Analysis of estrogen receptor modulation in human breast cells by immunofluorescence microscopy

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1 Abstract

Breast cancer is one of the highest ranked causes for death among all cancer diseases and despite decades of research, the linchpin of its development is not yet clear. Indeed, research focused on understanding the development of cancer is more meaningful if performed in healthy breast epithelial cells than in cancer cell lines in which changes leading to malignancy have already occurred. The research group of Prof. Shanaz Dairkee therefore studies the influence of estrogen and estrogen-mimicking reagents, called xenoestrogens, on breast cancer development using unique non-malignant cellular models of the human breast.

Recent results obtained by flow cytometry and western blots in the Dairkee lab have showed a rapid increase in the ERα receptor within healthy breast cells exposed to estrogen and xenoestrogens. ERα represents the target of tamoxifen, one of the most frequently used chemotherapeutic and chemopreventive for breast cancer. Although flow cytometry (FCM) and western blots (WB) were widely used by this research group for protein quantification after exposure, localization of these receptors and associated proteins within cellular compartments remained unknown.

The goal of my internship project was to establish an immunofluorescence (IF) protocol for microscopic visualization of proteins of interest and testing multiple primary antibodies to optimize cellular localization of ERα. First, validation of general IF protocol steps, such as fixation, permeabilization, washing, and antibody incubation was performed. This was followed by optimizing conditions of cell confluency, storage of fixed cells, antibody dilutions, incubation temperature and duration.

After generating a general IF localization protocol for indirect staining with primary antibodies and fluorophore-labeled secondary antibodies, the effort was extended to achieve greater sensitivity of detection by signal amplification using a biotin-labeled secondary antibody in combination with fluorophore-labeled avidin molecules. For some cellular protein combinations, double-immunostaining was established as described below.

Finally, IF results were compared to FCM results obtained with the same reagents. Although staining principles were similar, comparisons of IF and FCM illustrated differences in the level of sensitivity of each method, and provided opportunities for the analysis and interpretation of data collected by both techniques.

2 Introduction

2.1 Breast Cancer – Statistics

The most frequent cancer diagnosed in women develops primarily in the breast and is counted for the fifth cause of death from cancer overall. Whereas the incidence is significantly higher in more developed countries (**Figure 1**), the mortality rates differ only slightly between countries of different economic backgrounds due to the improved health care systems in higher developed countries (**Figure 2**).

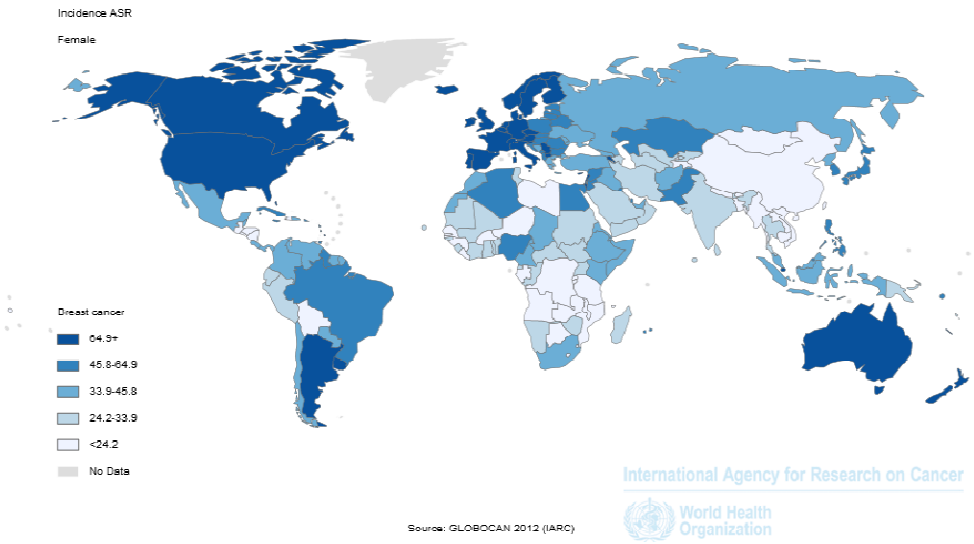


Figure 1 Map of breast cancer incidence with Age-standardised rates (ASR) (http://globocan.iarc.fr/Pages/Map.aspx (accessed Jul 10, 2015))

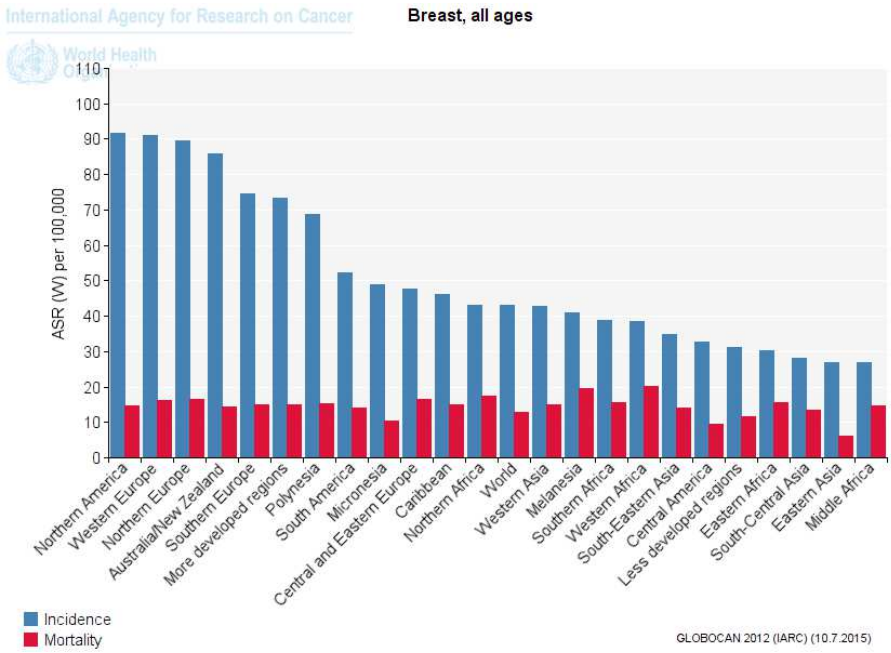


Figure 2 Incidence and mortality for breast cancer (http://globocan.iarc.fr/Default.aspx (accessed Jul 10, 2015))

2.2 Breast cancer – pathologies

Cancer can generally be classified in grades as rating system to compare cancer cells in growth behavior and abundance to tissue specific cells. The range starts with grade 1 describing well differentiated cancer cells with slow growth behavior and most likely better prognoses, followed by grade 2 for moderately differentiated cells and the highest grade 3 cells for poorly differentiated and disorganized cell behavior. Therefore, grade 3 cancer cell lines such as BT474M1, MCF7, T47D, SKBR3 and MDA231 are common models used in breast cancer research. Furthermore, stages were defined regarding localization, size of the tumor and metastasis based on removed tissue during surgery. Beside those macromolecular structures, breast cancer can be genetically distinguished and most likely affects receptor patterns for ERα (MCDONNELL & NORRIS, 2002; HUANG et al., 2014) ERβ (HALDOSÉN et al., 2014) and HER2 (which is also known as ErbB2; ROSS & FLETCHER, 1998) GPR30 (PROSSNITZ et al., 2007) and EGFR.

Diagnosis of the receptor status is especially of interest for the choice of therapy, since receptor dependent cancers can be treated with drugs blocking the receptor. For example, tamoxifen is used for the treatment of ER^+ cancers or trastuzumab against $HER2^+$ cancers (MORRIS & CAREY, 2006).

2.3 Research project Dairkee group

The research group of Prof. Dairkee focuses on hallmarks leading to the development of breast cancer. Therefore, influences of hormones such as estrogen or estrogen-mimicking compounds such as Bisphenol-A (BPA) and methylparaben (MP) were analyzed in healthy breast cells obtained by fine-needle aspiration from non-cancerous breast tissue (DAIRKEE et al., 2008; DAIRKEE et al., 2013). Indeed, investigating breast cancer development in healthy breast epithelial cells represents an advantage of understanding the mechanisms leading to cell rearrangements compared to cancer cell lines, in which cells reached already cancer specific behavior.

Although tamoxifen is widely used for ER^+ breast cancer chemotherapy (HEEL et al., 1978), the role of estrogen in breast cancer development remains to be fully defined (HUANG et al., 2014). Furthermore, xenoestrogens are more and more prevalent in synthetic bottles, cosmetics, food packages etc. and thereby people are exposed to estrogen-mimicking substances every day. Another hint for the influences of “environmental” hormones mimicking estrogens is the increasing occurrence of breast cancer in female as well as male (DEY et al., 2009; ANDERSON et al., 2010).

Previous experiments within the research group of Prof. Dairkee identified a role of estrogen on receptor changes and cell growth of healthy breast epithelial cells by western blot and

flow cytometry analysis. Both methods generate cell population data, not single cell analysis, which is why heterogeneity and cellular localization at the single cell level was undetermined. Thus, immunofluorescence microscopy of plated cells was used for the visual analysis of protein localization..

