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Exosomal communication between cells of the tumor microenvironment

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Eksomit ovat alle 100 nm kokoisia lipidirakkuloita, joita solut käyttävät solujen välisessä viestinnässä. Näissä rakkuloissa on osoitettu löytyvän lipidejä, proteiineja ja jopa RNA:ta. Vaikka eksosomien olemassa olo huomattiin yli 30 vuotta sitten, niitä on alettu tutkimaan kunnolla viimeisen 10 vuoden aikana. Nyt eksosomeista toivotaan vastausta siihen, miksi osa syöpäpotilaista lakkaa vastaamasta lääkehoitoihin.

Opinnäytetyön tarkoituksena on antaa yleiskuva, millaista on tutkia näitä äärimmäisen pieniä lipidirakkuloita. Työ toteutettiin viljelemällä syöpäsolulinjoja ja käsittelemällä niitä lääkkeillä, joita käytetään HER2 positiiviseen rintasyöpään, trastutsumabilla ja T-DM1:llä. Viljelyistä eristetiin eksosomit ultrasentrifuugin avulla. Eksosomeja käytetiin erilaisissa kokeissa, joissa selvitettiin trastutsumabin/ T-DM1 esiintymistä eksosomien pinnalla. Mittaukset tehtiin immonologisia leimausmenetelmiä ja virtaussytometriä käyttäen.

Tutkimustuloksista selvisi trastuzumabin ja T-DM1:n tasot eksomien pinnalla. Tulokset antoivat hieman yleiskuvaa eksomien luonteesta, mutta herätti muita myös lisää kysymyksiä. Aihetta tullaan edelleen tutkimaan tutkimusryhmässä eristämillämme eksosomeilla.

Avainsanat	Eksosomit, solujen välinen viestintä, rintasyöpä, HER2-reseptorit, Trastutsumab, T-DM1

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Exosomes are part of the large heterogeneous group of extracellular vesicles (EV's) which cells can use to exchange proteins, lipids and even RNA. Described for the first time in 1983, exosomes remained little studied until 2007. Extracellular vesicles and exosomes have gained a lot of attention in the past ten years both as mediators of intercellular communication and potential cancer biomarkers.

The thesis contains different protocols from cell culturing to exosome purification by using ultracentrifuge. We studied the effects of trastuzumab and T-DM1, drugs used for HER2 positive breast cancer, in different cell lines. Exosomes harvested from drug treated cells were used to measure if there is trastuzumab/ T-DM1 in their surface. This was done using different immonolabelings and flowcytometry.

The results gave us information about the nature of exosomes derived from cancer cells. We proved the measured vesicles were exosomes for sure. The subject will be studied further using the exosomes harvested during the execution of the thesis.

Keywords	Exosomes, communication between cells, breast cancer, HER2 receptors, trastuzumab, T-DM1

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1 Introduction

Breast cancer is the most common cancer type among women in industrialized countries (Puistola 2011). In the year 2011 31.8% of all the cancers diagnosed in Finnish women were breast cancer (Suomen Syöpärekisteri 2011). There has been a notable increase of breast cancer cases in the recent years. A part is explained by the aging effect of the main population, but it is thought that around 2/3 is explained by other factors. (Puistola 2011.)

Breast cancer is treated with a combination of surgical removal of the tumor, radiotherapy, chemotherapy and hormonal treatment. In cases of HER2 positive breast cancer trastuzumab is used with chemotherapy. (Puistola 2011.)

Trastuzumab emtansine (T-DM1) has been approved recently for the treatment of HER2-positive metastatic cancer. This new drug was more effective and less toxic compared to trastuzumab + chemotherapy in clinical trials (Verma 2012; Hurvitz 2010).

Heikki Joensuu's research team from the molecular oncology in the University of Helsinki (Biomedicum Helsinki) is researching the different mechanisms of cancer. One of the ongoing projects there, researched by Ph. D. Mark Barok, is to study how T-DM1 is bounded and carried in exosomes secreted by cancer cells, and how T-DM1 bounded exosomes affects cells in the tumor microenvironment.

A quick look to the Theseus database revealed that there is not a single thesis, which would deal about exosomes. The main objective of this thesis is to provide an insight of how such small and challenging nanovesicles can be studied.

The guiding scientific questions studied during the working process of this thesis were:

- Is there exosomes in the samples collected from cell cultures?
- Is there HER2 expression in exosomes?
- Is there T-DM1 on the surface of the exosomes prepared from T-DM1-treated cancer cells?

The subjects were studied using different methods, such as: cell culturing, preparation of exosomes by differential ultracentrifugation, characterization of exosomes by flow cytometry.

Mark Barok guided my thesis. Senior Lecture Hannele Pihlaja from Helsinki Metropolia University of Applied Sciences provided mentoring and feedback during the different phases of the thesis. Elisa Sonne provided peercomments in the last phase of the thesis.

2 Introduction to the topic

2.1 Extracellular vesicles and exosomes

Exosomes are part of the heterogeneous group of extracellular vesicles (EV's). The nomenclature can be quite confusing as the vesicles are referred to by many different names, such as: apoptotic bodies, ectosomes, exosomes, nanovesicles, matrix vesicles… The list goes on. In literature exosomes are often describe as particles smaller than 100 nm in diameter that are actively secreted from cells, while microvesicles are considered larger than 100 nm and often buds of the surface. (Witwer 2013; Lötvall 2015.)

2.1.1 A brief history

Described for the first time in 1983, these vesicles were pretty much designated just as cellular garbage cans acting to discard unwanted molecular components. They remained little studied for the next ten years. (Théry 2011.) The concept of exosomes surfaced again in 1996, when a research team showed that Epstein–Barr virus transformed B-lymphocytes secreted exosomes that bore molecules essential for the adaptive immune response. Later studies provided the basis for the hypothesis that exosomes could play an active role in intercellular communication. (Raposo 1996: 1161-1172.)

The big breakthrough came several years later in 2007, when the group of Jan Lötvall in Sweden provided convincing description, in a paper called "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange be-

tween cells", of the presence of mRNA and microRNA inside these vesicles. It was showed, at least in vitro, that some of that RNA present in the exosomes could be translated into proteins in the target cells. Ever since the breakthrough, the interest in these nanoparticles has spiked exponentially. (Théry 2011; Valadi 2007: 654- 670)

2.1.2 Composition, biogenesis and function

Exosomes are generated by both eukaryotic and prokaryotic cells (Buzas – György – Nagy – Falus − Gay 2014). They have been reported to be present in every biofluid and can travel to distant tissues from the original site they were excreted from (Witwer 2013; Tickner – Urquhart – Stephenson – Richard – O'Byrne 2014: 1). The production of nanovesicles is a highly conserved process. It has been showed that nanovesicles are present, for example in bacterias, plants, yeast and parasites (Lötvall 2015).

Exosomes contain a specific subset of proteins from endosomes, the plasma membrane and the cytosol. They form inside eukaryotic cells and are released to cell microenvironment. (Kowa – Tkach − Théry 2014.) They do not contain proteins of nuclear, mitochondrial, endoplasmic reticulum or Golgi- apparatus origin. When analyzed by whole-mount electron microscopy, exosomes appear as a flattened sphere that is limited by lipid bi-layer. (Théry – Zitgovel – Amigorena 2002.)

There is no consensus on the exact function of these nanovesicles, but there are some hypotheses. First of all, exosomes could bear combinations of ligands that would engage different cell-surface receptors simultaneously. Second, exosomes could bind to target-cell membranes, which would bear "new" surface molecule, and potentially acquire new adhesion properties. Third, exosomes could fuse with target cells and exchange membrane proteins and cytosol between the two cell types. (Théry − Zitvogel− Amigorena 2002) Nonetheless, it is agreed that they play an important role in cell to cell communication.

Extracellular vesicles and exosomes have gained a lot of attention in the past ten years both as mediators of intercellular communication and potential cancer biomarkers. Several reports indicate that cancer cells release more extracellular vesicles than normal cells and the biomolecular cargo (proteins, lipids and nucleic acid) is reflective to the cells origin. Knowledge about the content of extracellular vesicles derived from tu-

mor cells with differing stages of aggression could be used to establish new diagnostic approaches using patient-derived vesicles from body fluids. (Nawaz 2014.)

2.1.3 Role in tumor microenvironment

The tumor microenvironment is considered as comprised of a heterogeneous population of cancer cells, stroma cells and tumor-infiltrating immune-cells that all interact with each other contributing to tumor development and progression (Lu − Huang − Hanash – Onuchic − Jacob 2014).

Recent studies have shown that exosomes can have a bimodal role in cancer. Tumor derived exosomes can manipulate the local and systemic environment to aid cancer growth, angiogenesis and dissemination. They can also travel to distant sites to promote the generation of the pre-metastatic niche. Exosomes may also manipulate the immune system to elicit an anti–tumor response by recruiting cells to enhance tumor invasion, tumor angiogenesis and dissemination. (Kahlert − Kalluri 2013; Tickner 2014.)

Preliminary results from other studies suggest that exosomes' mediated transfer of proteins between cancer cells can lead to chemoresistance (Kahlert 2013: 433). For example, a recent study by Corcoran and al. concluded that exosomes from different docetaxel-resistant prostate cancer cell lines may, in part, confer chemoresistance to non-resistant prostate cancer cell lines (Corcoran 2012).

2.2 HER2 positive cancers

HER2 is the abbreviation of **h**uman **e**pidermal growth factor **r**eceptor 2 and is a member of the epidermal growth factor receptor (EGFR) family (Bang 2010). The amplification or overexpression of HER2 has been shown to play an important role on the development of certain types of breast and gastric cancers. It can be used as a biomarker and is commonly tested as it dictates how the cancer will be treated. HER2 positive cancer can also be diagnosed in gastric, gastroesophageal, ovarian, endometrium, bladder, and salivary duct cancers. Trastuzumab is commonly used to treat HER2 positive breast cancer. It is also used for the treatment of patients with HER2 positive metatasic gastric cancer. (Isola 2014.)

2.3 Trastuzumab emtansine, an anti-HER2 antibody-drug conjugate

Antibody-drug conjugates (ADCs) are structurally composed of a monoclonal antibody, a small chemical cytotoxic drug and a linker. The ADC is delivered specifically to the target cells by the antibody, the cytotoxic drug kills the target cells; the antibody and the cytotoxic drug are linked stably by the linker (Erickson 2006: 4426). Trastuzumab emtansine (Kadcyla®), shortly T-DM1 is an ADC that consist of trastuzumab as the monoclonal antibody and DM1 as the cytotoxic drug.

Trastuzumab (Herceptin®) is a monoclonal antibody that selectively binds with high affinity to the extracellular domain of the human epidermal growth factor receptor 2. Trastuzumab has many mechanisms of action such as down-regulation of HER2 in the plasma membrane, inhibition of HER2 ectodomain shedding and HLA-I-restricted antigen presentation. It also kills cancer cells by inducing apoptosis and inhibiting angiogenesis. (Barok 2014.) Despite its diverse mechanisms of action, a significant part of patients treated with trastuzumab either do not respond initially or relapse after experiencing a period of clinical response. (Phillips 2008: 9280.)

Emtansine, or DM1, is a chemotherapy drug that kills cancer cells by causing mitotic arrest, disrupting intracellular trafficking and inducing apoptosis. Even though DM1 has more pronounced anti-tumor effects, trastuzumab mediated effects should not be underestimated as the continuation of trastuzumab based therapies beyond breast cancer progression might be beneficial. (Barok 2014.) T-DM1 has been approved for the treatment of metastatic breast cancer in 2013 after proving its efficacy and safety in clinical trials (FDA 2013).

Even though primary resistance of HER2 positive metastatic breast cancer to T-DM1 seems to be relatively infrequent, most patients treated with the drug develop acquired drug resistance (Barok – Joensuu − Isola 2014).

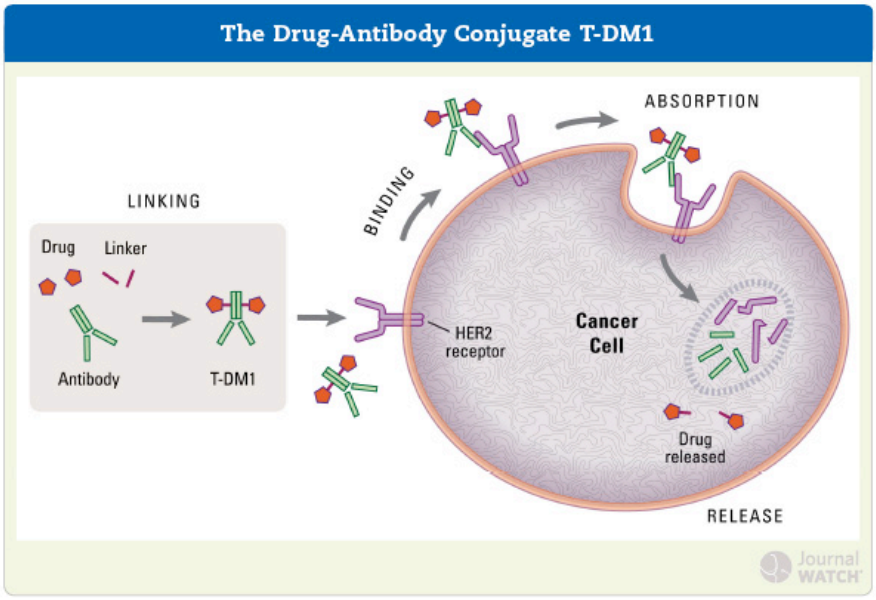


Figure 1. Trastuzumab emtansine and its internalization into a target cell (Jwatch 2012).

