Establishment of a test system for multiparameter analysis of intracellular signal pathways by the use of flow cytometry.

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Sammendrag

Det har vært en økende fokus på stam celle forskning det siste tiåret. Forskere tror stam celler kan brukes i behandling av mange sykdommer. Vår gruppe har fokusert på mesenkymale stam celler og deres respons på cellulært stress, inflammasjon og deres inhiberende effekt på adaptiv immunitet. For å kunne bruke stam celler i terapi må man forstå hvordan kombinasjoner av signaler påvirker stam celles opprettholdelse og differensiering. Å studere hvordan signal veier aktiveres og hvordan flere signal veier påvirker hverandre er tidkrevende og arbeidsomt ved bruk av vanlige immuno-assay teknikker. Et test-system som kan måle mange parametere samtidig vil være et kraftig verktøy i studier av slik signalering i stam celler.

Ved Immunologisk Institutt på rikshospitalet i Oslo, jobber vi med etablering av et multi parameter test system basert på fluorosens merkede partikkel populasjoner, som kan separeres i et flow cytometer. Fluorosens baserte ”immuno-sandwich” teknikker er brukt for å måle analytter i dette systemet, ved å bruke et array av forskjellig merkede partikkel populasjoner som er koblet til forskjellige oppfangende antistoffer. Analyttene blir i dette systemet bundet mellom et oppfangende antistoff og et detekterende antistoff i et ”sandwich” og dette måles via en indirekte farge-merking via et fluorokrom konjugert antistoff.

Hoved målet med master oppgaven var å etablere dette multi parameter test systemet og å verifisere at dette systemet kan diskriminere mellom nært beslektede analytter. Et del mål var å konstruere et array for å studere stress påvirkede signalering i stam celler, basert på introduksjons studier i stam celler. Disse introduserende studiene ble fokusert på cellulært stress indusert av gamma bestråling i stam celler. Bakgrunnen for disse studiene var upubliserte data fra vår lab som indikerte at gamma bestråling kunne indusere PGE2 produksjon.

Resultater viser at multiparameter test systemet kan diskriminere mellom beslektede analytter om antistoffer med riktig reaktivitet of egenskaper velges. Data fra test systemet og Western analyse viser god korrelasjon, og demonstrerer at noen antistoffer binder analyttene spesifikt, mans andre kryss reagerer med andre analytter. Det gjenstår mye arbeid for å etablere ferdige array som er til å stole på, men våre data har vist at test systemet har et poteniale som kan lede til fullt utviklede funksjonelle array.

Introduserende studier har vist at gamma bestråling induserer COX 2 i telomerase transduserte mesenchymale stam celler. Denne effekten er ikke indusert via reaktive oksygen species. PGE2, som dannes ved hjelp av enzymet COX 2, har blitt foreslått som effektor i den inhiberende effekten mesenkymale stam celler har på adaptive immunitet.

Anti-COX 2 antistoffer ble koblet til partikler og det ble vist at test systemets data og data fra Western analyse var sammenlignbare. Tiden tillot ikke videre utvikling av et array.

Abstract

The last decade the field of stem cell research has grown tremendously. Researchers believe stem cells may provide ways to treat many diseases. Our group has focused on mesenchymal stem cells, their response to cellular stress, inflammation and their inhibiting effect upon adaptive immunity. To use stem cells in therapy one needs to understand the combination of signals that affect stem cell homeostasis and differentiation. To study how signal pathways are activated and how different signal pathways interact, using conventional immunoassay techniques are time consuming and laborious. A test system that can read many parameters simultaneously will be a powerful tool in study of signaling events and pathway interaction in stem cells.

At the Institute of Immunology, Rikshospitalet Oslo, we are establishing a multi parameter test system based on fluorescence stained particle populations that can be distinguished in a flow cytometer. Fluorescence based immuno-sandwich techniques are used to detect analytes in this system, using an array of particle populations coupled to different capture antibodies. The analytes are sandwiched between particle bound capture antibodies and soluble detection antibodies. The sandwiches are then detected indirectly with a fluorochrome conjugated second step antibody.

The main objective of the master assignment was to establish this multiparameter test system and to and verify that this system could discriminate between closely related analytes. A part objective was to construct an array for studying stress induced signaling in stem cells, based on introductory studies in stem cells. The introductory studies were focused on the effect of gamma irradiation induced cellular stress in stem cells. This was due to unpublished results from our lab that indicated that gamma irradiation could induce PGE2 production in stem cells.

The main findings are that the multiparameter test system can distinguish closely related analytes if antibodies with the right reactivity and properties are selected for the immuno-sandwiches. The data obtained from the test system and Western data were comparable, showing that some antibodies reacted only to with their specific analyte, while others cross reacted with other analytes. A lot more work must be carried out to establish reliable multi

parameter arrays, but the findings are that the test system has a good potential that, with further development, can lead to reliable arrays.

Introductory studies have shown that gamma irradiation induces COX 2 in telomerase transduced mesenchymal stem cells. This induction is not mediated through reactive oxygen species. As the prostaglandin PGE2, which is synthesized by the COX 2 enzyme, has been suggested as the mediator of the inhibiting effect that mesenchymal stem cells have on adaptive immunity, the induction of COX 2 mediated by gamma irradiation raises important questions. Anti-COX 2 antibodies were coupled to particles and it was shown that the particle based test system gave similar results as the Western analysis. Time did not allow further development of an array.

Prologue

The work with this master thesis has been very exciting and filled with a lot of learning. It has encompassed many techniques and work in many fields, giving me a chance to learn a lot. I have been able to do a lot of the work independently including planning, practical work and research. Many have also been a good support and help along the way.