2.4 IF

Immunofluorescence describes the visualization of target molecules by the detection of fluorophore-labeled antibodies. Direct Immunofluorescence utilizes antibodies directly labeled with a fluorescent molecule, whereas indirect staining includes a secondary antibody binding to the Fc domain of an unlabeled primary antibody and therefore carries the fluorophore itself (**Figure 3** a). The latter strategy represents a signal amplification compared to direct immunofluorescence since several secondary antibodies can bind to the primary antibody (ODELL & COOK, 2013).

An even better signal amplification can be obtained by an extended indirect immunofluorescence using a biotin-labeled secondary antibody followed by fluorophore-labeled avidin detection. Additionally to the binding of secondary antibodies, multiple avidin molecules interact with biotin resulting in an extended ratio of fluorophore molecules per target molecule (**Figure 3** b).

Overall, immunofluorescence is a microscope-based technique, which was chosen as additional technique in the research group of Prof. Dairkee for addressing questions of the role of xenoestrogens in breast cancer development.

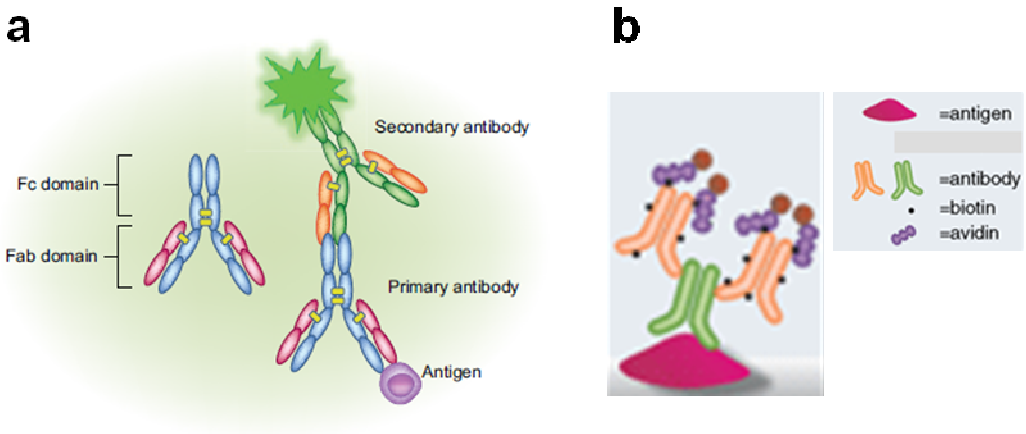


Figure 3 Indirect staining strategies in IF (modified (ODELL & COOK, 2013))

2.5 Aim of the studies

The aim of the internship was to apply the IF localization protocol for studying proteins of interest in the Dairkee Lab, and their modulation in the presence or absence of estrogen. Therefore, signal amplified strategies were chosen and protocols adapted to cell lines, materials, incubation times and temperatures as well as antibody concentrations.

Breast-cancer is described as initially estrogen-dependent disease and estrogen-signaling mainly occurs *via* the two receptors ERα and ERβ. Thereof, ERα appears as the better studied receptor, as drugs against it are used successfully for chemotherapy. Despite its relevance in diagnosis and therapy, the knowledge about mechanisms leading to breast cancer development and alternate strategies in case of drug resistance asks for further studies on ERα and estrogen influences (HUANG et al., 2014).

The role of ERβ in breast cancer development and its potential for new drugs is less good defined than for ERα due to technical lacks regarding specific antibodies in the past (HALDOSÉN et al., 2014). Thus, its influences on breast cancer development and the interplay between the receptors still need to be explored.

Since not all $ERα^+$ breast cancer patients respond to the widely used chemotherapeutic tamoxifen, further studies aim for the identification of additional biomarkers for better outlooks of therapy usage and outcome. In that regard, phosphorylated ERα-S118 levels were studied in relation to tamoxifen therapy effects (KOK et al., 2009).

G protein-coupled receptor 30 (GPR30) is shown to be highly expressed in ER-negative breast cancer (CHEN & RUSSO, 2009) exerting cell growth-inhibiting influences after binding of its agonist G-1 (WEI et al., 2014).

4EB-P1 is part of the eukaryotic translation initiation factor 4E-binding protein 1 (EIF_4EBP_1), a complex that regulates protein translation and represents a downstream molecule of the mTOR pathway. Active mTORC1 phosphorylates 4EB-P1, which releases its binding partner elF-4E in order to initiate cap-dependent translation. Proteins resulting of these translations are most likely players in cell proliferation and growth processes. Phosphorylated 4EB-P1 was shown to be increased in cancer tissue and is therefore of interest for studies about carcinogenesis. The group of Prof Dairkee discovered the induction of 4EB-P1 in HRBEC samples after the exposure to BPA. Furthermore, the influences of the mTOR inhibitor and drug rapamycin on BPA-exposed breast epithelial cells were analyzed (GOODSON et al., 2011).

The ribosomal protein RPS6 is another member of the mTOR pathway and exhibits a comparable impact on protein synthesis and translation as 4EB-P1. Exposure of HRBEC's to

BPA yielded likewise increased concentrations of RPS6 as well as of its phosphorylated form (GOODSON et al., 2011).

Cathepsin D is a lysosomal aspartyl protease overexpressed in and secreted by breast cancer cells of estrogen receptor positive as well as negative phenotypes (GARCIA et al., 1996; RADISKY, 2010). While the increased levels of Cathepsin D in $ERα^+$ are provoked by estrogen, mechanisms leading to high expression in $ERα^-$ breast cancer cells remain unknown. Anyways, the misregulation of Cathepsin D was shown to facilitate epithelial cancer cell growth and metastasis.

34βE12 represents an antibody for high molecular cytokeratins, which are present in epithelial cells, and was used to confirm that the human samples being studied were epithelial in origin.

ErbB2, also named HER2, is a cell membrane-bound tyrosin kinase expressed on epithelial cells. Overexpression of this protein occurs in 25% of all breast cancer incidences and is target of the antibody drug trastuzumab (MORRIS & CAREY, 2006).

Cyclin D1 represents a key molecule in cell cycle regulation being overexpressed in 35% of all breast cancer diseases. It is a subprotein of a larger complex, which phosphorylates the tumor suppressor protein BRCA1, which interacts for example with ERα. Defect genes for those proteins were also used as prognostic factor for the development of breast cancer (KEHN et al., 2007).

3 Experimental Procedure

3.1 IF staining

The preparation of cell cultures for Immunofluorescence was performed using Millicell EZ 8-well glass slides (Merck Millipore) as well as 11 x 22 micron coverslips (Thomas Scientific).

3.1.1 Cell preparation

Two main cell types were prepared for Immunofluorescence staining, with HRBEC's as cells to address scientific questions and cancer cell lines as internal controls for the IF itself. The control cell lines were chosen corresponding to the primary antibody of interest. Since growth behavior between the non-malignant cells (PA"#") and cancer cell lines differs, the amount of seeded cells was validated to the needs of cell lines and incubation times.

Cover glasses were placed in 100 x 15 mm tissue culture treated dishes (Santa Cruz Biotechnology) and tested for cell amounts of 0.5×10^6 and 1×10^6 cells for PA024, PA025 and PA115 in regard to an incubation time of 8 days. Cancer cell lines were tested for 0.5×10^6 and 0.25×10^6 cells for incubation times of 3 days.

PAs were seeded on microscope slides at 5000 and 10,000 cells/well, whereas cancer cell lines were seeded at 2500, 5000 and 10,000 cells/well with the same incubation times of 8 days and 3 days, respectively.

All mass cultures were maintained as 75 cm^2 or 25 cm^2 tissue culture flasks (Santa Cruz Biotechnology). PAs were incubated at 37 °C with 0.5% CO_2 in LC 2% medium, whereas cancer cell lines were incubated at 37 °C at 5% CO_2. RPMI (Sigma Aldrich) + 10% FCS (JR Scientific Inc.) was used for the cell lines MDA231, SKBR3, BT474M1 and T47D, DMEM (Sigma Aldrich) + 10% FCS for MCF7 cells. All cells are adherent cells and split in a dilution of 1:10 for culture maintenance.

Spent medium was removed from the flasks before washing once with PBS 1x and adding trypsin to detach cells from flask bottoms. Trypsinization was stopped by the addition of the respective medium and cells in suspension were collected for counting in 15 ml or 50 ml falcons (Corning Centri Star). Calculated volumes of cell suspensions were transferred to new falcons and centrifuged at 1800 rpm for 5 min at 18 °C. Supernatants were discarded and cell pellets resuspended in corresponding amounts of media followed by seeding on microscope slides or in plates on cover glasses. Whereas medium for cancer cell lines was supplemented with 10% FCS, PAs were seeded in 0.2% LC medium. Incubation conditions were kept the same as for cell line maintenance.