In T-DM1, an average of 3.5 DM1 molecules are bound to one molecule of trastuzumab via a non-reducible thioether linker. In this ADC, trastuzumab is responsible to carry the cytotoxic drug (DM1) to the HER2-positive cancer cells. After binding to cell-surface HER2 receptors, T-DM1 is internalized by the target cancer cells which is followed by the intracellular degradation of the trastuzumab and linker parts of T-DM1 resulting of the release of the free DM1 inside the cancer cells (Figure 1.). (Barok 2014.)

3 Methods

The size of exosomes (30-100 nm) falls below the optical resolution of conventional light microscope, which makes a conventional light microscope useless to image exosomes. To prove that exosomes are present in a sample, different approach is needed. As mentioned before, in this project exosomes will be studied by cell culture, exosome purification and characterization.

Every exosome harvesting protocol used by researches is slightly different from the other. The difference between these protocols may lead to the purification of different subtypes of exosomes. That is why it is important to always mention what kind of materials, centrifuges and rotors were used during the purification. (Lötvall 2015.)

3.1 Cell culture

Cell culture means growing cells artificially in a tube or in a specific container. Cells need a medium that contains essential organic and inorganic compounds, such as: glucose, vitamins, amino acids and inorganic ions. The exact cultivation protocol varies from cell type to cell type. (Saarela 2013.)

3.1.1 Aseptic technique

Using aseptic techniques is important to avoid infections of the cells when culturing them. The cells can only grow properly in an environment free of other microorganisms, such as: bacteria, viruses, fungi. Nonsterile supplies, medium, reagents and dirty work surfaces as well as air borne particles are the main source of contaminants and microorganisms. Incubators are seldom completely sterile, but keeping them clean with regular decontamination procedures and using sealed, vented cap culture vessels should be enough to keep the cells uninfected (Ryan 2008).

Aseptic technique is designed to provide a barrier between microorganisms in the environment and the cell culture. The elements in aseptic technique are sterile work area, good personal hygiene, sterile reagents and media and sterile handling.

To keep surfaces clean from contaminants, it should always be wiped with 70% ethanol before and after use. Everything that is put inside the laminar flow hood has to be also cleaned with ethanol. Commercial media and reagents undergo strict quality control to ensure their sterility. Pouring liquid directly from flasks or bottles should be avoided to keep their sterility. Sterile work should always be done inside a disinfected laminar flow hood.

Good personal hygiene means using proper protecting equipment, such as laboratory coats and gloves, keeping long hair tied in the back and your hands clean. This reduces the probability of contaminating the working surfaces with skin flakes. It also protects from any spillages from the cell culture, which is important when working with biohazardous materials. (Aseptic technique 2015.)

3.1.2 Contaminations in cell cultures

Although cell cultures are very popular among life science researchers, they can be very difficult tool to work with in the laboratory. Cell cultures make a dynamic, ongoing use of living things and often respond to our mistakes not only by erratic behavior but by dying as well. (Ryan 2008.)

Erratic culture behavior can take many forms; unusual growth patterns or inconsistent, spotty, and uneven cell attachment are the most common problems. A serious and very widespread problem that alters the characteristic growth, metabolism, morphology, attachment, and membranes of the cell cultures is mycoplasma. These bacteria-like microbes are the smallest self-replicating organisms known (0.3-0.8 μm in diameter), they lack a cell wall making them almost invisible to microscopic observation and they are fastidious in their growth requirements. Unlike other bacterial infections, they do not cloud the growing media or leave any other visible signs of contamination- not even when growing in high density. Mycoplasma spreads easily through contaminated surfaces and media. (Ryan 2008.)

3.1.3 Used cell lines

In this study we used four types of cell lines: TIME, SNU-216 and SKBR3 and JIMT-1. TIME and SKBR3 cell lines are from American Type Culture Collection (abbreviated ATCC), SNU-216 is from Korean Cell Line Bank and JIMT-1 from German Collection of Microorganisms and Cell Culture.

TIME cells are human endothelial cells originally derived from the foreskin of a neonatal. TIME cells have been immortalized by using a retrovirus, thus making the cells biohazardous. (ATCC 2014.) However, it has been showed previously in this study that the virus is not present in this cell line, and it should be safe to handle the culture under normal, sterile conditions.

SNU-216 is a human gastric cancer cell line established from a nodal metastasis of stomach cancer, has moderate amounts of HER2 receptors and is trastuzumab-resistant. (KCLB 2014.)

SKBR3 is a human breast cancer cell line derived from the primary tumor of a breast cancer patient and is HER2-positive and trastuzumab-sensitive. It is much more sensitive to T-DM1 than trastuzumab. (ATCC 2014; Barok – Tanner – Köninki – Isola 2011)

JIMT-1 is also a human breast cancer cell line which has been established from the pleural effusion of patient with grade 3 invasive ductal breast cancer. It is described to carry HER2 oncogene amplification and to be insensitive to trastuzumab, however, it is sensitive to T-DM1. (DSMZ 2015; Barok 2011.)

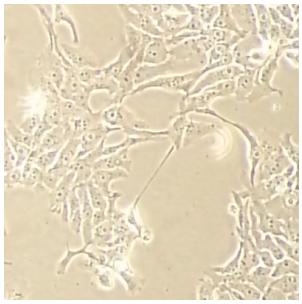


Figure 2. JIMT-1

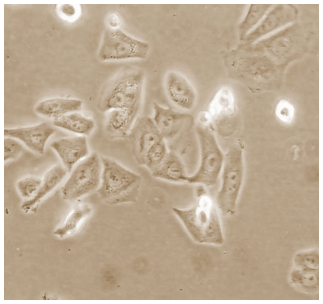


Figure 3. SKBR3

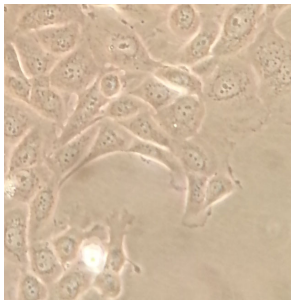


Figure 4. SNU-216

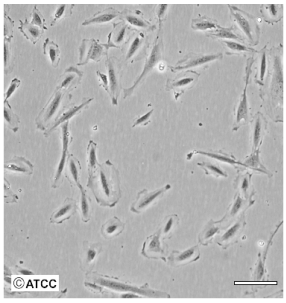


Figure 5. TIME (picture from ATCC)

Figures are the light microscopy images of the cell lines in different states of confluence. Figures 2-4 are self taken, and figure 4 is from ATCC official webpage.

3.1.4 Protocol

Despite of the difference between the cell lines, the cells were cultured using the same washing and centrifuge protocols. Only the culture medium differed from cell line to cell line. The materials are described in detail in the following table.

Table 1. Material list in detail.

Materials	Other information	Company
Media:		
DMEM	High glucose, without glutamine	Lonza
RPMI 1640		Gibco by Life Technologies
Vascular cell basal	Low glutamine	ATCC
Supplements:		
Blasticidin		Invitrogen Life Technologies
Penicillin-Streptomycin – Glutamine		Gibco Life Technologies
Others:		
Phosphate buffered saline (PBS)		Lonza
Trypsin- EDTA		Lonza
Trypsin 10x		Lonza
Fetal bovine serum (FBS)		Gibco Life sciences
Conical tubes (Falcon)	50 ml	Corning
Cell culture vessels	160 ml, 75 ml	NUNC
Multi layered culture vessel	10 layered	Corning
Incubator	Autoflow IR direct heat CO_2 incubator	Nuaire
Waterbath	SW23	Julabo
Cell culture hood	Clean Wizard W130	Kojair
Pipets		Biohit
Centrifuge	IEC Centra CL2	Thermo Electron Corporation

Table 2. Used supplements in commercial media

Cell line	**Used commercial medium:**	**Supplemented with:**
TIME	Vascular cell basal medium	Blasticidin
SNU-216	RPMI	10% FBS, 1% PS-Glut
SKBR3	DMEM	10%FBS, 1% PS-Glut
JIMT-1	DMEM	10%FBS, 1% PS-Glut

First the wanted the cells to be separated from the mother culture. The mother culture medium is first discarded and the cells are washed with phosphate buffered saline (PBS) as it will be referred as later on. Detaching the cells from the bottles growing surface happens by adding of trypsin to the culture. The cultures are incubated for 10-15 minutes at 37 °C.

After making sure the cells are completely detached from the growing surface by using a light microscope, the activity of trypsin-EDTA is inhibited with culture medium. The

volume of used culture medium should be twice the amount of trypsin. The cells are then collected to a Falcon tube and centrifuged with 1000 rpm for 7 minutes.

The supernatant is thrown away and the pellet is suspended in some culture medium. The cell-suspension is added to a new culture bottle and cultured in an atmosphere of 5% CO_2 and 37 °C. Every 2- 3 days the cells have to be subcultivated.

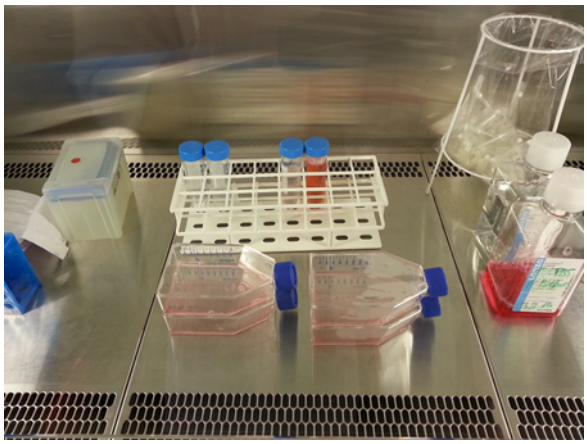


Figure 6. Cell culturing in a hood

3.1.5 Testing mycoplasma from the cell lines

One of the cell lines were having troubles to grow properly while the others had problems in attachment. This lead to the thought, that there might be something contaminating the cells. After a bit of research I decided to test them with Venor GeM by Minerva Biolabs, a mycoplasma detection kit for PCR.

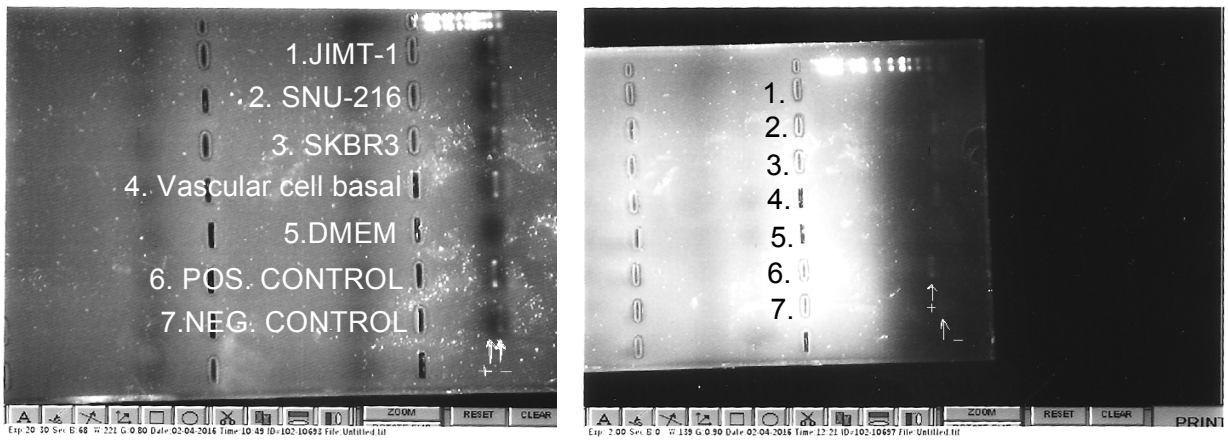


Figure 7. Mycoplasma PCR testing kit results after electrophoresis.

The results were negative. As it can be seen from the figure, the 30 minute run was not enough to clearly confirm the results. After 30 more minutes the bands on the agar gel became separated clearly enough to read the results. The reason why DMEM did not give a band is probably because of the serum presented in the sample, which inhibits the sample.

After an extensive talk with the members of the research group, it was decided to throw away the cell cultures, disinfect the working areas that were in contact with the cell lines and restart the cultures again anyway. Although time consuming and expensive, it is better to be sure the cells are not infected with mycoplasma, as it might interfere with the exosomes.

3.1.6 Standardizing Hyperflask bottles (Corning)

Preparing enough cells for drug treatments requires a lot of culture flasks. Initially we had to use ten 160 ml Nunc culture flasks by Thermo Fisher to split them later on into 30 160 ml Nunc flasks. The limited space of the incubator in use provided a challenge: the large amounts of giant sized flasks required almost all space in the incubator, making it impossible to culture other cell lines simultaneously.

We found a temporary solution to this problem by trying the 10 layered Hyperflask by Corning. This flask took the space of one 160 ml Nunc flask and allowed us to incubate several cell lines at the same time.

I was able to standardize a handling protocol for the Hyperflask. During this work we were able to grow successfully JIMT-1 and SKBR3 cells.

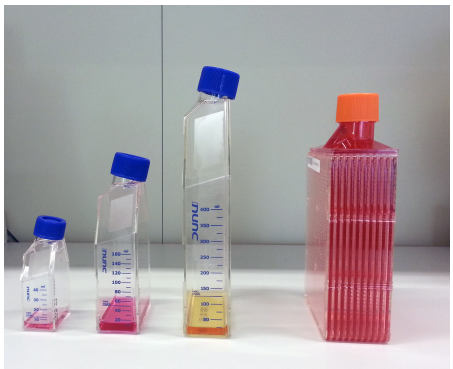


Figure 8. Comparison of cell culture flask sizes. Far right is the Hyperflask.

3.1.7 Treating the cell with T-DM1/trastuzumab for exosome preparations

One of the most important works during this thesis was harvesting exosomes from trastuzumab/ T-DM1/ PBS treated cells. The exosomes will be used later on in this study.

