogram plots showing comparison between PA115 control and treatment of it by Annexin V and PI. a) black curve indicates PA115 control and orange curve indicates PA115 H_2O_2 0.5mM b) black curve indicates PA115 control and red curve indicates PA115 H_2O_2 1mM c) black curve indicates PA115 control and brown curve indicates PA115 TAM1 d) black curve indicates PA115 control and light red curve indicates PA115 TAM2 e) blue curve indicates PA115 control and orange curve indicates PA115 TAM3

5. Discussion

5.1 Testing primary and secondary antibody

The differences between ER α HC20 and ER α G20 that ER α HC20 tends to bind unspecifically to the cells thus the lab group decided to use new polyclonal rabbit antibody ER α G20 for further experiments. The experiment testing four serial dilutions of ER α G20 (1:100, 1:250, 1:500, and 1:1000) was to decide an appropriate concentration of the primary antibody for upcoming experiments. Hereby both dilutions of 1:100 and 1:250 gave nuclear signal. The serial dilutions of 1:500 and 1:1000 didn't give any nuclear signal and it can be explained by insufficient antibodies in the dilutions. The appropriate dilution of the primary antibody was 1:250. Besides, ER β 14C8 was tested on the MCF7 and PA115 to determine the concentration. The acceptable dilution of the antibody was 1:50 that could demonstrate the valuable intensity. It was able to perform double staining Immunofluorescence determining the suitable primary antibody concentrations. Comparing to AF488 to ABC complex that PA115 observed brighter and stronger nuclear signal by ABC complex. Concerning on the double staining IF experiment the ABC complex was the suitable secondary antibody to observe better results and data for the analyzes.

5.2 Reproducibility of the experiments

It is possible to lose the fluorescence of the cover slips that were used on the previous experiments. It can be explained by that fluorescence dye can be bleached by the laser when the laser hits the cover slips to excite the fluorescence. On the other hand, if the cover slips were kept in bright or light absence place it tend to lose its fluorescence. Therefore, the experiment was to check the experimental procedures between the experiments further to check the equivalent results between the experiments.

The experiments achieved the expected results as it can be seen from the figure 17. However there were blobs found in the center of the pictures the results prove that experimental procedures were done according to the IF protocol and no mistakes were made.

5.3 Double staining Immunofluorescence

The double staining of the Immunofluorescence was dedicated to work on the patient samples that was harvested by periareolar fine needle aspiration. Hereby MCF7 is ER α and ER β positive breast cancer cell line thus PA115 could correlated to the MCF7. Therefore ABC was chosen to perform the experiment as a result of more desirable nuclear signals on the PA115. Both primary antibodies (ER α G20; ER β 14C8) localized its receptors further both secondary antibodies (ABC; AF594) detected the primary antibodies. Avidin is green fluorescence labeled dye whereas AF594 is red fluorescence labeled secondary antibody. Hence the co-staining IF was succeeded, the PA115 was exposed to Estrogen and XEs continuously for 6 days. The concentrations of the treatments were E_2L $5*10^{-6}$M, E_2I $5*10^{-5}$M, $E_2 10^{-4}$M, MIXL BPA 10^{-6} + MP 10^{-5} + PFOA 10^{-6}, MIXI BPA 10^{-5} + MP 10^{-4} + PFOA 10^{-5}, and MIXH BPA 10^{-4} + MP 10^{-3} + PFOA 10^{-4}. The area of the intensity was expanded by both ERs further the range of intensity of ER β was greater than the ER α. It explains that the estrogen mimicking hormone XEs influence the breast cancer growth. On the other hand, the visualization result would be accurate if the quantification of the intensities were done. The quantification of the ratio of ER α and ER β could not optimized due to the issue of the microscopy. The treatment was successful comparing to the control of PA115 (PA115 LC 2%).

5.4 Tamoxifen treatment

The most of the cells went through early apoptosis by the treatment of TAM on MCF7. The TAM inhibited the estrogen signaling pathway by persisting the cells in the G0/G1 phases. However the hydrogen peroxide treatment was 1 hour of incubation, the results were relatively identical to the TAM (table 1). It explains that H_2O_2 is extremely strong chemical substance. On the other hand, the treatment with TAM on the PA115 was not accurate to the expected results. Principally, the outcome of the value of treatment with TAM should be higher than the PA115 control (g,d,e; table 4). The negative values of the results can be explained by due to mistakes during the experimental procedures. The gating of the histograms

were made previously saved template of the work. Overall, the least amount of cells were discovered in the S phase of the cell cycle.

New validation of the Immunofluorescence protocol was optimized to conserve the reagents. The amount of the secondary Ab and Avidin (FITC) were 1 ml on previous validated IF protocol. After discussion of the changes in protocol, the amounts of the solutions were minimized till 50 µl for secondary Ab and 100 µl for Avidin (FITC). These volume were sufficient to perform the experiments. Moreover, the IF photos were taken at the magnification of 40x beforehand, although the amount of the cells in the field of the photo were not sufficient to be quantified thus the magnification was changed to 20x.

There were several trouble shootings occurred during the experiments. Firstly the anti-rabbit Biotinylated secondary was not working on the SKBR3 and MDA231. We could observe nuclear signals from the SKBR3, MDA231 which are triple negative breast cancer cell lines thus it should not have estrogen signaling pathway. It can be explained by that the anti-rabbit biotinylated secondary antibody was contaminated thus was giving the artificial signal. On the other hand, SKBR3 has Erbb2 so that primary antibody were crosslinking binding. In this manner the newly purchased anti-rabbit biotinylated secondary antibody was supplied moreover SKBR3 was held back from the experiments. Further there was issue on the analyzing the immunofluorescence photos at the microscopy using the Zen Pro software. The software was counting the background signal as a positive signal thus it was interrupting the quantification. Moreover the FITC channel of the microscopy was making blob in the center of the pictures which explains that the filter of the FITC was broken.

6. Conclusion

Understanding the signaling pathway of the XEs in the breast cell (HRBECs) can improve the targeted therapy further to avoid the risk of the breast cancer development. It is able to determine the ration of ER α and ER β in the patient samples utilizing the double staining IF. Overall, the experiments worked successfully with great guides.

7. References

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