3.1.1.1 Treated PA's

After 24 h of incubation time, different plates of each PA cell line were treated in the following way:

- Plate (a): Estrogen at a final concentration of 50 nM
- Plate (b): Mixture of the xenoestrogens
    - BPA of 10 nM
    - MP of 100 nM
    - PFOA of 10 nM
- Plate (c): 5 days in 0.2% LC medium; medium change on day 6 to 2% LC medium
- Plate (d): Control plate with 0.2% LC medium only

Fixation of all prepared cells was performed after 8 days of seeding. However, fixation of cancer cell lines took place after 3 days of incubation.

3.1.1.2 PAs in LC 2% medium for 3 days

In order to identify the influence of growth medium and incubation times on estrogen receptor expression in spontaneously immortalized cells, another IF of ERα staining was performed with PA115 seeded and grown in LC 2% medium for 3 days.

3.1.2 Fixation

Methanol fixation and formaldehyde fixation are known as the common procedures to secure antigens within the cells while retaining the cellular and subcellular structure. Methanol represents a harsher fixation and is recommended for stainings of cytoskeletal structures. However, the crosslinking reagent paraformaldehyde preserves cell structures better compared to methanol fixation and therefore represents the first choice of a fixation method for soluble proteins. Since the latter method is less harsh, a permeabilization of cell membranes is necessary to enable antibodies diffusing into the cell.

3.1.3 Storage

Appropriate storage of microscope slides and cover slips was investigated by testing two different strategies following the fixation step. Fixed cells were washed once with PBS 1x and were either air-dried or covered with PBS 1x before stored at 4 °C.

3.1.4 Permeabilization

Dry stored cells did not show fluorescent signals and were therefore tested for different incubation times of 10, 15 and 20 minutes during permeabilization. Cells were covered with PBS 1x + 0.2% Triton and incubated at RT. In PBS 1x stored cells were permeabilized only for 10 minutes with the same buffer at RT.

3.1.5 Blocking

In order to decrease background fluorescence, non-specific binding sites were blocked with PBS 1x + 10% FCS for 1 h at RT.

3.1.6 Primary Antibody Reaction

Different antibody concentrations were tested to define the range of the best signal outcome (data not shown). All primary antibodies used and their final concentrations are listed in Table 1. Incubations were tested at 4°C overnight, at 18°C for 2 h, or at 37°C for 2 h (data not shown). Antibodies were diluted in PBS 1x + 1% BSA. Double staining was performed for antibodies of different species. Subsequent to incubation, cells were washed twice with PBS 1x + 0.5% BSA + 0.05% Triton and twice with PBS 1x while rocking.

Table 1 Primary Antibodies with final concentrations.

Antibody/clone	Company	Species	Dilution	Localization (IF)
ER alpha (HC20)	Santa Cruz	rabbit	1:100	Nuclear
ER beta (14C8)	Genetex	mouse	1:100	Nuclear
Erb B2 (EP1045Y)	Genetex	rabbit	1:100	Membrane
RPS6 (H-4)	Santa Cruz	mouse	1:100	Cytoplasmic
4EBP1 (P1)	Santa Cruz	mouse	1:25	Nuclear
GPR 30 (N-15)-R	Santa Cruz	rabbit	1:50	Membrane
pER alpha (Ser118)	Santa Cruz	goat	1:25	Nuclear
34βE12 (Pan-Keratin)		mouse	1:10,000	Cytoplasmic
Cathepsin D (EPR3057Y)	Genetex	rabbit	1:50	Lysosomes
Cyclin D1 (A-12)	Santa Cruz	mouse	1:100	Nuclear

3.1.7 Secondary Antibody Reaction

Respective secondary antibodies (Table 2) were diluted in PBS 1x + 1% BSA. Incubation lasted for 45 min at 18 °C. Fluorescently-labeled antibody reactions were kept in the dark. In the next step, cells were washed 3 times with PBS 1x while rocking. Biotin-labeled antibodies were detected by a fluorescently-labeled Avidin in a subsequent step. Double staining was performed with different fluorescent molecules to allow signal separation.

3.1.7.1 Titration of Antibodies

New batches of secondary antibodies were tested for differences in fluorescence compared to previously used antibody and the right concentrations performing a titration using flow cytometry (FCM). Suitable antibodies were used as reference control. Frozen and fixed cell pellets of different cell lines were used considering cell specific influences on the staining. Fixed cell pellets of BT474M1 and PA151 (stored at -20 °C) were centrifuged at 3000 rpm for 3 min at 18 °C. The supernatant was discarded before cells were washed with PBS 1x + 0.5% BSA and 0.05% Triton for 2 min at 18 °C followed by another centrifugation step using

previously described settings. Subsequently, cell pellets were resuspended in 0.5 ml/falcon permeabilization buffer with PBS 1x + 0.2% Triton and incubated for 10 min at 18 °C. After addition of 2 ml PBS 1x/falcon and centrifugation as described above, unspecific binding sites were blocked with 0.5 ml/falcon of PBS 1x + 10% FCS. Washed and centrifuged cell pellets were resuspended in 560 µl PBS 1x + 0.5% BSA and splitted in 7 tubes à 80 µl. Mouse anti-RPS6 was chosen as primary antibody in a stock dilution of 1:50 and added in amount of 80 µl/tube resulting in a final dilution of 1:100. One cell pellet was kept as no-antibody control. Primary antibody incubation took place overnight at 4 °C in the dark while rocking. Cell suspensions were washed first, then incubated with the secondary antibody AF488 goat-anti-mouse (Life Technologies) diluted in the following titration steps: 1:100, 1:250, 1:500, 1:1000 and 1:2000; for 1 h in the dark. Furthermore, cells were incubated with the reference antibody AF488 donkey-anti-mouse in a dilution of 1:2000. Samples were further diluted with 300 µl PBS 1x/ falcon followed by the measurements using the Accuri flow cytometer (BD Biosciences).

Final secondary antibody dilutions are listed in Table 2.

Table 2 Secondary antibodies with final concentrations.

Antibody/clone	Company	Species	Dilution
AF488 (bb)	Life technologies	Goat-anti-rabbit	1:200
AF488	Invitrogen	Donkey-anti-goat	1:2000
AF488 (new)	Life technologies	Goat-anti-mouse	1:400
AF488	Invitrogen	Donkey-anti-mouse	1:2000
DL594	Vector Lab.	Horse-anti-mouse	1:200
DL594	Vector Lab.	Goat-anti-rabbit	1:200
DL594	Vector Lab.	Donkey-anti-goat	1:2000
Biotin	Vector Labs	Goat-anti-rabbit	1:500
Biotin	Vector Labs	Anti mouse	1:500

3.1.8 Avidin Reaction

Biotin labeled secondary antibodies were detected by fluorescently-labeled Avidin diluted in PBS 1x + 1% BSA. Incubation was performed for 30 min at 18 °C in the dark. After incubation, cells were washed 3 times with PBS 1x while rocking.

3.1.9 Mounting

Cells were mounted with mounting medium containing DAPI as nuclear staining dye (Vector Labs).

3.1.10 Microscopy

All IF pictures were taken with a Zeiss AX10 Observer Z.1 equipped with Axiocam 506 mono and 503 color and a HXP120C as light source. Zen pro was used as software.

3.2 FCM: comparison IF staining

Strategies of signal amplification within antibody staining for IF as well as for flow cytometry are for example the usage of higher antibody concentrations or the signal amplification by Biotin-Avidin reactions. Both strategies were tested for the primary antibody against ERα detected either by a secondary antibody labeled with AF488 or with Biotin and followed by a FITC-labeled Avidin.

3.2.1 FCM

Fixed cell pellets of MCF7 and SKBR3 grown in full medium, PA115 grown in 0.2% LC medium or in 2% LC medium and Estrogen-treated PA115 were prepared for the staining with ERα as described in Chapter 3.1.7. Before the primary antibody was added, cell pellets were resuspended in 150 µl PBS 1x + 0.5% BSA and split into two. One series of cell pellets was used as no-antibody control whereas the other series was incubated with ERα in a final dilution of 1:100. All pellets were treated overnight at 4 °C while rocking.

Subsequently, cell pellets were washed with PBS 1x, centrifuged at 3000 rpm for 3 min at 18 °C. Supernatants were discarded and cell pellets resuspended in 300 µl PBS 1x + 0.5% BSA. An amount of 100 µl cell suspension was incubated either with the secondary antibody AF488 goat-anti-rabbit in a final dilution of 1:500, Biotin-anti-rabbit in a final dilution of 1:500 or Biotin-anti-rabbit in a final dilution of 1:100. Secondary antibody solutions incubated for 1 h at 18 °C in the dark.