The confluence of the cell culture has to be ~70% before they can be treated with the drugs. Cells are washed with PBS for 3 times to make sure the growing medium is gone. Then the cells are incubated for 5 minutes in ice cold PBS. This is to stop the cells from internalizing the drugs right away. The cells are treated with trastuzumab or T-DM1 and incubated for 20 minutes.

The cells are once again washed three times with PBS and incubated in ice cold PBS for 5 min. Since we planned to harvest exosomes from the treated cell cultures, we added exosome depleted medium to make sure the vesicles harvested from the medium were originated from the cells. The cells are incubated for 24 hours.

3.1.8 Collecting exosomes

When collecting exosomes from cell cultures, you have to keep in mind that we are collecting extremely tiny particles (<100 nm). Cells secrete exosomes to their surroundings and can be harvest directly from the culturing media.

To make sure the collected nanovesicles are solely from the cells, it is important to culture cells in an exosome depleted media. FBS is often used to supplement culturing media as a nutrient to the cells. Despite of being filtered, commercially available FBS contains a lot of EV's that would interfere with the exosomes harvested for experiments. (Shelke – Lässer – Gho – Lötvall 2014.) There is commercially available exosome free FBS, but it is possible to prepare it in the laboratory as well. First a 20% FBS, 1% PS-Glut medium is prepared. The media is then ultracentrifuged overnight in 100 000 x g and 4°C for 18h. After centrifugation, the media is collected. Avoiding the formed pellet in the tubes, the exosome depleted medium is collected to a container. Then, through a 0,4 um sterile filter, the media can be aliquot for later use.
In purposes of exosome harvest, the cells were incubated in exosome depleted medium at least for 3 days.

3.2 Purification

Exosomes are harvested from cultured medium of cancer cells. The best way to obtain large amount of exosomes is by using a series of centrifugation and ultracentrigation. Ultrafiltration is also used to purify large amounts of exosomes. Exosomes can also be purified by trapping them on beads bearing an antibody specific for exosomal surface molecules. This easy to use method is and can be used to characterize exosomes roughly, but it is not suited for purification of large amounts of exosomes. In this project exosomes will be acquired by ultracentrifugation. (Théry 2006.)

3.2.1 Used protocol

The centrifugation protocol used in this study is modified version from the one described in the publication “Isolation and characterization of exosomes from cell culture supernatants and biological fluid, 2006” by Théry and al. We modified mainly the centrifugation times mentioned in the article.

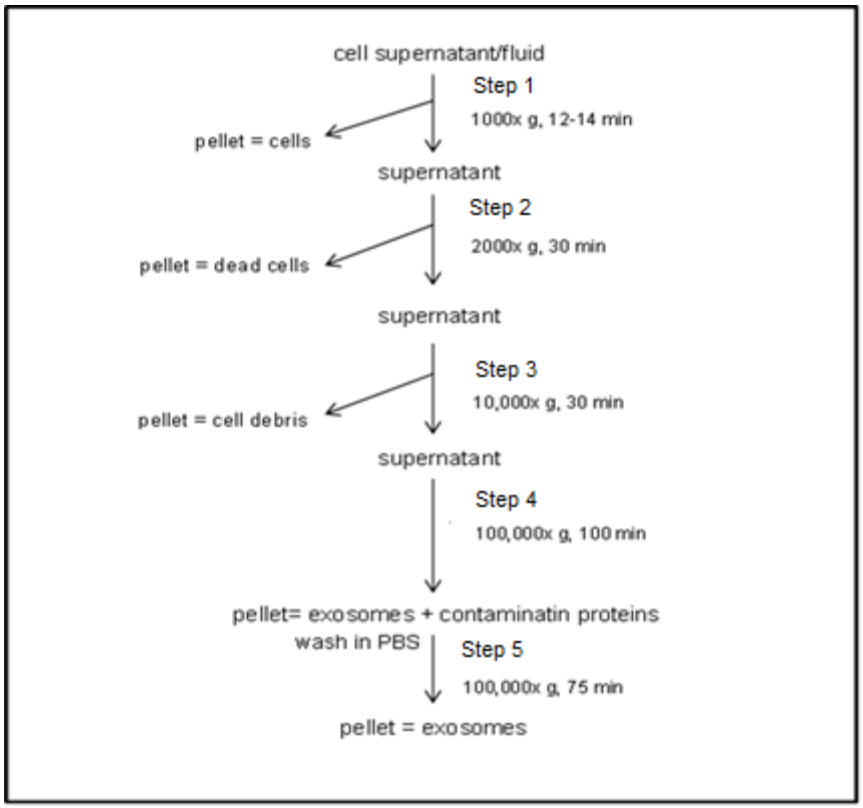


Figure 9. Flow chart of centrifugation. All centrifugation are done at 4°C

The flow chart provides the protocols used to purify the exosomes from the harvested media. In steps 1-3 the supernatant is centrifuged and the pellet discarded. In steps 4-5 the pellet is kept and suspended in a large volume of PBS. The purified pellet is suspended in 400 µl of PBS and thawed into Eppendorf Lo-Bind tubes. The samples are stored in -80 °C.

Table 3. Centrifuges and models used in exosome purification

Centrifuge steps	Centrifuge model	Company	Rotor type	Bottle type
1-2	Varifuge 3.0R	Heraeus Sepatech	8080 C	Conical tubes 50 ml (Falcon)
3	7780	Kubota	AG-6512C	
4-5	XL 90 Ultracentrifuge / Optima XL-80 Ultracentrifuge	Beckman Coulter	50.2 Ti	Polycarbonate tubes 26.3 ml (Beckman Coulter)

Appendix 3 provides the ultracentrifuge protocol after the three first centrifugation steps.

3.2.2 Can 2 ultracentrifuges remove unbound T-DM1?

Protein quantitation assay provides information on how the T-DM1 behaves during ultracentrifugation. During this thesis we used Easy- Titer™ Human IgG (H+L) Assay Kit by Thermo Scientific. This kit was used in a couple of experiments to measure the IgG concentrations of samples. The kit contains monodispersed polystyrene beads coated with anti-IgG antibodies. When the beads are mixed with a sample containing IgG, they aggregate and cause decreased absorption of light. This means that low IgG concentrations yield high absorbance values and high IgG concentrations yield low absorbance values. (Easy-Titer IgG Assay Kits 2015.)

The objective of this experiment was to confirm that two ultracentrifugation steps are enough to remove the unbound T-DM1 from the samples.

T-DM1 was added from the store stock into PBS to a final concentration of 50 µg/ml, 10 µg/ ml and 1 µg/ ml. The tubes went through the same two ultracentrifugation steps that were done when extracting exosomes from harvested media. A small 100 µl sample was taken before ultracentrifugation and after ultracentrifugation. The exact protocol for this experiment can be seen in Appendix 4.

The T-DM1 concentration was measured with Easy-Titer™ kit according to the manufactures' instructions. The absorbance at 405 nm was measured by using Multiskan EX by Thermo Electron Corporation.

3.3 Characterization

Identification and characterization of exosomes requires morphological analysis. Characterization involves different methods that reveal different properties of exosomes. (Thèry 2006.) In this project, the characterization will be done using flow cytometry.

Flow cytometry is a technology that simultaneously measures and analyzes multiple physical characteristics of single particles, usually cells. The properties measured are the particles relative size, granularity or internal complexity and fluorescence intensity. The characteristics are measured using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser lights and emits fluorescence. (Introduction to Flow Cytometry 2000.) In the following explained experiments we used Accuri C6 Flow Cytometer.

3.3.1 Measurement of the HER2 level of cells

This experiment was done to test T-DM1 binding to cells. SKBR3, SNU-216 and TIME cells were collected and sorted into test tubes. JIMT-1 cells were left from this test, as M. Barok had done this experiment to this cell line in his previous studies (Barok 2011). The labeling was done with two antibodies. T-DM1 was used as the primary antibody and binds only to HER2 receptors. Alexa-488-GAHIG (Goat Anti-human IgG) was used as the secondary antibody. Alexa 488 is a fluorescent dye conjugated to GAHIG. Since the trastuzumab part of T-DM1 is a human IgG, GAHIG binds to T-DM1.

T-DM1 binds with high affinity to the HER2 receptors of the cancer cells. To decrease the internalization of T-DM1 by the cells, the labeling has to be done on ice. The exact used protocol can be seen in Appendix 1.

3.3.2 Measuring T-DM1 from SNU-216 exosomes

In this experiment exosomes were collected from the SNU-216- cell cultures that were treated with 1 µg/ml of trastuzumab or T-DM1. The culture medium was changed to exosome-depleted medium, then drugs were added to the cells followed by a 24 h incubation of the cells at 37 ºC and 5 % CO_2.

Exosomes are too small to be reliably analyzed by direct cell sorting. To overcome this problem, exosomes are bound to latex beads of a size that is in the detection range of a flow cytometer. After a series of washing and centrifuging the samples were labeled with primary antibodies: T-DM1, Alexa-647-anti-CD63 and Alexa-647-isotype control. Alexa-647 is the fluorescent dye conjugated to the antibody against CD63. Alexa-488-GAHIG was used as the secondary antibody, which should bind into T-DM1. To detect the fluorescent intensities we used FL-1 and FL-4 channels on the flow cytometer. Fl-1 was used for detecting the fluorescent intensity of Alexa-488 and FL-4 used for detecting the fluorescent intensity of Alexa-647.

Table 4. Used primary antibodies and their description

Primary Antibody	Produced in	Properties
A647-anti-CD63 (BD Pharmigen, material number: 561983)	Mouse	Targets to CD63. Exosome marker.
A647-isotype control (BD Pharmigen, material number: 557783)	Mouse	Negative control. Designed to measure non-specific background signal caused by the primary antibody. No specificity for target protein (Bioss Antibodies 2015).
T-DM1	Trastuzumab part (T) is a humanized IgG	Used to label HER2- sites.

Table 5. Used secondary antibody and its description

Secondary Antibody	Produced in	Properties
A488-GAHIG (Jackson Immunoresearch, 109-606-088)	Goat	Binds to human IgG

3.3.3 Measuring T-DM1 from SKBR3 exosomes

This experiment was very similar to the one made with SNU-216 cells. The exosome preparation from the SKBR3 cells, the labeling protocol and the guiding questions were the same as the experiment done with the SNU-216 cells. The cells were treated with 1 µg/ml of trastuzumab or T-DM1. The treatment protocol can be seen in Appendix 2.

Due to the preliminary results of this experiment, the test was done twice. The second time we used three other exosome markers and another secondary antibody, Alexa-488-GAMIG, as well. The labeling protocol and the flow cytometry channels were the same as in the first round.

Table 6. Additional primary antibodies used in this experiment.

Primary Antibody	Produced in	Properties
FITC-anti-CD81 (Sigma-Aldrich, SAB4700233)	Mouse	Reacts with CD81, a member of tetraspanin family, expressed in the majority of cells (Sigma-Aldrich 2015). Can be used as an exosome marker. FITC is the fluorescent dye, detectable in FL-1.
Anti-CD9 (Sigma-Aldrich, SAB4700092)	Mouse	Targets to CD9. Exosome marker.
Anti-TSG101 (Sigma-Aldrich, SAB2702167)	Mouse	Binds into ubiquitinated cargo proteins. Is required for the sorting of endocytic ubiquitinated cargos into multivesicular bodies (MVBs). (Abcam 2015.) Can be used as an exosome marker.

Table 7. Secondary antibodies and their properties

Secondary Antibody	Produced in	Properties
A488-GAHIG (Jackson Immunoresearch, 109-606-088)	Goat	Binds to human IgG
A488-GAMIG (Jackson Immunoresearch, 115-546-062)	Goat	Binds to mouse IgG

3.4 Electron microscope

Electron microscopes use a beam of charged particles instead of light and use electromagnetic or electrostatic lenses to focus the particles. The resolution is quite astonishing comparing to light microscopies as they can see features as small as tenth of a nanometer. This kind of resolution is enough to visualize exosomes with great detail. There are different kinds of electron microscopes, each using a different method to visualize samples. (FEI Company 2010:4.)

Exosomes can be visualized by using the electron microscope; samples were negative stained or immunolabeled with exosome marker anti-CD63, and then with secondary antibodies anti-mouse IgG and anti-human IgG. Anti-mouse IgG was used against anti-CD63 antibody, since anti-CD63 was produce in a mouse. Anti-human IgG is against the trastuzumab or T-DM1, since these are human IgGs. The secondary antibodies were gold-conjugated.

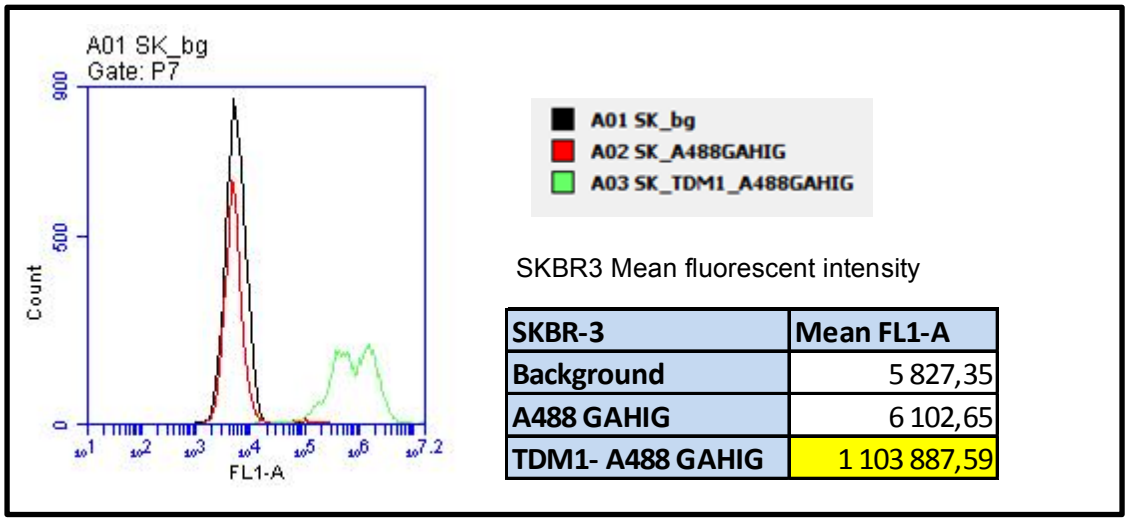
Negative staining was done to investigate the size and morphology of the vesicles while immunostaining was done to examine whether the vesicles have exosome markers and whether trastuzumab and T-DM1 is bound to exosomes.

The exosomes were pictured using a transmission electron microscopy Jeol JEM-1400 (Jeol Ltd., Tokyo, Japan). This EM's magnification range goes from x50 to x800000. EM-work was carried out with the assistance of Ph.D. Maija Puhka and with the help of the EM-Unit resources (Electron Microscopy Unit, Institute of Biotechnology, University of Helsinki).

4 Results

4.1 Flow cytometry results of HER2 levels on the cells

The results of this flow cytometry test were analyzed by using the Accuri sofware. The histograms are one of the many formats in which flow cytometry results can be presented. The horizontal axis represents the fluorescence intensity (FI) detected in the appropriate channel (FI of FITC and Alexa-488 was detected in FL-1, FI of Alexa-647 was detected in FL-4) and the vertical axis represents the detected events.

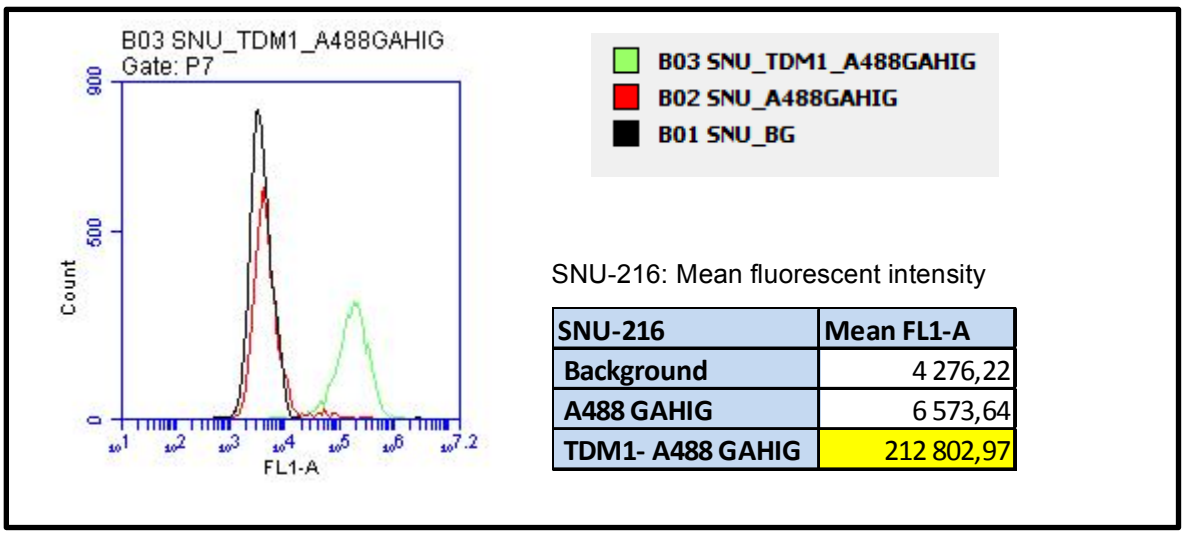


SKBR3 Mean fluorescent intensity

SKBR-3	Mean FL1-A
Background	5 827,35
A488 GAHIG	6 102,65
TDM1- A488 GAHIG	1 103 887,59

Figure 10. SKBR3 data.

The high fluorescent levels of T-DM1- A488 GAHIG confirmed that T-DM1 does bind with high affinity to the SKBR3 cells, meaning there are HER2 receptors on the cells. In the histogram the mean fluorescent intensity of Alexa488-GAHIG is a bit higher than the background's fluorescent level (see the table above). This means there is some binding of Alexa488- GAHIG to the cells that were not labeled with T-DM1 before. This is the unspecific binding to the cells and provides a background to the T-DM1 – Alexa488-GAHIG labeled cells. The green T-DM1– Alexa488-GAHIG is detected further in the horizontal axis, meaning there is T-DM1 binding.

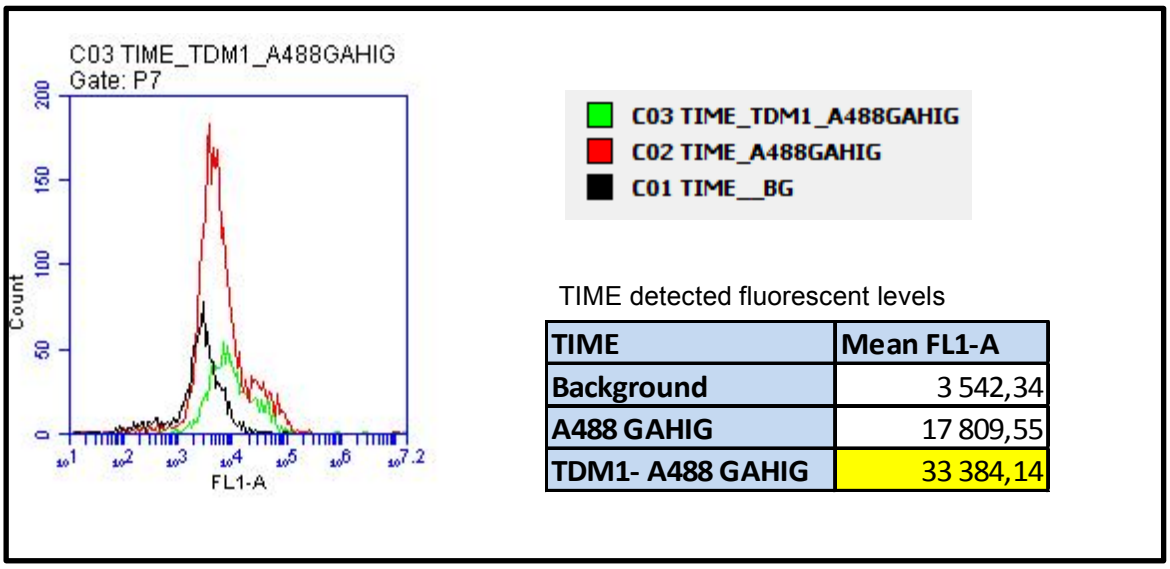


SNU-216: Mean fluorescent intensity

SNU-216	Mean FL1-A
Background	4 276,22
A488 GAHIG	6 573,64
TDM1- A488 GAHIG	212 802,97

Figure 11. SNU-216 data.

The mean fluorescent of T-DM1 – Alexa488-GAHIG labeled SNU-216 cells is lower than that of SKBR3 cells (212 802, 1 103 887, respectively). This indicates that SNU-216 cells have lower HER2 levels than SKBR3 cells. Again, the histogram is similar to the one of the SKBR3-cells.



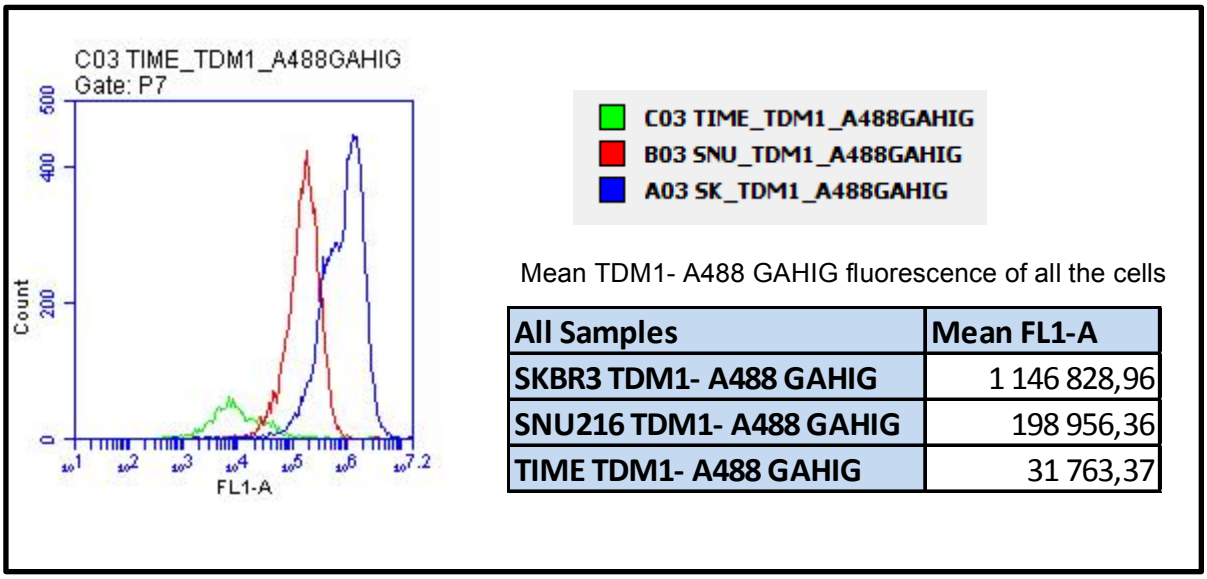
TIME detected fluorescent levels

TIME	Mean FL1-A
Background	3 542,34
A488 GAHIG	17 809,55
TDM1- A488 GAHIG	33 384,14

Figure 12. TIME data.

Surprisingly the TIME cells also bind some T-DM1. As seen from the table above, the mean FL1-A of T-DM1- A488- GAHIG is higher than that of A488- GAHIG (17 809.55, 33 384. 14, respectively).

This can be explained by the nature of the TIME cells. TIME cells were established from the foreskin epithelia cells of a neonatal. It is described in literature that neonatal cells express Fc receptors, also known as the Brambell receptor. Its function is evident in early life in the transport of IgG from the mother to fetus and neonate for passive immunity (Kou 2010: 777). Since T-DM1 is essentially an IgG with a drug attached to it, it is possible that it binds to the Fc receptors as well. Other possible explanation is that TIME cells do express HER2 receptor after all, even though insignificantly comparing to the other cell lines (33 384.14, 1 103 887.59 respectively from the SKBR3 data table).



Mean TDM1- A488 GAHIG fluorescence of all the cells

All Samples	Mean FL1-A
SKBR3 TDM1- A488 GAHIG	1 146 828,96
SNU216 TDM1- A488 GAHIG	198 956,36
TIME TDM1- A488 GAHIG	31 763,37

Figure 13. Conclusive data.

The conclusive table shows all the histograms and mean fluorescent intensities from this experiment. As it was concluded earlier, the SKBR3 cells bind the most T-DM1 and thus have the highest HER2 receptor level. The SNU-216 cells also have HER2 receptors, although the HER2 receptor expression is less compared to SKBR3 cells (see the table above). In comparison TIME cells do binds T-DM1 only a little. The histogram concludes the same thing: the shift in horizontal FL1-A axis means shows that HER2 of SKBR3 is higher than SNU-216 and TIME. The further the histogram stands in the axis, the more the measured sample has HER2.

4.2 FC results of T-DM1 from SNU-216 exosomes

In the following chapters the results of this experiment will be discussed by using charts and tables containing the numeric values of the immunolabeled exosomes.

The results of this flow cytometry test were analyzed by using the Accuri software. Charts were made using Excel.

4.2.1 Exosomes harvested from PBS treated SNU-216 cell

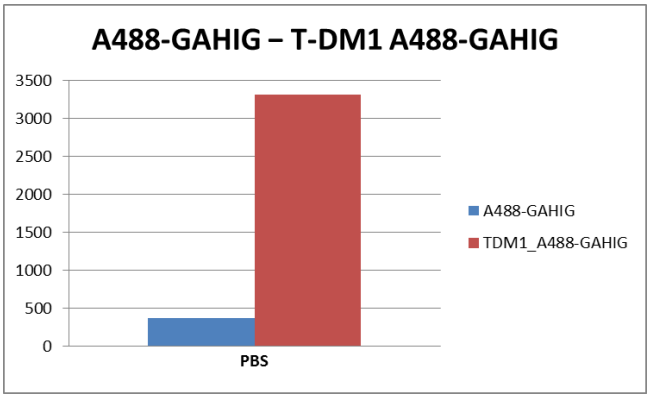


Figure 14. T-DM1 levels.

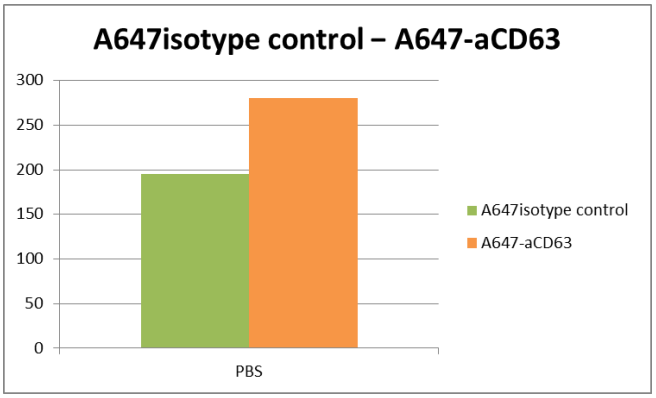


Figure 15. Exosome marker.