Samples with Biotin-labeled antibodies were washed again with PBS 1x, centrifuged and resuspended in PBS 1x + 0.5% BSA with Avidin Fluorescein D in a final dilution of either 1:500 or 1:200. After another incubation time of 30 min at 18 °C in the dark, all samples were prepared for measurements in a 96 flat-bottom well plate. Measurements were performed in duplicate using the Accuri flow cytometer (BD Biosciences).

3.2.2 IF

Cover glasses with fixed cells of MCF7 and SKBR3 grown in full medium as well as PA115 grown in 2% LC medium were chosen to perform a comparable staining to the above mentioned FCM protocol. Basic steps followed the standard protocol as described in Chapter **4.1.** The primary antibody ERα stayed the same as in the FCM staining with a final dilution of 1:100. However, a slightly different concentration was chosen for the secondary antibody AF488 goat-anti-rabbit with a dilution of 1:100 instead of 1:200. Biotin-anti-rabbit was diluted in 1:100 and detected by an Avidin Fluorescein D in a dilution of 1:200.

4 Results

4.1 Protocol Optimization

A proper confluency of cells for IF was obtained for cancer cell lines seeded in amounts of 0.25 x 10^6 cells/100 x 15 mm tissue culture treated dishes on cover glasses and 5000 cells/well in Millicell EZ slide 8-well glasses during a 3 days incubation time. However, non-malignant cell lines needed to be seeded in doubled cell amounts of 0.5 x 10^6 cells/ 15 mm tissue culture treated dishes on cover glasses and 10,000 cells/well in Millicell EZ slide 8-well glasses. Furthermore, incubation of non-malignant cells including the treatment with Estrogen, xenoestrogens, or control LC 2 % medium lasted 8 days.

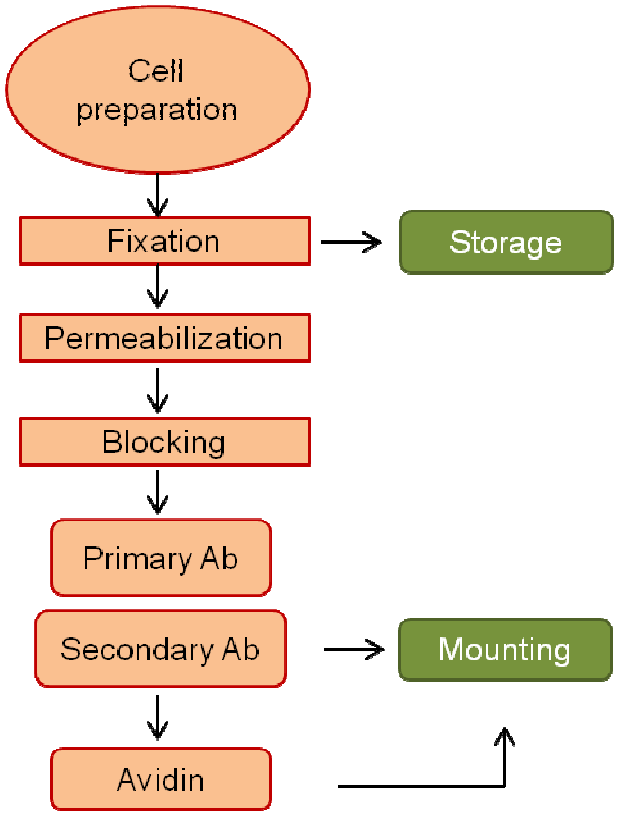


Figure 4 Scheme for the established IF protocol pointing out the main steps during the stainin.

Cells were washed with PBS 1x before the fixation was performed using a 4% formaldehyde solution for 15 min at 18 °C. After another washing step with PBS 1x, fixed cells were either stored in PBS 1x at 4°C or further processed for an IF. In the latter case, cells were permeabilized with PBS 1x + 0.5% Triton for 10 min at 18 °C.

Methanol fixation resulted in cell loss of T47D cells in microscope slides (data not shown) and thus further fixation was performed with formaldehyde only. Immunofluorescence in fixed and dry-stored cells yielded signals for cytoplasmic signals for example of RPS6 and Cathepsin D but failed in nuclear staining like for ERα or 4EB-P1. Different incubation times for the permeabilization of MCF7 grown and fixed either on cover glasses or on microscope slides were tested to obtain nuclear staining signals for ERα (**Figure 5**). Cells following the same fixation but stored in PBS 1x were used as reference staining. Fluorescent signals were obtained for all conditions regarding incubation times for the permeabilization as well as material for cells stored in PBS 1x (**Figure 5, left site**). However, dry-stored cells showed no ERα signal for none of the different permeabilization times (**Figure 5, right site)**.

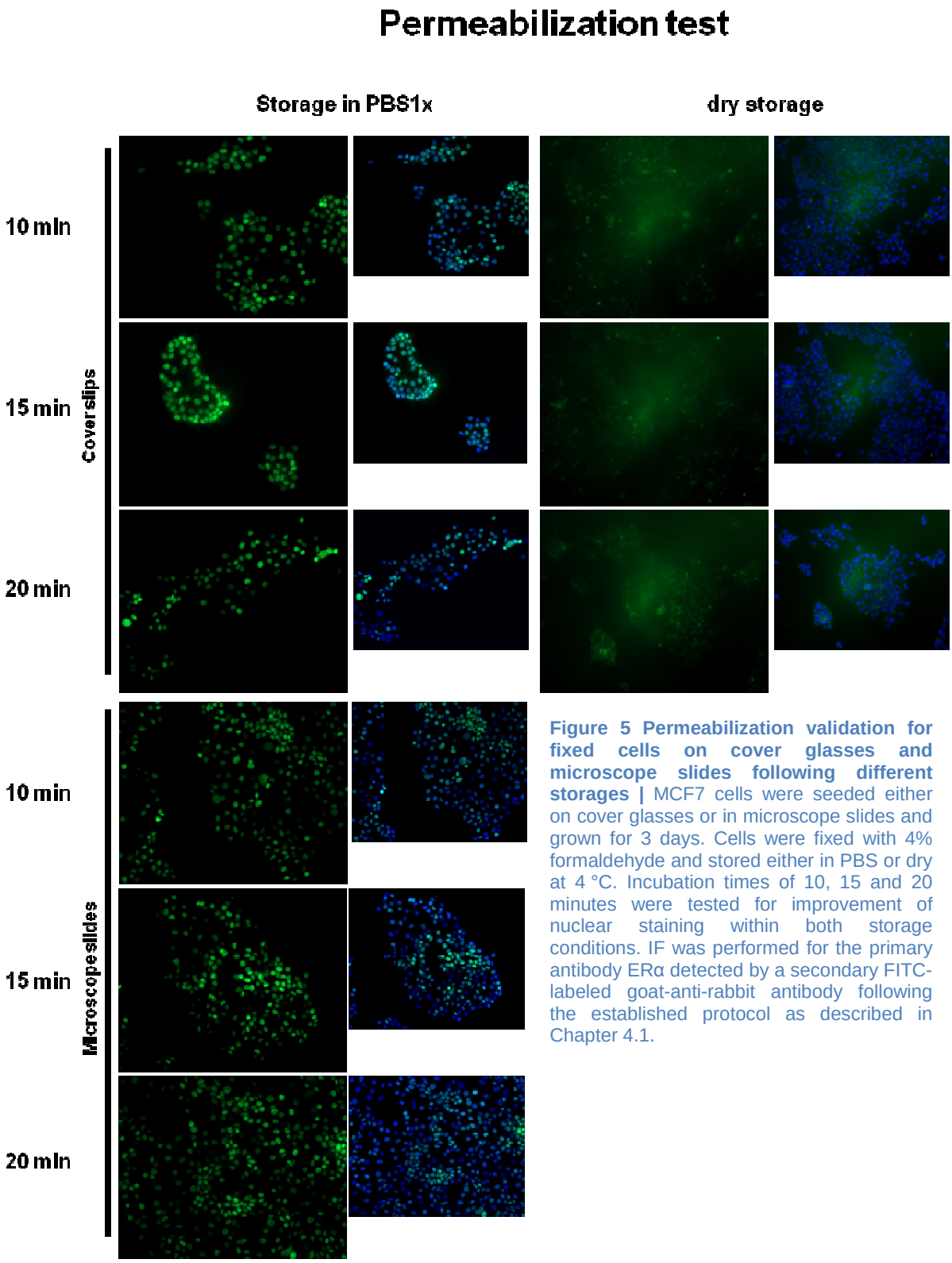


Figure 5 Permeabilization validation for fixed cells on cover glasses and microscope slides following different storages | MCF7 cells were seeded either on cover glasses or in microscope slides and grown for 3 days. Cells were fixed with 4% formaldehyde and stored either in PBS or dry at 4 °C. Incubation times of 10, 15 and 20 minutes were tested for improvement of nuclear staining within both storage conditions. IF was performed for the primary antibody ERα detected by a secondary FITC-labeled goat-anti-rabbit antibody following the established protocol as described in Chapter 4.1.