Table 8. Exosomes harvested from PBS-treated SNU-216, numeric values

		Mean FL1-A	Mean FL4-A
Exosomes prepared from PBS-treated SNU-216 cells	Bg	257,01	196,03
	A647isotype control	256,48	**195,16**
	A488-GAHIG	**368,92**	195,69
	A647-aCD63	256,93	**280,03**
	A647-aCD63_A488-GAHIG	294,71	254,78
	TDM1_A488-GAHIG	**3 308,50**	194,01
	A647-aCD63_TDM1_A488-GAHIG	2 304,14	249,4

T-DM1 binds only to the HER2-receptors. Alexa488-GAHIG binds to T-DM1. Thus, the exosomes treated only with Alexa 488-GAHIG, would provide a background on how the secondary antibody behaves, while the exosomes labeled with both T-DM1 and Alexa488-GAHIG reveals the HER2-levels of the exosomes.

The same goes with the Alexa 647 isotype control: as mentioned earlier the A647 isotype control is designed to measure non-specific background signal caused by the primary antibody and has no specificity for target protein. It sets the background from which the exosome marker anti CD63 can be compared to

In figure 14 low fluorescent levels of A488-GAHIG indicate that there is no T-DM1 in the sample. It is only natural, as exosomes that were derived from the PBS treated cells should not have T-DM1 at all on their surface. High fluorescent levels of T-DM1 − A488-GAHIG means there are HER2- receptors on the exosomes. In figure 14 we can see that there is some CD63 binding in the samples, indicating that the vesicles in the sample are probably exosomes. The difference between Alexa647-isotype control and Alexa647 – antiCD63 is not very high though.

4.2.2 Exosomes harvested from trastuzumab treated SNU-216 cells

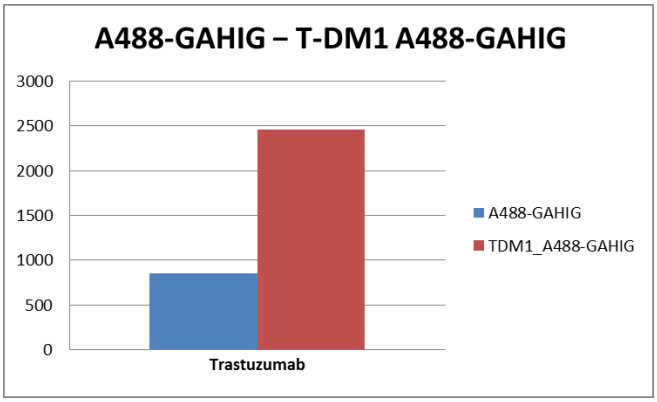


Figure 16. T-DM1 levels.

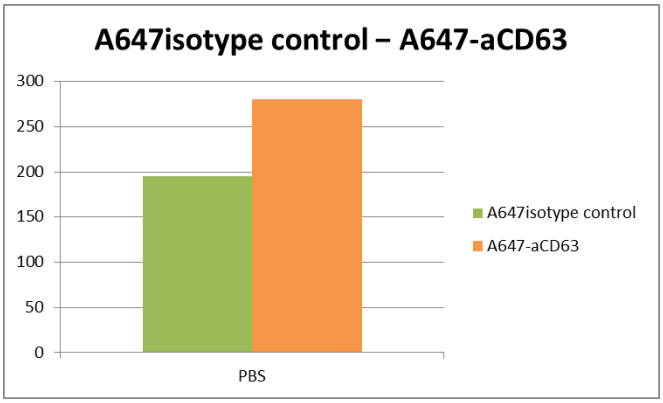


Figure 17. Exosome marker.

Table 9. Exosomes harvested from trastuzumab-treated SNU-216 cells, numeric values

		Mean FL1-A	Mean FL4-A
Exosomes prepared from trastuzumab-treated SNU-216 cells	Bg	251,68	179,09
	A647isotype control	252,14	**174,42**
	A488-GAHIG	**849,22**	178,52
	A647-aCD63	252,82	**507,3**
	A647-aCD63_A488-GAHIG	1083,54	495,43
	TDM1_A488-GAHIG	**2458,04**	177,97
	A647-aCD63_TDM1_A488-GAHIG	2223,62	512,63

In figure 16 we can see that the level of Alexa488-GAHIG in this sample is higher than the one on the PBS treated. This supports the hypothesis that there is trastuzumab on the surface of the exosomes. After labeling the exosomes with both T-DM1 and Alexa 488-GAHIG, the fluorescent levels rise. This indicates that there are unsaturated, free binding sites for HER2 receptors on the surface of the exosomes.

4.2.3 Exosomes harvested from T-DM1 treated SNU-216 cells

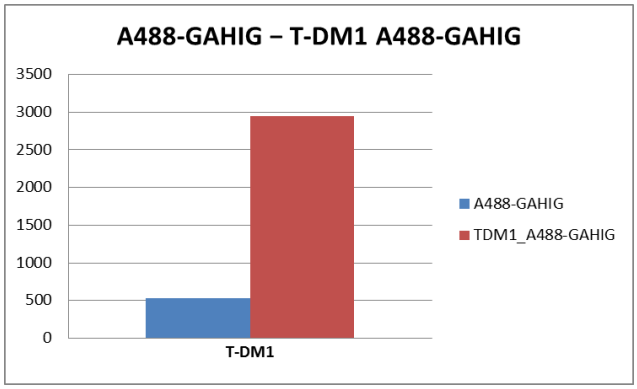


Figure 18. T-DM1 levels.

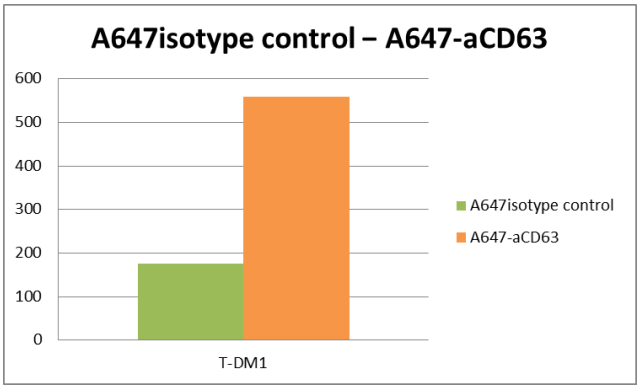


Figure 19. Exosome marker.

Table 10. Exosomes harvested from T-DM1- treated SNU-216 cells, numeric values

		Mean FL1-A	Mean FL4-A
Exosomes prepared from TDM1-treated SNU-216 cells	Bg	261,22	174,32
	A647isotype control	257,2	**174,86**
	A488-GAHIG	**525,07**	174,08
	A647-aCD63	248,81	**559,56**
	A647-aCD63_A488-GAHIG	496,16	599,75
	TDM1_A488-GAHIG	**2943,29**	176,21
	A647-aCD63_TDM1_A488-GAHIG	2 780,22	487,65

The vesicles were positive for an exosome marker (CD63), in order to get a confirmation that the measured particles are truly exosomes, the samples must be tested further with different exosome markers or by visualizing them with electron microscopy.

4.2.4 Conclusive data on SNU-216 exosomes

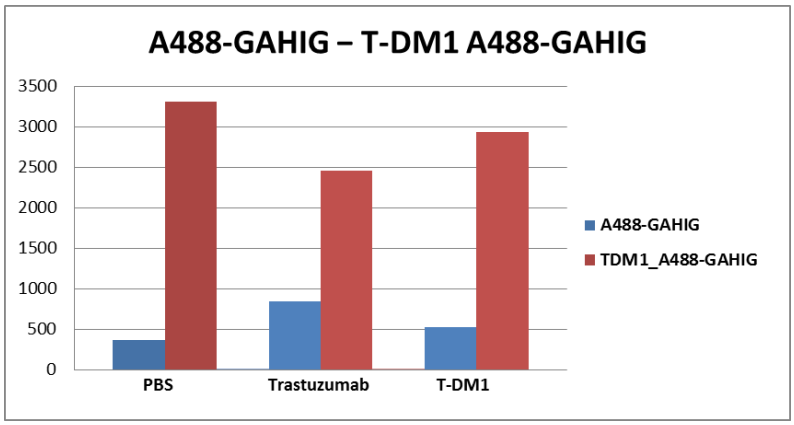


Figure 20. T-DM1 levels, conclusive chart.

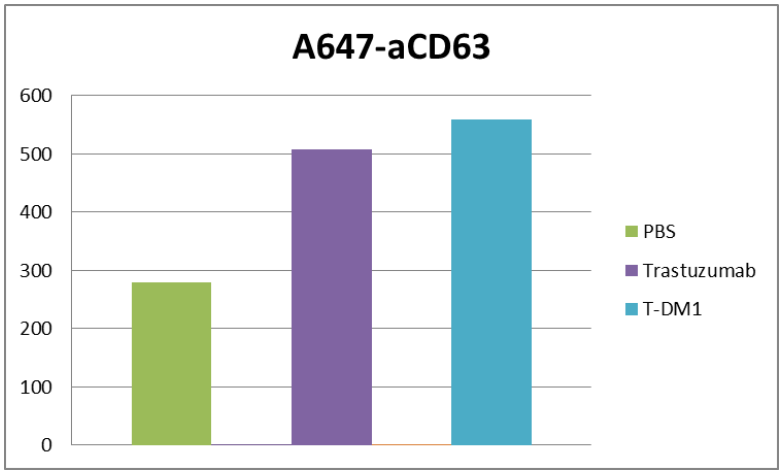


Figure 21. Exosome markers, conclusive chart.

In figure 20 we can see the comparison between A488- GAHIG labeling and T-DM1-A488-GAHIG labeling. Exosomes harvested from the trastuzumab and T-DM1 treated cell labeled with A488 GAHIG have a higher fluorescent level than in case exosomes from PBS-treated cells: this indicates that these exosomes have trastuzumab and T-DM1 on their surface (368 – 849 – 525 from the tables 8-10). Interestingly the amount of saturated HER2-receptors is initially higher in the trastuzumab treated cells, while the PBS and T-DM1 treated have very similar amounts (compare the blue bars). In the case of all the three treatments we can see also a high amount of free unsaturated HER2 receptors that can bind significant amounts of T-DM1, which can be seen as the difference between the red and blue bars.

Figure 21 provides a comparison between the detected exosome marker CD63 in the samples. This might indicate that the trastuzumab and T-DM1 treatment can increase the CD63 level of the exosomes.

4.3 FC results of exosomes harvested from PBS/trastuzumab/T-DM1-treated SKBR3 cells

Surprisingly the results in this test were for the part of the exosomes very different to the results of SNU-216 cells. We did not get any significant increase in FL-4 channels when measuring A647-aCD63 sample, as the SNU-216 cells established.
In other words, the particles we measured might not be exosomes. To test this we labeled the samples with other known exosome markers.

On the other hand, we did get a high increase in TDM1-A488GAHIG, which means there is clear T-DM1 binding.

4.3.1 Results from the first testing

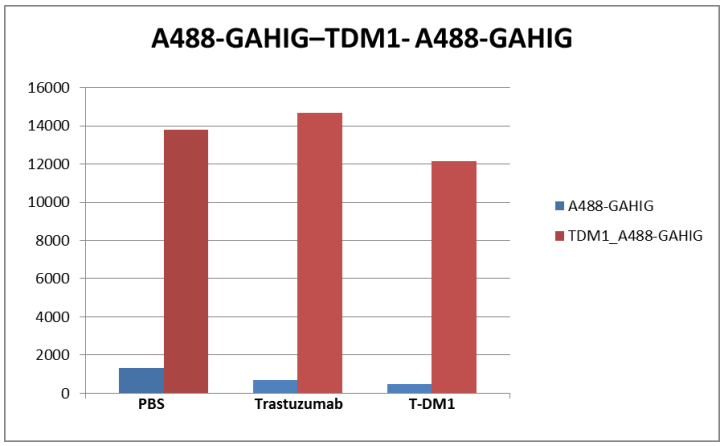


Figure 22. T-DM1 levels, conclusive chart

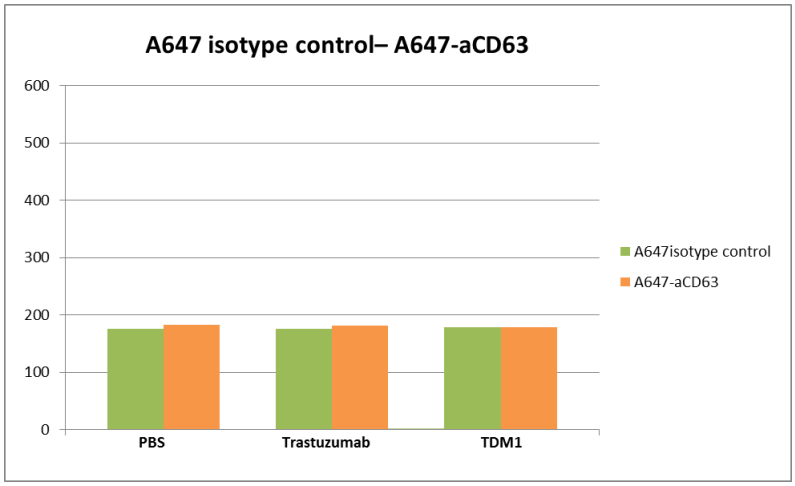


Figure 23. Exosome marker, conclusive data

Figures 22-23 shows the results from the exosomes only labeled with the same primary and secondary antibodies as the SNU-216 exosomes in a conclusive chart.

In figure 22 we can see that the T-DM1 binding follows pretty much the same pattern established by the SNU-216 cells. The blue bar shows Alexa488-GAHIG binding while the red shows the T-DM1 – Alexa488-GAHIG. The comparison between the two of them shows the amount of free T-DM1 binding sites in the exosomes.

As it can be seen in figure 23, the exosome marker CD63 does not react the same way as with the exosomes from SNU-216 cells. Both green and orange bars stay in the same level.

Table 11. Exosomes harvested from PBS treated SKBR3 cells, numeric values

		Mean FL1-A	Mean FL4-A
Exosomes prepared from PBS treated sKBR3 cells	Bg	262,82	175,6
	A647isotype control	409,17	**176,29**
	A488-GAHIG	**1300,19**	180,03
	A647-aCD63	376,81	**182,83**
	A647-aCD63_A488-GAHIG	322,87	177,72
	TDM1_A488-GAHIG	**13784,73**	176,8
	A647-aCD63_TDM1_A488-GAHIG		179,27

Table 12. Exosomes harvested from trastuzumab treated SKBR3 cells, numeric values

		Mean FL1-A	Mean FL4-A
Exosomes prepared from trastuzumab treated SKBR3 cells	Bg	252,43	176,09
	A647isotype control	289,05	**175,88**
	A488-GAHIG	**709,38**	176,25
	A647-aCD63	277,03	**181,63**
	A647-aCD63_A488-GAHIG	620,44	176,98
	TDM1_A488-GAHIG	**14 673,95**	176,72
	A647-aCD63_TDM1_A488-GAHIG	11 273,29	176,72

Table 13. Exosomes harvested from T-DM1 treated SKBR3 cells, numeric values

		Mean FL1-A	Mean FL4-A
Exosomes prepared from TDM1 treated SKBR3 cells	Bg	269,87	175,8
	A647isotype control	325,25	**178,09**
	A488-GAHIG	**489,76**	178,51
	A647-aCD63	294,38	**178,41**
	A647-aCD63_A488-GAHIG	533,82	175,24
	TDM1_A488-GAHIG	**12 140,82**	176,51
	A647-aCD63_TDM1_A488-GAHIG	9 293,41	178,31

4.3.2 Exosomes harvested from PBS-treated SKBR3 cells

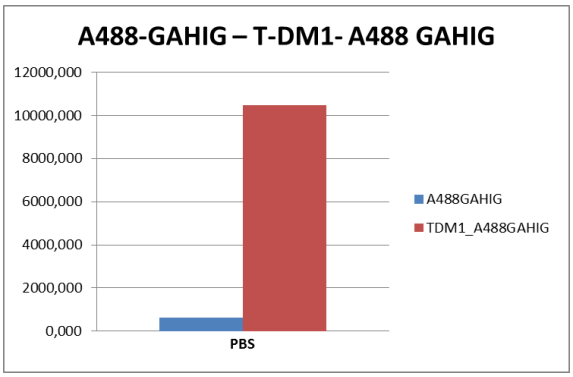


Figure 24. T-DM1 levels

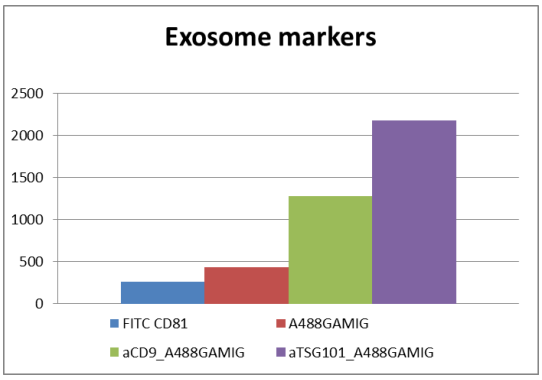


Figure 25. Exosome markers

In figure 24 we can see, what the SNU-216 cells already stated: the exosomes from the PBS treated cells did not have T-DM1 on their surface. In figure 25 we can see the different exosome markers used in this test. The samples were positive for TSG101 exosome marker as well as they showed some CD9 positivity. Fluorescent levels of both FITC and Alexa-488 were detected in the FL-1 channel of the flow cytometer.

Table 14. Exosomes harvested from PBS treated SKBR3 numeric values

		Mean FL1-A
Exosomes preapared from PBS treated SKBR3 cells	BG	255,49
	A488GAHIG	613,600
	TDM1_A488GAHIG	10473,160
	FITC CD81	268,23
	A488GAMIG	779,34
	aCD9_A488GAMIG	790,68
	aTSG101_A488GAMIG	2851,22

4.3.3 Exosomes harvested from trastuzumab treated SKBR3 cells

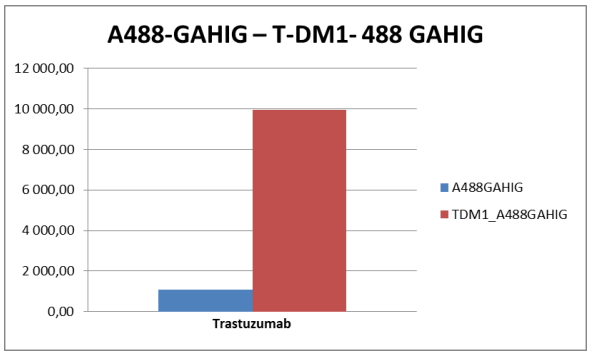


Figure 26. T-DM1 levels

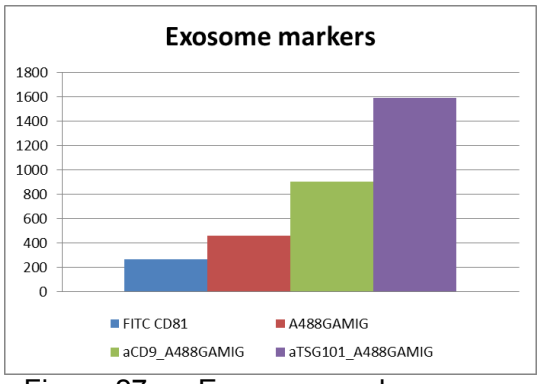


Figure 27. Exosome markers

The exosomes harvested from the trastuzumab treated cells followed the same pattern as the exosomes harvested from the PBS treated cells. These exosomes showed slight CD9 positivity and a very high TSG101 positivity.

Table 15. Exosomes harvested form trastuzumab treated SKBR3 cells, numeric values

		Mean FL1-A
Exosomes preapared from Trastuzumab treated SKBR3 cells	BG	332,34
	A488GAHIG	1 088,60
	TDM1_A488GAHIG	9 959,79
	FITC CD81	267,67
	A488GAMIG	458,05
	aCD9_A488GAMIG	902,96
	aTSG101_A488GAMIG	1 589,38

4.3.4 Exosomes harvested from T-DM1 treated SKBR3

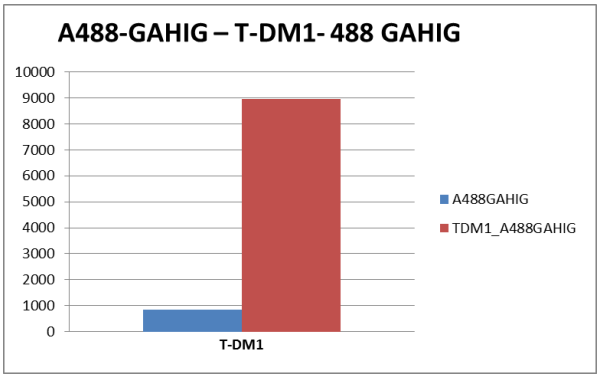


Figure 28. T-DM1 levels

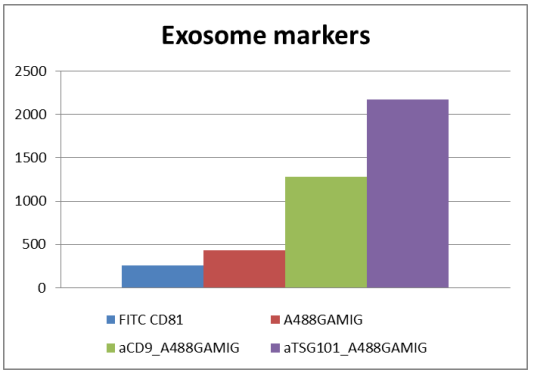


Figure 29. Exosome markers

The exosomes harvested from the T-DM1 treated cells followed the same pattern set by the PBS/trastuzumab treated cells.

Table 16. Exosomes harvested from T-DM1 treated SKBR3, numeric values

		Mean FL1-A
Exosomes preapared from T-DM1 treated SKBR3 cells	BG	255,23
	A488GAHIG	834,07
	TDM1_A488GAHIG	8 961,96
	FITC CD81	256,1
	A488GAMIG	429,63
	aCD9_A488GAMIG	1 278,38
	aTSG101_A488GAMIG	2 174,60

In the case of T-DM1 treated cells, the exosomes also seem to express CD9 marker better than the other treated cells. The TSG101 positivity was very clear in these exosomes as well.

4.3.5 Conclusive data on SKBR3 exosomes

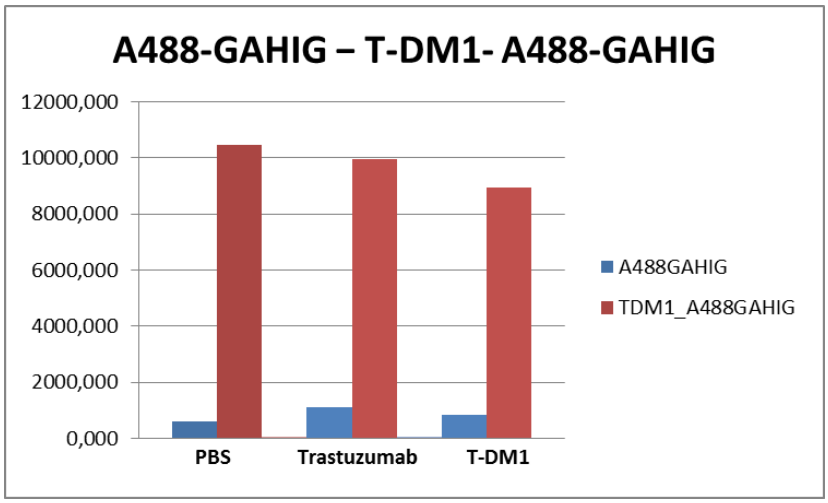


Figure 30. T-DM1 levels, conclusive chart

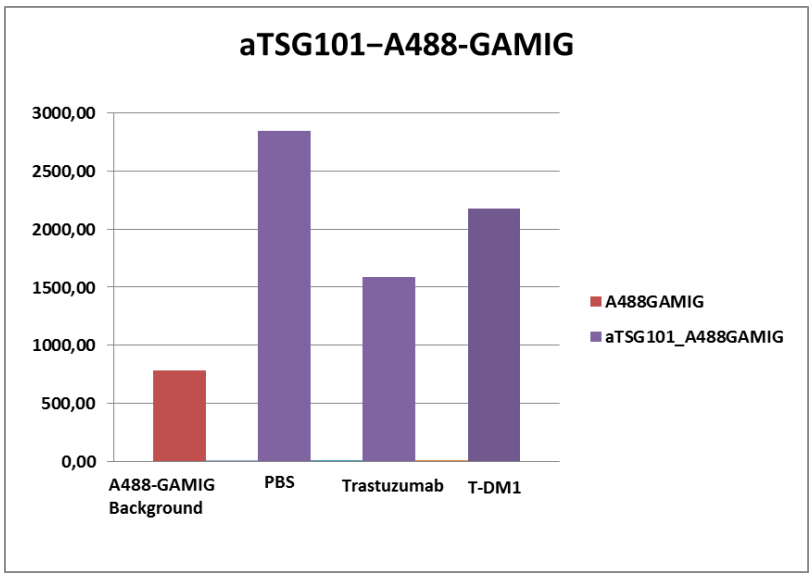


Figure 31. TSG101 levels, conclusive chart

The figures 30-31 conclude the information above and make a comparison between the differently treated cells. As seen in figure 30 the exosomes harvested from the trastuzumab treated SKBR3 cells seem to have the most saturated HER2 receptors (see the blue bars from figure 30).

Figure 31 shows the levels of the exosome marker TSG101 in comparison of the highest Alexa488-GAMIG.

4.4 Results of T-DM1 quantitation assay

The results gave expected results. As it was anticipated, the ultracentrifugation protocol is enough to remove T-DM1 from the sample bellow measurable quantities.

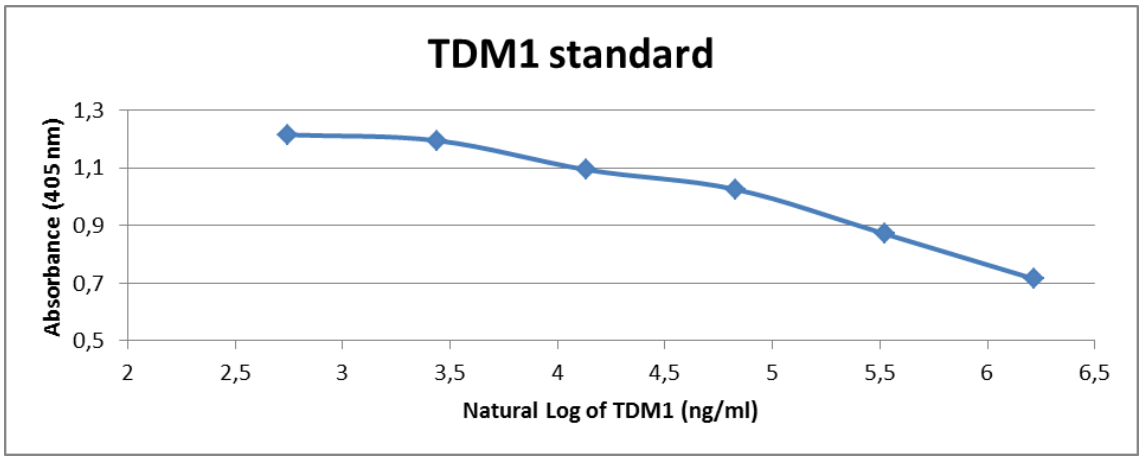


Figure 32. Standard curve for T-DM1.

The standard curve was generated by using the data on table 6.