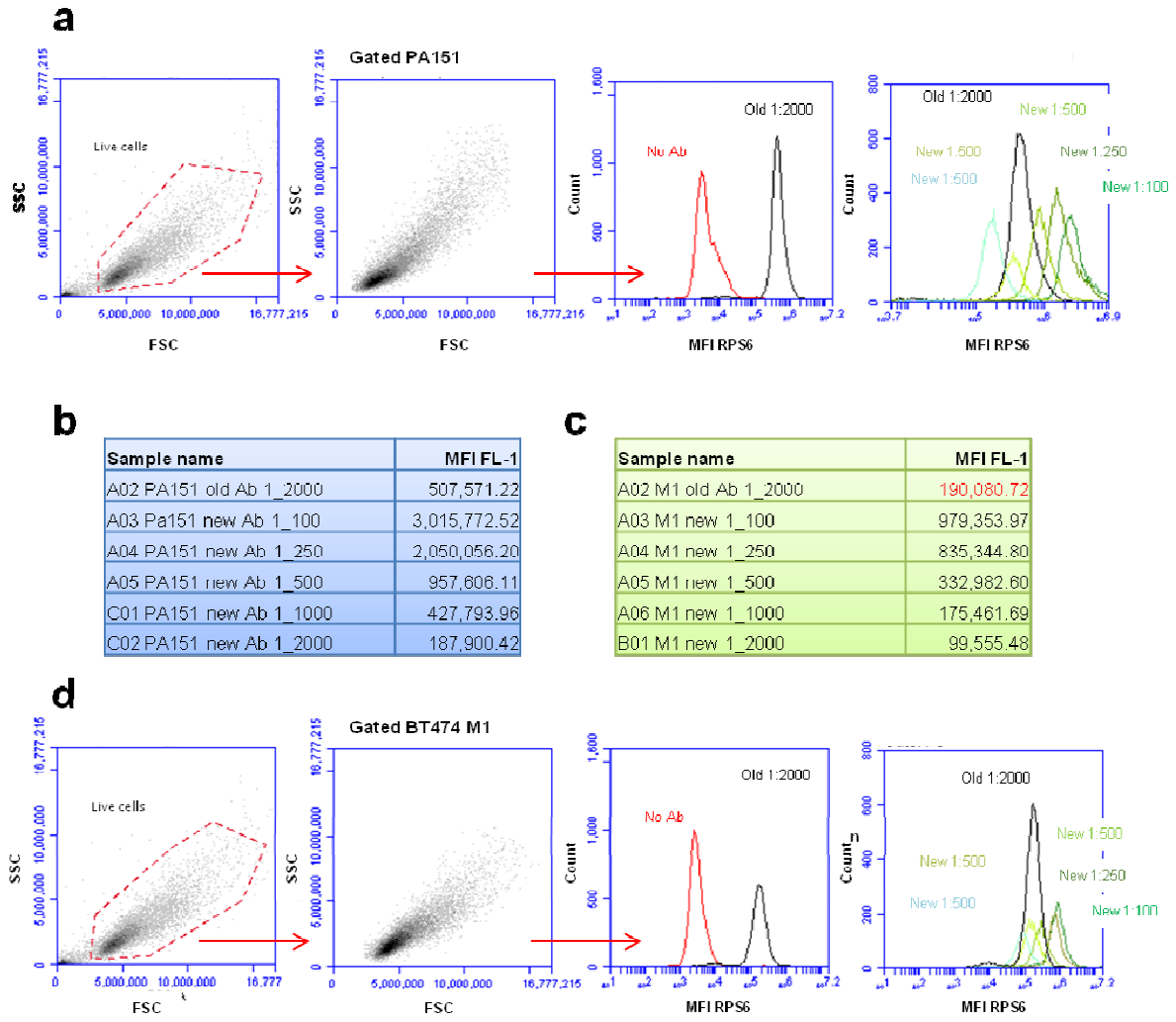
Since the nuclear staining for dry-stored cells was not improved with adjustment of the permeabilization, further troubleshooting experiments were performed. Incubation of dry-stored cells in PBS 1x at 37 °C overnight before all further protocol steps followed starting with permeabilization, showed a comparable fluorescent signal for nuclear ERα staining compared to in PBS 1x stored cells (data not shown).

Subsequently, cells were washed 2 times with PBS 1x + 0.5% BSA + 0.05% Triton and once with PBS 1x for 5 min each. Unspecific binding sites were blocked with PBS 1x + 10% FCS for 1 h at 18 °C while rocking. Another washing step with PBS 1x followed, before the primary antibody incubation was performed with respective antibodies diluted in PBS 1x + 1% BSA. Cells were incubated at 37 °C for 2 h and washed thoroughly with PBS 1x + 0.5% BSA + 0.05% Triton (twice) and PBS 1x only (twice) for 5 min each, before the secondary antibody reaction was performed. Secondary antibodies were diluted in PBS 1x + 1% BSA and incubated in the dark for 45 min at 18 °C while rocking. Another washing procedure of 3 x 5 min with PBS 1x followed. Cells stained using the indirect IF with fluorescent-labeled secondary antibodies were prepared for microscopy by mounting and covering with glasses. IF staining with Biotin-labeled secondary antibodies were processed with another step of detection between fluorescent-labeled avidin binding to biotin, before mounting and covering was performed as well.

4.2 Secondary Antibody Titrations

In order to determine the corresponding fluorescence intensity of a new ordered antibody compared to the former used antibody, a titration using flow cytometry was performed. Cells of two different cell lines, namely PA151 and BT474M1, were prepared for a no-antibody control, the respective concentration of the previous used antibody and a 5 step titration of the unknown antibody. The abundant cytoplasmic protein RPS6 was used as primary mouse antibody for the detection by an AF488-labeled goat-anti-mouse secondary antibody. Titration steps started with a lowest dilution of 1:100, followed by 1:250, 1:500, 1:1000 and the highest dilution of 1:2000. Since the older antibody was used in the upper limit of 1:2000 as lowest dilution recommended, no less concentrated steps were included in the titration of the new antibody. Live cells were gated (**Figure 6**; first two plots from left) for the Mean Fluorescence Intensity (MFI) quantification within all conditions.

The control yielded a significant lower MFI of 5,725 in PA151 and 3,289 in BT474M1 compared to the 1:2000 dilution of the old antibody with a MFI of 507,571 and 190,080, respectively. A comparable MFI of the titrated new antibody was observed in the dilution range of 1:1000 and 1:500 in both cell lines (**Figure 6**).



Sample name	MFI FL-1
A02 PA151 old Ab 1_2000	507,571.22
A03 Pa151 new Ab 1_100	3,015,772.52
A04 PA151 new Ab 1_250	2,050,056.20
A05 PA151 new Ab 1_500	957,606.11
C01 PA151 new Ab 1_1000	427,793.96
C02 PA151 new Ab 1_2000	187,900.42

Sample name	MFI FL-1
A02 M1 old Ab 1_2000	190,080.72
A03 M1 new 1_100	979,353.97
A04 M1 new 1_250	835,344.80
A05 M1 new 1_500	332,982.60
A06 M1 new 1_1000	175,461.69
B01 M1 new 1_2000	99,555.48

Figure 6 AF488 goat-anti-mouse titration | PA151 and BT474M1 were stained with a primary antibody against RPS6 (1:100) followed by the previous used secondary donkey-anti-mouse AF488 in a dilution of 1:2000 and the titration dilutions 1:100, 1:250, 1:500, 1:1000, 1:2000 of the new goat-anti-mouse AF488 antibody, respectively. Live cells were gated for MFI quantifications.

4.3 Double staining

All double staining followed the established IF protocol described in Chapter 4.1 regarding the basic steps. Variables are represented by the combination of the primary antibodies and their detection by different fluorescent-labeled secondary antibodies.

4.3.1 ERα/4EB-P1 double staining

An ERα/4EB-P1 double staining was validated for the cancer cell line MCF7 as ERα^{+}/4EB-P1^{+} cells compared to SKBR3 as ERα^{-}/4EB-P1^{+} cells. MCF7 cells without primary antibodies

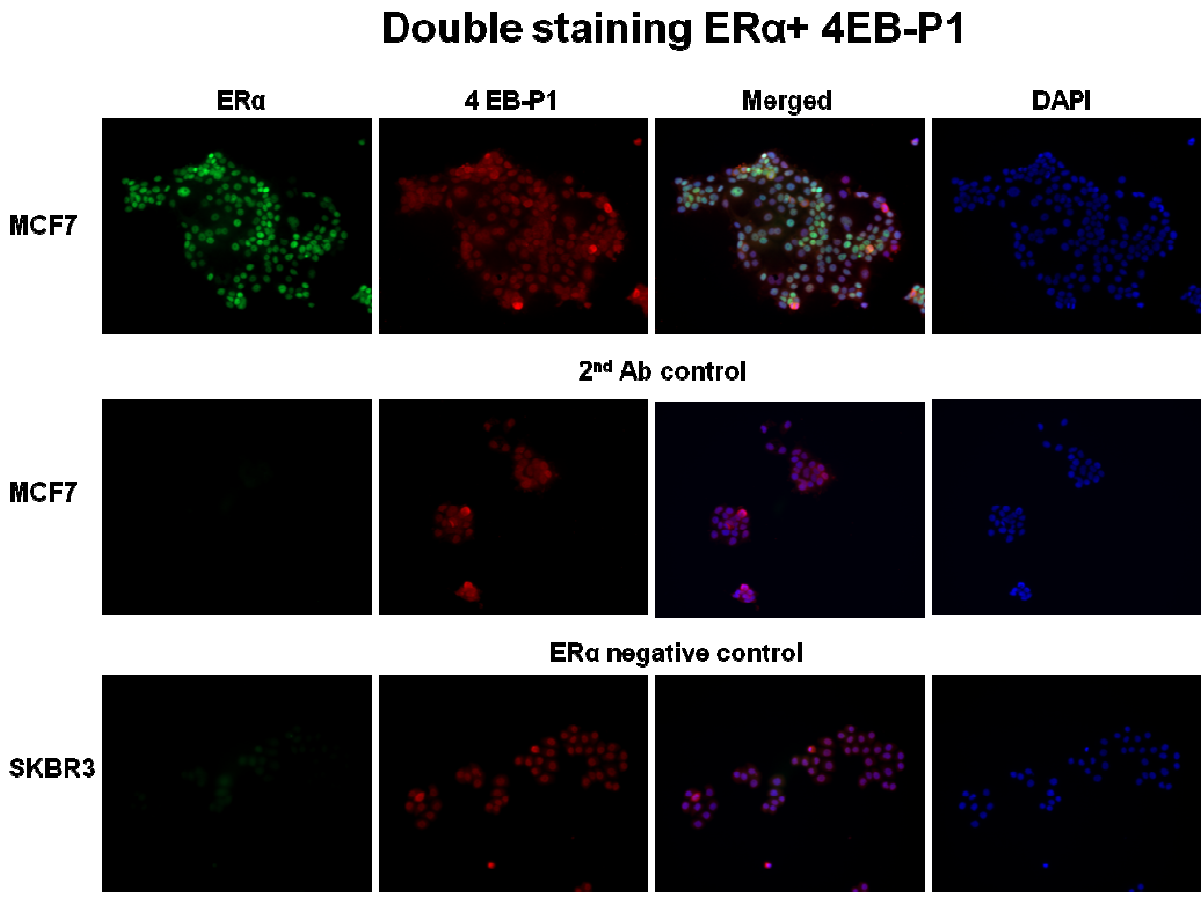


Figure 7 ERα/4EB-P1 double staining in MCF7 and SKBR3 cancer cell lines | Cells were stained with the primary rabbit-ERα and mouse-4 EB-P1, followed by detection of an AF488 goat-anti-rabbit and DL594 horse-anti-mouse, respectively. MCF7 cells missing primary antibody staining were used for detection of fluorescence background signals, whereas SKBR3 was an internal control of ERα specific staining. DAPI was used as nuclear reference staining added with the mounting medium.

but following secondary antibody staining steps were included for background fluorescence signals. Dilutions of primary antibodies were chosen as described in Chapter **3.1.6** with 1:100 for ERα and 1:25 for 4EB-P1. ERα was detected by an AF488-labeled goat-anti-rabbit antibody in the dilution of 1:200, whereas 4EB-P1 was followed by a DL594-labeled horse-anti-mouse antibody in the dilution of 1:200. Pictures were taken with the same exposure time as well as set for the same contrast and brightness adjusted to the secondary Ab control. MCF7 showed a clear specific signal for ERα with little fluorescence background. However, DL594 resulted in high unspecific signals as shown by MCF7 secondary antibody control (**Figure 7** second row). Additionally, pictures were improved in fluorescence background of DL594 with more harsh adjustments in contrast and brightness compared to the AF488 background. Merged pictures with DAPI as nuclear reference staining approved the localization of ERα and 4EB-P1.

4.3.2 ERβ/ErbbB2 double staining

An ERβ/ErbB2 double staining was validated for the cancer cell line BT474M1 as ERβ$^+$/ErbB2$^+$ cells. BT474M1 cells without primary antibodies but following secondary antibody staining steps were included for background fluorescence signals. Dilutions of primary antibodies were chosen as written in Chapter **3.1.6** with 1:100 for ERβ (mouse) and 1:100 for ErbB2 (rabbit). ErbB2 was detected by an AF488-labeled goat-anti-rabbit antibody in the dilution of 1:200, whereas ERβ was followed by a DL594-labeled horse-anti-mouse antibody in the dilution of 1:200. Pictures were taken with the same exposure time as well as set for the same contrast and brightness adjusted to the secondary antibody control (**Figure 8**).

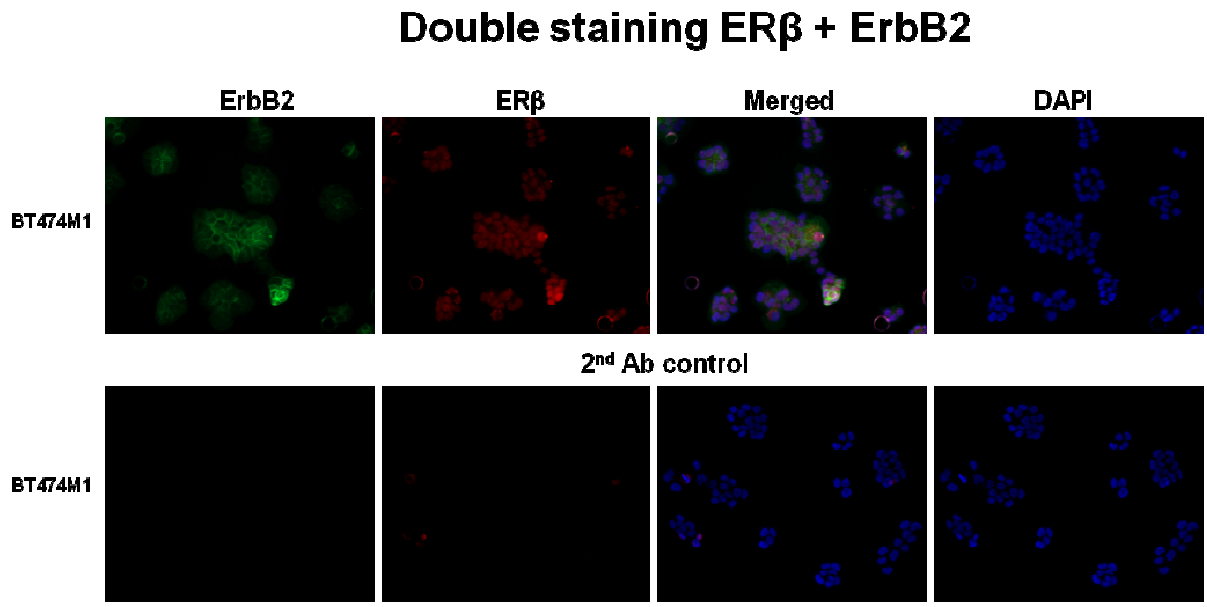


Figure 8 ERβ/ErbB2 double staining in BT474M1 cancer cells | Cells were stained with the primary rabbit-ErbB2 and mouse-ERβ, followed by detection of an AF488 goat-anti-rabbit and DL594 horse-anti-mouse, respectively. BT474M1 cells missing primary antibody staining were used for detection of fluorescence background signals. DAPI was used as nuclear reference staining added with the mounting medium.

Both stainings showed a specific fluorescent signal and were located as mentioned in literature with ErbB2 on membranes and ERβ in nuclei. Contrast and brightness adjustment led to erasure of fluorescence background without losing significant staining signals.

4.3.3 Cathepsin D/RPS6 double staining

A Cathepsin D/RPS6 double staining was validated for the cancer cell line MCF7 as Cathepsin D$^+$/RPS6$^+$ cells. MCF7 cells without primary antibodies but following secondary antibody staining steps were included for background fluorescence signals. Dilutions of primary antibodies were chosen as written in Chapter **3.1.6** with 1:50 for Cathepsin D (rabbit) and 1:100 for RPS6 (mouse). Cathepsin D was detected by an AF488-labeled goat-anti-rabbit antibody in the dilution of 1:200, whereas RPS6 was detected by a DL594-labeled horse-anti-mouse antibody in the dilution of 1:200. Pictures were taken with the same

exposure time as well as set for the same contrast and brightness adjusted to the secondary antibody control (**Figure 9**).