Table 17. T-DM1 standard curve values

	Mean absorbance	Concentration (ng/ml)	Ln (concentration)
A	**0,715**	500	6,214608098
B	**0,871**	250	5,521460918
C	**1,025333333**	125	4,828313737
D	**1,093666667**	62,5	4,135166557
E	**1,195**	31,2	3,440418095
F	**1,215**	15,6	2,747270914
G	1,275666667	0	

Table 18. Day 1 results

	Mean	Concentration (ng/ml)
D1/1	1,200666667	717,6
D1/2	0,566333333	> 500 ng/ml
D1/3	1,237666667	< 15,6 ng/ml
D1/4	1,240333333	< 15,6 ng/ml
D1/5	1,256666667	< 15,6 ng/ml
D1/6	1,138333333	204,5593985
D1/7	1,108	52,734375
D1/8	1,294	< 15,6 ng/ml
D1/9	1,272666667	< 15,6 ng/ml
D1/10	1,297333333	< 15,6 ng/ml

Table 19. Day 2 results

	Mean	Concentration (ng/ml)
D2/1	1,066	5807,622505
D2/2	0,623	>500 ng/ml
D2/3	1,189	33,3112782
D2/4	1,173666667	38,70676692
D2/5	1,219666667	< 15,6 ng/ml
D2/6	1,240333333	< 15,6 ng/ml
D2/7	0,945333333	195,8874459
D2/8	1,242666667	<15,6 ng/ml
D2/9	1,255333333	< 15,6 ng/ml
D2/10	1,258	< 15,6 ng/ml

Table 20. Day 3 results

	Mean	Concentration (ng/ml)
D3/1	0,565666667	> 500 ng/ml
D3/2	0,777	400,6410256
D3/3	1,305	<15,6 ng/ml
D3/4	1,324333333	< 15,6 ng/ml
D3/5	1,271333333	<15,6 ng/ml
D3/6	0,612666667	> 500 ng/ml
D3/7	0,668333333	< 500 ng/ml
D3/8	1,172666667	38,07631579
D3/9	1,232	< 15,6 ng/ml
D3/10	1,282333333	<15,6 ng/ml

The tables above show the results of this test. This test was done on three separate days. The orange color on the tables shows the concentration of the first samples before the first ultracentrifugation. The green is for the T-DM1 concentration of the samples after the second ultracentrifugation. The detection limits of the used kit ranges from 15,6 ng/ml to 500 ng/ml, as shown in table 17. The labeling of the samples in tables 18-20 means D(day)/sample number. For further information on the samples, refer to appendix 5.

In conclusion the results were every time the same, no matter what the starting concentration was. Samples 1 and 6 showed that there is present T-DM1, after two ultracentrifugations the amount of the T-DM1 in samples 5 and 10 was below the measurable range of the kit.

4.5 Electron microscopy results

Electron microscope was used to see if the particles harvested from SNU-216 cells were truly exosomes. Indeed, the scanning revealed there were nanovesicles fitting the description of exosomes.

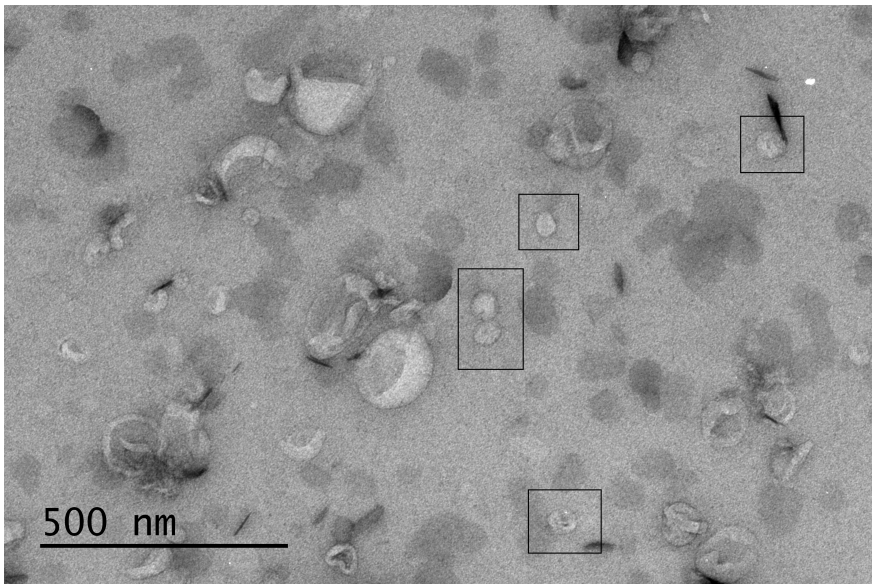


Figure 33. Negative staining of exosomes harvested from PBS treated SNU-216 cells. The vesicles inside boxes are exosomes. (Picture by Mark Barok)

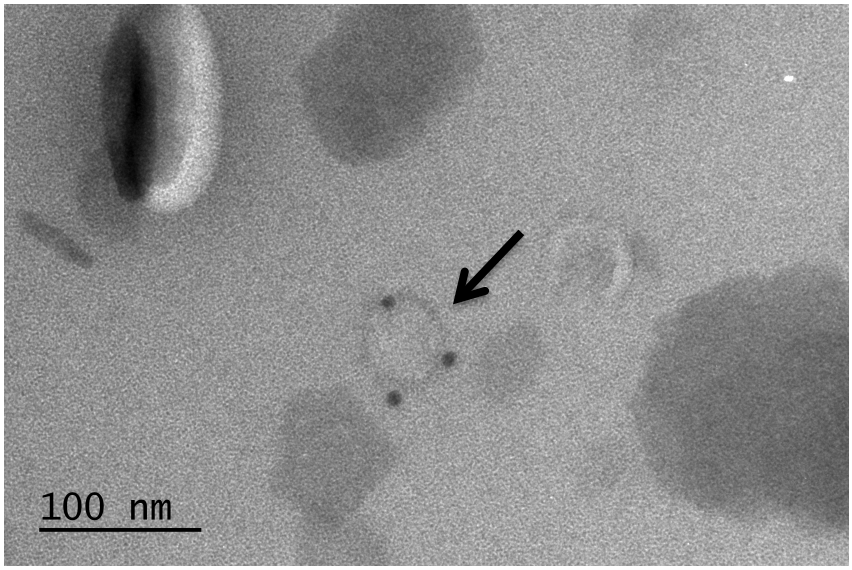


Figure 34. Immunostaining of exosomes harvested from trastuzumab-treated SNU-216 cells. (Picture by Mark Barok)

This sample was immunostained with anti-CD63 antibody, then with the gold conjugated secondary antibody. The dots seen in the surface of the vesicle are from the gold-bound secondary antibody.

5 Discussion

5.1 Permissions and contracts

Thesis topics realized in the Finnish health and social services has to have its permissions in check. When dealing directly with patients, theses will need also a permission from The National Advisory Board on Social Welfare and Health Care Ethics ETENE. The permissions of this project have been taken care of already, since the project has already started, and generally research teams in Biomedicum Helsinki have this things set. The research permission has been applied for from the University of Helsinki. A contract between the research team, the university and me was signed.

5.2 Ethics and morality

There were no contradictions of ethics or morality in this project. There was no patient contact whatsoever in this project and no one's identity was compromised. No animals or humans were harmed. The project was purely technical.

Presenting results as they are, without embellishing them to suit better the needs of the study is one of the most important ethical guidelines of a biomedical scientist. The results presented in this thesis are shown just as we got them.

5.3 Thoughts about the project

I started this project with little knowledge about what exosomes are. As the project went on, I became to understand how complex was this subject. Even though the subject itself exosomes is quite old, its only the recent interest on them that has taken this field forward.

The thesis answered well the scientific questions set in the beginning and more. We were able to get crucial data from the experiments done during the execution of this thesis. More over, we extracted a lot of exosomes for later use in this project. What stroke me the most was how laborious it was to collect and purify exosomes from the starting culture. It took several weeks from the first cultivation steps to the final product.

5.4 About the results

The results on the HER2 levels provided us with the information we did not previously knew for sure. We had the assumption that the TIME cell did not have any HER2 receptors in their surface. Surprisingly, we got some T-DM1 binding in the TIME cells, which were suppose not to bind T-DM1, as it should not have HER2 receptors at all. After doing some research, this was explainable with the neonatal Fc-receptors.

The flow cytometry experiment results on the exosomes collected form the PBS/trastuzumab/T-DM1 treated SNU-216 and SKBR3 cells were quite informative and gave us some preliminary results on the composition of the exosomes derived from the treated cells. It was surprising to see how differently the exosomes were from one cell line to another. The experiments proved that exosomes do vary a lot depending where they were originally secreted from. These experiments will be continued in the future by increasing the concentration of trastuzumab/T-DM1 the cells will be exposed to.

To conclude very briefly the results of this thesis regarding to the guiding scientific questions asked in chapter 1:

- Is there exosomes in the samples collected from cell cultures? Yes, there was exosomes in the samples collected from the cell cultures.

- Is there HER2 expression in exosomes? Yes, the T-DM1- Alexa488- GAHIG binding in the flow cytometry experiments proved that.

- Is there T-DM1 on the surface of the exosomes prepared from T-DM1-treated cancer cells? Yes, there is, but not as much as it was presumed before testing the exosomes.

5.5 Reliability of the results

There is a lot of variables in this project, the biggest being cell cultures. We followed the different aseptic methods strictly to avoid any kind of contamination. Unfortunately, during the moving process of the cell laboratory from one floor to another, we managed to possibly get our cells contaminated. This was a major setback and required a lot of work to reculture the cells in the amounts we had prior contamination.

On the other hand, this was perhaps also a good thing. We got to start from surely clean working areas, cultures and materials. The exosomes extracted during this project were all collected after the great cleanup.

Another major variable was centrifuging. The first three steps were mostly uneventful, but as soon as we moved to the ultracentrifuge phase, things took a tricky turn. The polycarbonate bottles did not survive centrifuging after a couple times of use. They would crack badly and spill some of its contents to the rotor. Mostly the pellet formed in these tubes was salvageable, but it makes think if it has any effect on the subset of exosomes collected from the cracked bottles.

Throughout the different experiments, the strict pipetting protocols were a challenge. As always, pipetting mistakes made by humans are not uncommon. Different people have different ways of pipetting, and that small difference might interfere with the results. Only by repeating the experiments, will the results be accurate enough to be presentable.

5.6 Future

The future of this project is promising. While the experiments answered some questions on the nature of these exosomes, new ones resurged. Some of the future plans will include testing the exosomes on macrophages and eventually even get some exosomes derived from patients with T-DM1/trastuzumab treated breast cancer.

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15/01/2015
Labeling protocol for flow cytometry measurement of the HER2 level of cells

Cells: SKBR-3, SNU-216; TIME
Primary Ab: TDM1, labeling concentration: 50 ug/ml
Secondary Ab: Alexa-488-GAHIG (goat anti human IgG)

TDM1 store stock: 20 mg/ml = 20 000 ug/ml
labeling cc = 50 ug/ml
dilution: 400X
D1=predilution: 50X: 2 ul TDM1 + 198 ul BSA-PBS
then 6,3 ul prediluted TDM1 + 43,7 ul sample

Labeling protocol

1. Remove the supernatant from the bottle containing the cells
2. Wash cells with 5ml PBS
3. Add trypsin to the bottle (3 ml trypsin in case of medium size bottles)
4. Put the bottle back into the thermostat, wait until the cells are up
5. Add cell culture medium (or 10%-FBS-PBS in case of TIME cells) into the bottles to stop trypsin and remove the cells.
6. Put the cells into 50 ml Falcon tubes
7. Centrifuge 1100 rpm, 7 min
8. Remove supernatant, keep the pellet (pellet = cells)
9. Suspend the cells in 1 ml 2%-BSA-PBS
10. Add 10 ml PBS and 10 ml 2%-BSA-PBS into the Falcon tube
11. Centrifuge 1100 rpm, 7 min
12. Remove supernatant, keep the pellet (pellet = cells)
13. Suspend the cells in 150 ul 2%-BSA-PBS

14. Add 43,7 ul cell suspension into 3 sorter tubes, put the tubes into ice, and keep the cells on ice during the whole labeling, use ice cold PBS for washing

    Tube 1: background,
    Tube 2: GAHIG background
    Tube 3: sample

15. Tube 1: add 6,3 ul BSA-PBS, incubation: on ice, 20 min
    Tube 2: add 6,3 ul BSA-PBS, incubation: on ice, 20 min
    Tube 3: add 6,3 ul prediluted TDM1, incubation: on ice, 20 min

16. Wash the samples: add 4 ml PBS into the tubes, centrifuge: 4 C, 1100 rpm, 7 min
17. Discard the supernatant, keep the pellet
18. Wash the samples: add 4 ml BSA-PBS into the tubes, centrifuge: 4 C, 1100 rpm, 7 min
19. Discard the supernatant, keep the pellet

20. Tube 1: add 1,1 ul BSA-PBS, incubation: on ice, 20 min
    Tube 2: add 1,1 ul Alexa-488 GAHIG, incubation: on ice, 20 min
    Tube 3: add 1,1 ul Alexa-488 GAHIG, incubation: on ice, 20 min

After adding the fluorescent dye-conjugated Ab to the cells, keep the samples in a dark place.

21. Wash the samples: add 4 ml PBS into the tubes, centrifuge: 4 C, 1100 rpm, 7 min
22. Discard the supernatant, keep the pellet
23. Wash the samples: add 4 ml PBS into the tubes, centrifuge: 4 C, 1100 rpm, 7 min
24. Discard the supernatant, keep the pellet
25. Suspend the samples in 250 ul 1%-formaldehyde-PBS
26. Keep the samples at 4 C, and a dark place.