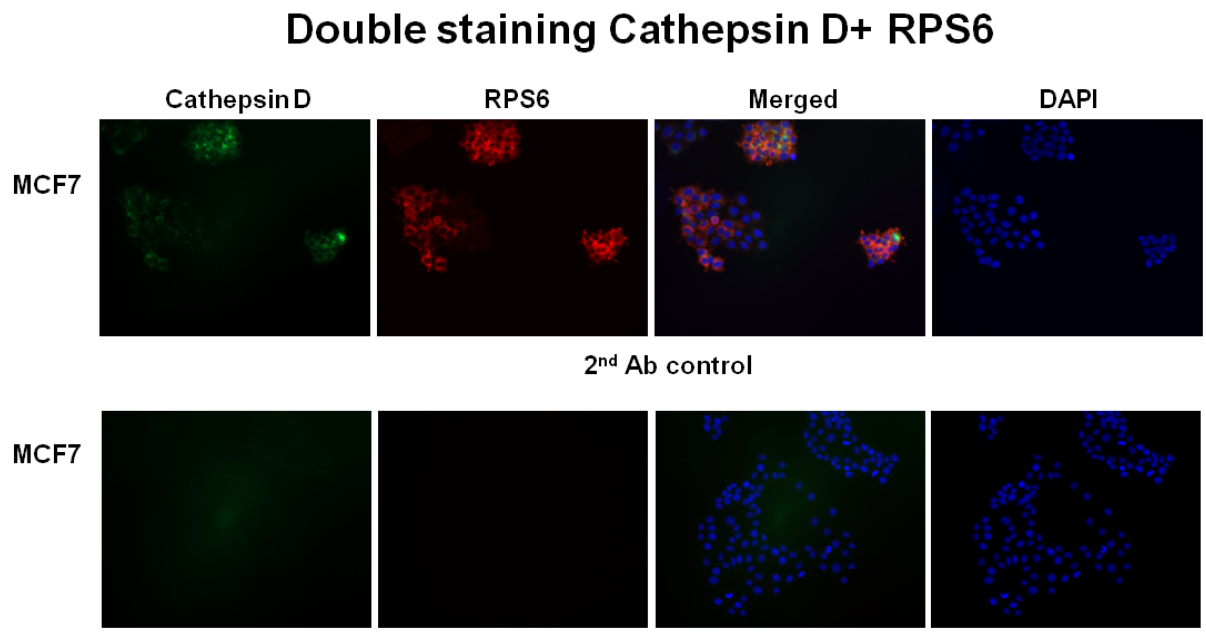


Figure 9 Cathepsin D/RPS6 double staining in MCF7 cancer cells | Cells were stained with the primary rabbit-Cathepsin D and mouse-RPS6, followed by detection of an AF488 goat-anti-rabbit and DL594 horse-anti-mouse, respectively. BT474M1 cells missing primary antibody staining were used for detection of fluorescence background signals. DAPI was used as nuclear reference staining added with the mounting medium

Immunofluorescence staining for Cathepsin D yielded a dotted staining as it is expected for a lysosomal localization. RPS6 detection yielded cytoplasmic staining surounding nuclear DAPI staining.

4.4 Signal Amplification by Biotin-Avidin staining

IF staining of ERα in PA cells grown for 7 days in 0.2% LC medium did not yield detectable fluorescence signals). In comparison to cancer cell lines such as MCF7 and T47D, PAs showed significant lower concentrations of ERα in western blot and FCM analyses and therefore we hypothesized that signal amplification using a Biotin-Avidin strategy might lead to fluorescence signal detection.

Therefore, PA115 were seeded in 2% LC medium on cover slips and fixed after 3 days of incubation with formaldehyde. Cover slips were directly processed following the established protocol as described in Chapter 4.1. The primary antibody ERα was either detected by a secondary AF488 goat-anti-rabbit in a dilution of 1:200 or by a Biotin-anti-rabbit in a dilution of 1:500. Avidin Fluorescein D in a dilution of 1:500 was used for fluorescent detection of Biotin-labeled secondary antibodies. Cover glasses processed without primary antibody incubation represented fluorescence background controls.

Both staining strategies resulted in fluorescent signals after subtraction of background fluorescence produced by unspecific binding of secondary antibodies (**Figure 10**).

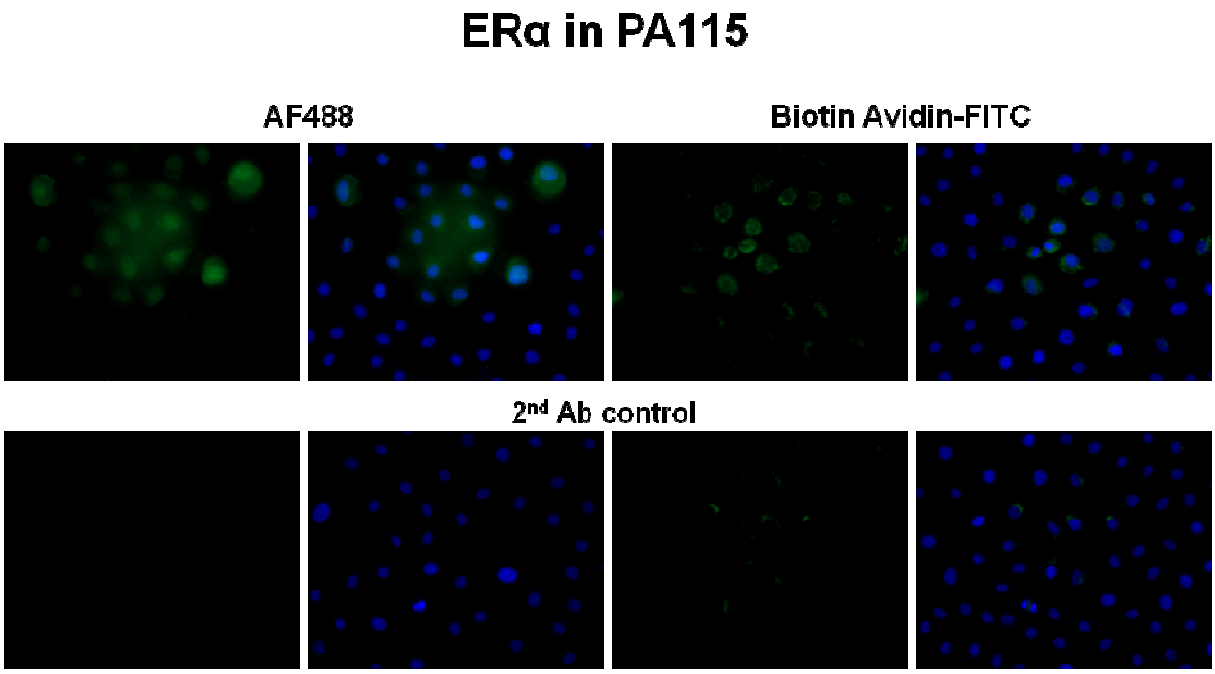


Figure 10 Comparison of indirect IF and the signal amplified strategy of biotin-avidin staining

4.5 Comparison of staining by IF and FCM

Flow cytometry and immunofluorescence were compared in order to analyze background:signal ratios for each technique.

Regarding the flow cytometry experiment, MCF7, SKBR3 and different grown and treated PA115 (LC medium 0.2%, LC medium 2%, Estrogen treated) were incubated with the primary antibody against ERα followed by either a FITC-labeled secondary antibody, a biotin-labeled antibody in the dilution of 1:500 or a biotin-labeled antibody in the dilution of 1:100. FITC-labeled Avidin in a dilution of 1:500 was combined with the 1:500 diluted biotin-labeled secondary antibody, whereas a 1:200 diluted FITC-labeled Avidin was used for the higher concentrated Biotin-labeled antibody.

The best signal-to-noise ratios were seen in the indirect staining using a secondary FITC-labeled antibody in all cells and conditions tested (**Figure 11**). Values of $ERα^+$ cell lines ranged from 5.07 to 9.45 compared to the $ERα^-$ SKBR3 with a ratio of 2.92. Lower ratios and therefore a higher background was obtained by the Biotin-Avidin strategy in the combination of 1:100 dilution for Biotin and 1:200 dilution for Avidin with ratios between 2.38 for MCF7 to 3.23 for PA115 grown in 2% LC medium. Highest estrogen receptor levels were found in PA115 2% cells, followed by PA115 0.2% among all staining strategies tested. SKBR3 as $ERα^-$ cells showed a baseline fluorescence with signal ratios of 1.51 (Biotin 1:100-Avidin

1:200) – 2.92 (2nd FITC) compared to the positive signal ratios of 2.38 – 5.07 for the ERα+ MCF7 cells.

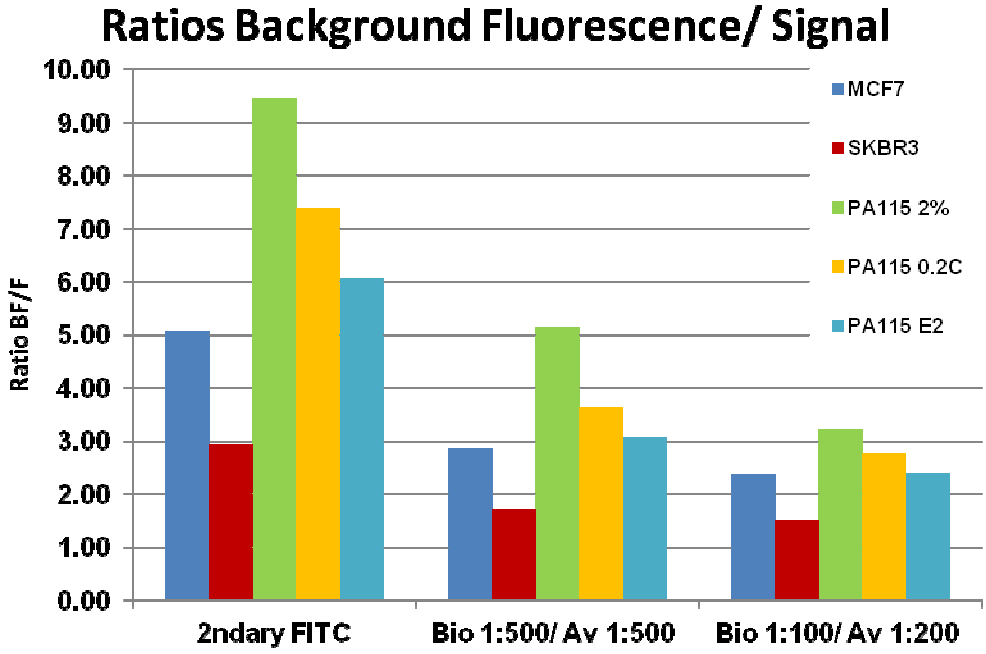


Figure 11 Signal-to-noise ratios of different staining strategies | Fixed cell pellets of MCF7, SKBR3, PA115 2% LC medium, PA115 0.2% LC medium and estrogen-treated PA115 were stained using 3 different strategies: (1) ERα (rabbit) – FITC-labeled goat-anti-rabbit; (2) ERα (rabbit) – Biotin-anti-rabbit(1:500) – FITC-labeled Avidin (1:500); (3)) ERα (rabbit) – Biotin-anti-rabbit (1:100) – FITC-labeled Avidin (1:200). MFI were measured using the flow cytometer Accuri (BD) and calculated for signal-to-noise ratios.

Immunofluorescence was performed for MCF7, SKBR3 grown in 10% medium and PA115 grown in 2% LC medium using a staining combination of the primary antibody against ERα detected by either a secondary antibody labeled with FITC in a dilution of 1:100 or a biotin-labeled secondary antibody in the dilution of 1:100 followed by a FITC-labeled avidin in a dilution of 1:200 (**Figure 12**). Cells without primary antibody incubation but following secondary antibody incubations were chosen as fluorescence background. Subsequently, settings for exposure time, contrast and brightness of positive signals were set corresponding to the demands of secondary antibody controls.

Indirect immunofluorescence with FITC-labeled secondary antibodies showed relatively weak fluorescence signals after adjustments of controls within the PA115 2% staining (**Figure 12**) compared to MCF7, where SKBR3 cells were the negative control (data not shown). In comparison to the former mentioned combination of antibodies, clear fluorescence signals were yielded within the biotin-avidin staining.

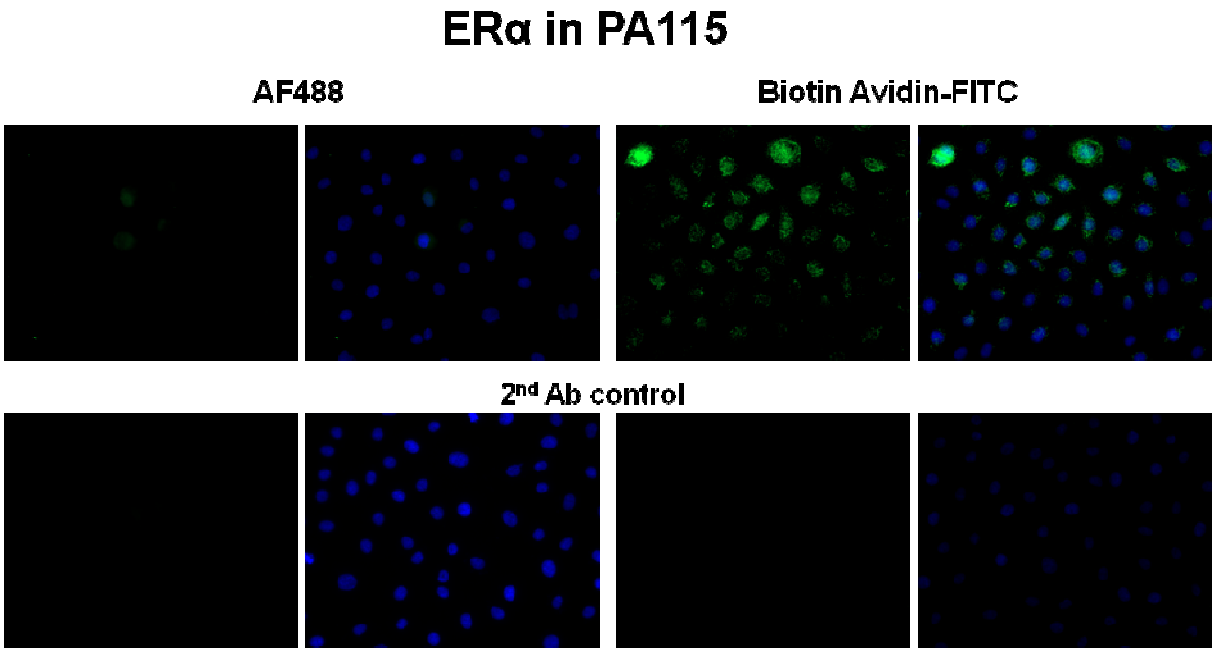


Figure 12 Comparison of 2 different indirect IF stainings for detection of ERα | MCF7, SKBR3 (data not shown) and PA115 2% LC medium were stained for ERα using different secondary antibodies: Either FITC-labeled goat-anti-rabbit in a dilution of 1:100 or Biotin-anti-rabbit (1:100) followed by a FITC-labeled Avidin (1:200) was used to detect the primary anti-ERα antibody. Secondary antibody controls followed the same protocols except for missing primary antibody incubations. Settings of exposure time, contrast and brightness were adjusted to the control cells MCF7 and SKBR3 as well as for secondary antibody controls.

5 Discussion

5.1 IF protocol validation

An IF protocol has been optimized by a step-by-step adaptation of general staining procedures for proteins of interest in Prof. Dairkee's research group. Conditions for optimal confluency of cancer cell lines as well as non-malignant cells for IF on cover slips as well as on microscope slides were defined. Cell metabolism and therewith the regulation of receptors is regulated by extracellular signals, which can be of soluble origin or cell contact signals. Subsequently, cell confluency is not only important for visual aspects within IF but also for analyses outcome of receptor specific staining. Each cell line and type exhibits different cell growth regarding time and spatial positioning, for example in cluster or single, and therefore needs to be optimized. This optimization has been performed for the HRBEC's and cancer cell lines used and is described in Chapters **3** and **4**.

The fixation method was chosen due to the results of a comparing experiment between methanol or formaldehyde usage (Chapter **3.1.2**). Since formaldehyde yielded better cell preservation and supported the maintenance of organic cell structures, it became the primary choice of fixation.

Different incubation times and temperatures for the permeabilization were analyzed and set to the shortest time tested with 10 minutes at a temperature of 18 °C. Neither cell loss nor improvement of ERα staining was observed with longer permeabilization times or higher temperatures in MCF7 cancer cells. Indeed, extended permeabilization should be avoided in regard to destroying influences of the detergent on cell structures.

Antibody concentrations have been determined individually, balancing concentration in relation to signal outcome. A temperature of 37 °C was chosen to improve the protein-protein interactions of antibody and target molecule, which yielded staining improvements especially for the antibody against ERα (clone HC-20).

Further efforts were performed to enhance the IF protocol using the signal amplification strategy combining indirect IF with a biotin-avidin step. Here, different concentrations of the respective reagents were as well tested for signal-to-noise ratios and determined for a staining of ERα.

The optimized protocol and tested antibody concentrations can be used to analyze the influence of environmental compounds like xenoestrogens or estrogen itself on molecular changes in breast epithelial cells.

Further improvements of specificity and localization might be obtained by different antibody clones for example of ERα. Since staining results can differ between the usage of different antibodies and clones, further adaptations or even the establishment of a new protocol might be necessary.

The signal amplifying IF staining with biotin-avidin detection might be also of interest for the antibodies against Cyclin D1, Cathepsin D and phosphorylated ERαS118. IF staining using those antibodies yielded weak fluorescent signals in positive control cells containing abundant amounts of the respective proteins (not all data shown). In case of non-malignant breast epithelial cells, signals apparently need to be amplified to obtain visible fluorescence in microscopy.

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