NOTE: The protocol was written by Mark Barok

Drug treatment protocol

PBS/tratuzumab/TDM1-treatment of SKBR-3 cells before extracellular vesicule preparation
Treatment: 25-26/2/2015

23_febr	24_febr	25_febr	26_febr	27_febr
M	Tu	W	Th	F
10 multilayers: 30 giants		treatment	medium harvesting	

1.	Plate the cells into **30 giant flasks**	Monday
2.	Wait until confluence reaches ~ 70 %	
3.	Wash the cells with PBS, 3X, ice cold PBS (7 ml), incubation time with PBS: 5 min.	Wednesday
4.	Add 6 ml ice cold PBS containing:	
	a. 10 ug/ml TDM1	
	b. 10 ug/ml trastuzumab	
	c. PBS	
5.	Incubate on ice for 20 min	
6.	Wash the cells with PBS, 3X, ice cold PBS (8 ml), incubation time with PBS: 5 min.	
7.	Add **exosome-deplated medium** to the cells (15 ml)	
8.	Incubate the cells for 24 h	
9.	Harvesting medium for exosome preparation	Friday

10*6 ml PBS = 60 ml

2X (30,25 ml PBS + 15,125 ul TDM1)
2X (30,25 ml PBS + 14,43 ul trastuzumab)

trastuzumab:
intended cc: 10 ug/ml
store stock: 21 000 ug/ml
add 2,857ul TDM1 into 6 ml medium
add 28,57 ul TDM1 into 60 ml medium
add 3,809 ul TDM1 into 8 ml medium
add 38,09 ul TDM1 into 80 ml medium

TDM1:
intended cc: 10 ug/ml
store stock: 20 000 ug/ml
6 ml * 1 ug/ml = 6 ug
20 000 ug/ml * X = 6 ug
X = 0,0003 ml = 0,3 ul
add 3 ul TDM1 into 6 ml medium
add 30 ul TDM1 into 60 ml medium
add 4 ul TDM1 into 8 ml medium
add 40 ul TDM1 into 80 ml medium

27 ul TDM1 (store stock) had been added into 54 ml PBS
25,75 ul trastuzumab (store stock) had been added into 54 ml PBS
27,25 ul TDM1 (store stock) had been added into 54,5 ml PBS
26 ul trastuzumab (store stock) had been added into 54,5 ml PBS

(Protocol written by Mark Barok)

Ultracentrifugation protocol after three first centrifugation steps

Exosome-preparation from PBS/trastuzumab/TDM1-treated JIMT-1 cells – 02/03/2015

Exosome-preparation from PBS/trastuzumab/TDM1-treated SKBR-3 cells – 03/03/2015

1. Three preliminary centrifugations have already done, samples are stored at – 80 C
2. Melt the samples
3. Put the samples into UC tubes
4. Mark the UC tubes
5. First UC: 28 800 RPM, 100 min, 4 C
6. Pour the supernatant, keep the pellet (pellet = exosomes), hold the tube vertically and remove the supernatant from the aperture of the tube with vacuum
7. Resuspend the pellet in 2 ml PBS
8. Transfer all the resuspended pellet from the same treatment into 1 "common" UC tube
    a. 1 "common" tube for the PBS-treated
    b. 1 "common" tube for the t-treated
    c. 1 "common" tube for the TDM1-treated
    d. balance
9. Add 2 ml PBS into the tubes which you emptied, resuspend, transfer the PBS into the "common" tube
10. Add 2 ml PBS into the tubes which you emptied, resuspend, transfer the PBS into the "common" tube
11. Fill up the "common" UC tube with PBS (max volume = 25 ml)
12. Mark UC tubes
13. Balance tube
14. Second UC: 28 800 RPM, 75 min, 4 C
15. Pour the supernatant, keep the pellet (pellet = exosomes), hold the tube vertically and remove the supernatant from the aperture of the tube with vacuum
16. Resuspend the pellet in 400 ul PBS
17. Aliquot the samples into 8 (8 X 50 ul) Protein LoBind Eppendorf Tubes
18. Label the tubes:
    a. exosomes from PBS-treated JIMT-1 cells, date
    b. exosomes from trastuzumab-treated JIMT-1 cells, date
    c. exosomes from TDM1-treated JIMT-1 cells, date
19. Store the Eppendorf tubes at – 80 C.

(Protocol written by Mark Barok)

ANALYSIS OF EXOSOMES BY FACS OF LABELED EXOSOMES BOUND TO BEADS (Note: this protocol is from the paper "Isolation and characterization of exosomes from cell culture supernatants and biological fluid" by Théry and al.)

Materials

Purified exosomes (Basic Protocol 1, step 12; or Basic Protocol 2, step 8; or Basic Protocol 3)
3.9-μm latex beads, surfactant-free aldehyde/sulfate, 4% solids (Interfacial Dynamics 12-4000; *http://www.idclatex.com*)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
PBS/1 M glycine
PBS/0.5% (w/v) BSA
Fluorochrome-conjugated primary or secondary antibodies
Test tube rotator wheel for 1.5-ml microcentrifuge tubes
Microcentrifuge
Flow cytometer (e.g., FACScan; BD)

1. ***To analyze exosomes purified by ultracentrifugation***: Incubate **5 μg** purified exosomes as measured by Bradford assay (Support Protocol 9) with **10 μl** latex beads
15 min at room temperature, in a 1.5-ml microcentrifuge tube.
A nonspecific adsorption of all exosomal proteins to the latex beads occurs in this step.

2. Add **PBS to a final volume of 1 ml**, and **incubate on a test tube rotator wheel 2 hr at room temperature.**
Overnight incubation at 4◦C is also possible.

3. Add **110 μl of 1 M glycine** (i.e., 100 mM final), **mix gently and let stand on the bench at room temperature for 30 min.**
The purpose of this step is to saturate any remaining free binding sites on beads.

4. **Microcentrifuge 3 min at 4000 rpm, room temperature. Remove the supernatant and discard.**

5. **Resuspend the bead pellet in 1 ml PBS/0.5% BSA and microcentrifuge 3 min at 4000 rpm, room temperature. Remove the supernatant and discard.**
If not enough proteins are used to coat beads, the bead pellet may be spread along the side of the tube. In this case, resuspend the beads by flushing the side of the tube with PBS/0.5% (w/v) BSA; the pellet will become visible after the next centrifugation

6. **Resuspend the bead pellet and microcentrifuge as in step 5 to wash <u>two more times</u>.**

7. **Resuspend beads in 0.5 ml PBS/0.5% BSA.**

8. Incubate 10 μl coated beads with **50 μl anti-exosomal protein antibody diluted in PBS/0.5% BSA 30 min at 4◦C.**

9. **Wash twice with 150 μl PBS/0.5% BSA**.
If necessary, incubate with 50 μl secondary antibody diluted in PBS/0.5% BSA 30 min at 4◦C. Wash twice in PBS/BSA, **resuspend in 200 μl of PBS/BSA.**
Use fluorophore-coupled primary antibodies, or noncoupled primary antibodies followed by fluorophore-coupled secondary antibodies. *Always perform negative-control staining on another 10 μl of beads, using an irrelevant isotype-matched primary antibody.*
When analyzing exosomes purified with antibody-coated beads (Basic Protocol 3), use directly conjugated fluorescent antibodies for FACS, rather than antibodies requiring a secondary antibody step.

If the fluorescence signal is weak with antibodies specific for abundant exosomal proteins (e.g., MHC II on dendritic cell exosomes), try to increase the amount of exosomes and/or *decrease the amount of beads* in step 1.

10. **Analyze antibody-stained exosome-coated beads on a flow cytometer.**
Adjust the forward scatter (FSC) and side scatter (SSC) to see both single beads and bead doublets. Gate on both single and doublet beads to analyze fluorescence. Compare fluorescence obtained with specific antibody and with irrelevant isotype control.
When performing flow cytometric analysis of exosome-coated Dynal beads it is important to be aware that these paramagnetic beads may cause blockages in certain flow cytometric instruments. This is due to the use of electro-magnetic valves controlling the fluidics of certain instruments. This may present a problem for some instruments including BD FACScalibur range and BD FACScanto. The authors have never experienced any such problems when using BD FACScan.

Labeling protocol

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3/07/2014

Exosomes were prepared from the supernatant of 42 flasks of SKBR-3 cells; 96h incubation

1. 8 ul beads + 5 ul exosomes + glycine + **resusp in 200 ul BSA-PBS**
2. 8 ul beads + 5 ul exosomes + glycine + **resusp in 195 ul BSA-PBS** + 5 ul A647-isotype control
3. 8 ul beads + 5 ul exosomes + glycine + **resusp in 200 ul BSA-PBS** + 1,2 ul A488-GAHIG
4. 8 ul beads + 5 ul exosomes + glycine + **resusp in 195 ul BSA-PBS** + 5 ul A647-anti-CD63
5. 8 ul beads + 5 ul exosomes + glycine + **resusp in 195 ul BSA-PBS** + 5 ul A647-anti-CD63 + **resusp in 200 ul BSA-PBS** + 1,2 ul A488-GAHIG
6. 8 ul beads + 5 ul exosomes + glycine + **resusp in 175 ul BSA-PBS** + 25 ul TDM1 + **resusp in 200 ul BSA-PBS** + 1,2 ul A488-GAHIG
7. 8 ul beads + 5 ul exosomes + glycine + **resusp in 170 ul BSA-PBS** + (5 ul A647-anti-CD63 + 25 ul TDM1) + **resusp in 200 ul BSA-PBS** + 1,2 ul A488-GAHIG

TDM1 store stock: 20 mg/ml = 20 000 ug/ml
labeling cc = 50 ug/ml
dilution: 400X
D1=predilution: 50X: 1 ul TDM1 + 49 ul BSA-PBS
then 25 ul prediluted TDM1 + 175 ul sample

T-DM1 quantity assay ultracentrifugation protocol

1. day

TDM1 concentration: 10 ug/ml	
Add 10 ml PBS into a UC tube	
Add 5 ul TDM1 in to 10 ml	**Sample 1**
Do the 1. UC	
Take 100 ul sample from the supernatant	**Sample 2**
Discard the supernatant	
Resuspend in 10 ml PBS	
Take 100 ul sample	**Sample 3**
Do the 2. UC	
Take 100 ul sample from the supernatant	**Sample 4**
Discard the supernatant	
Resuspend in 10 ml PBS	**Samle 5**
Store the samples at - 80 C	

TDM1 concentration: 1 ug/ml	
Add 10 ml PBS into a UC tube	
Add 0,5 ul TDM1 in to 10 ml	**Sample 6**
Do the 1. UC	
Take 100 ul sample from the supernatant	**Sample 7**
Discard the supernatant	
Resuspend in 10 ml PBS	
Take 100 ul sample	**Sample 8**
Do the 2. UC	
Take 100 ul sample from the supernatant	**Sample 9**
Discard the supernatant	
Resuspend in 10 ml PBS	**Samle 10**
Store the samples at - 80 C	

TDM1 store stock: 20 mg/ml = 20 000 ug/ml
D = 20 000/10=2000

1. UC: 60 min, RPM: 28800
2. UC: 60 min, RPM: 28800

2. day

TDM1 concentration: 10 ug/ml	
Add 10 ml PBS into a UC tube	
Add 5 ul TDM1 in to 10 ml	**Sample 1**
Do the 1. UC	
Take 100 ul sample from the supernatant	**Sample 2**
Discard the supernatant	
Resuspend in 10 ml PBS	
Take 100 ul sample	**Sample 3**
Do the 2. UC	
Take 100 ul sample from the supernatant	**Sample 4**
Discard the supernatant	
Resuspend in 100 ul PBS	**Samle 5**
Store the samples at - 80 C	

TDM1 concentration: 1 ug/ml	
Add 10 ml PBS into a UC tube	
Add 0,5 ul TDM1 in to 10 ml	**Sample 6**
Do the 1. UC	
Take 100 ul sample from the supernatant	**Sample 7**
Discard the supernatant	
Resuspend in 10 ml PBS	
Take 100 ul sample	**Sample 8**
Do the 2. UC	
Take 100 ul sample from the supernatant	**Sample 9**
Discard the supernatant	
Resuspend in 100 ul PBS	**Samle 10**
Store the samples at - 80 C	

TDM1 store stock: 20 mg/ml = 20 000 ug/ml
D = 20 000/10=2000

1. UC: 100 min, RPM: 28800
2. UC: 75 min, RPM: 28800

			Dilution	
Sample1	10 ug/ml	10 000 ng/ml	40	250 ng/ml
Sample 6	1 ug/ml	1 000 ng/ml	4	250 ng/ml

3. day

TDM1 concentration: **10 ug/ml**	
Add **4997,5 ul PBS** into a UC tube	
Add 2,5 ul TDM1 in to 10 ml	**Sample 1**
Do the 1. UC	
Take 100 ul sample from the supernatant	**Sample 2**
Discard the supernatant	
Resuspend in 10 ml PBS	
Take 100 ul sample	**Sample 3**
Do the 2. UC	
Take 100 ul sample from the supernatant	**Sample 4**
Discard the supernatant	
Resuspend in 100 ul PBS	**Samle 5**
Store the samples at - 80 C	

TDM1 concentration: **50 ug/ml**	
Add **4987,5 ul PBS** into a UC tube	
Add 12,5 ul TDM1 in to 10 ml	**Sample 6**
Do the 1. UC	
Take 100 ul sample from the supernatant	**Sample 7**
Discard the supernatant	
Resuspend in 10 ml PBS	
Take 100 ul sample	**Sample 8**
Do the 2. UC	
Take 100 ul sample from the supernatant	**Sample 9**
Discard the supernatant	
Resuspend in 100 ul PBS	**Samle 10**
Store the samples at - 80 C	

TDM1 store stock: 20 mg/ml = 20 000 ug/ml
D = 20 000/10=2000

1. UC: 100 min, RPM: 28800
2. UC: 75 min, RPM: 28800

		Dilution	
10 ug/ml	10 000 ng/ml	50	200 ng/ml
50 ug/ml	50 000 ng/ml	200	250 ng/ml

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