I would first like to thank Professor Tor Lea, my supervisor at the Institute of Immunology (IMMI), Rikshospitalet in Oslo. He has been a very good support, both through counseling and teaching. He is a living library of information, and has been enthusiastic about our project.

I would also like to thank Dr. Fritjof Lund-Johansen at IMMI for his instructions and enthusiasm through our work. He provided much of the platform that my master took part in developing further.

I also thank bioengineer Ellen Karlstrøm and PhD student Charlotte Ramstad from my group at IMMI. They have both been patient and helpful during the practical work in my master, providing instructions and experience.

Many others at IMMI and in our group have been helpful and enthusiastic, and I am thankful for all the support and help I have received.

My wife and family have also been a good support from home, providing encouragement and interest.

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1. Introduction

The human body is a complicated meshwork of specialized cells, organized into tissues and organs. The cells communicate with each other to perform their functions; including production, movement, defense, storage, metabolism, and distribution. Stem cells are a group of cells that sustain and repair organs and tissues. It is believed that stem cells have many potential uses in therapy of diseases, and the studies of stem cells have grown tremendously. One must decipher the language of the stem cells to understand the signals and combinations of signals that govern their role in the body. Our laboratory group has focused on a type of stem cell from the bone marrow called mesenchymal stem cells. Our goal is to better understand the signals that govern these cells, especially in situations where they experience cellular stress.

The language of the cells consists of signal molecules, ligands, which when transferred from one cell to another leads to a change in the receiving cell. Multiple ligands may affect a given cell and lead to a response. Each signal is typically passed on inside the cell through a series of events called a signal transduction pathway. The combination of signals involves interaction between pathways. To understand how cells work together it is essential to understand the signals and how they make a change in the receiving cells. A major mechanism in regulating the flow of signals and combination of signals inside a cell is through phosphorylation and dephosphorylation of signal transducing proteins. Antibodies against phosphorylated amino acids that is characteristic for activated signal transducing proteins has provided a powerful tool to study these events.

Immunoassays such as Western blotting have long been used to study the level of phosphorylation-activity in signal pathways in the cell. These assays require many cells per sample and are limited in the amount of analytes that can be measured in each sample. This makes it hard to study many signaling molecules or pathways

simultaneously. Often the immunoassays are time consuming compared to the amount of results obtained.

To decipher how a ligand or a combination of different ligands affects signaling pathways inside a cell, one needs a measuring system that can detect multiple parameters, and a way of targeting them so they can be detected. This has been achieved for studies of the DNA and mRNA in the cell. DNA microchip arrays can encompass over a thousand parameters. They provide researchers with a systematic tool to survey DNA and RNA variation. While this gives a lot of information on transcription, it gives little direct knowledge of how signaling pathways function and interact.

There are many types of multiparameter assays for phosphoprotein studies under development. Recently a concept called multiplex analysis has been developed that allows multiparameter detection. The principle is based on particles with characteristic fluorescence, with up to 100 different particle fluorescence levels in one assay. Particles of one fluorescence level are coupled with antibodies specific for a given analyte, such as a phosphoprotein. After incubation with a sample solution containing the actual analytes, the binding of analytes to the specific particles are detected by adding specific fluorochrome coupled antibodies. The result is then measured in an instrument containing two lasers that enables the detection of particle fluorescence and analyte fluorescence simultaneously.

The commercially available assays are very expensive and often require additional and expensive equipment to analyze samples. The assays have a varying amount of parameters depending upon what is measured, but potentially up to 100 parameters can be read at once. For phosphoproteins, the assays are still small compared to the potential of 100 analytes.

At the Institute of Immunology (at Rikshospitalet in Oslo) we are establishing a method for fluorescence-coupling populations of particles so that a standard analytical flow cytometer can be used to register and distinguish particle populations. By coupling

antibodies to these fluorochrome stained particles, it will theoretically be possible to construct arrays with more than 100 parameters. The flow cytometer is an instrument that can measure multiple parameters on single particles or cells at a very high speed. It is a common instrument in many laboratories. By making a multiplex assay for flow cytometers the need for specialized expensive multiplex instruments will be surpassed. We believe an assay like this will be a powerful tool in studying signal transduction and interaction in stem cells, and other cell types.

The objective of my master is to establish a multiparameter array for detecting cell signaling components in a flow cytometer. I will investigate the potential an array of multiple particle populations, which can be measured in a flow cytometer, has to distinguish and detect signaling analytes. To achieve this I will test a group of antibodies that are specific for closely related cell signaling components. I will test the same antibodies using conventional Western blotting to verify and compare the results. After testing the potential of the array I will, if time allows it, begin introductory studies of signal pathways in stem cells with a focus on the cellular response to gamma irradiation-induced cellular stress. Having first used conventional techniques to investigate stress induced signaling and responses in our cells, I will proceed to establish a small array with the same antibodies used in the introductory studies, to measure the same stress induced analytes.

Thus my objectives to establish a multiparameter array include both the establishment of a multiparameter test system and the investigation of the potential in this system, before constructing an array based on introductory studies in stem cells that can be used for further studies in stem cells.

2. Theory

2.1. Cells and stem cells

2.1.1. Cells – the building blocks of the body

The adult body contains hundreds of different specialized cell types that perform specific functions. They make up the internal organs, such as heart and liver, and tissues like skin, muscles, and bones. They must function correctly to maintain body health.

Normally, specialized cells cannot change into a different type of cell. They have a finite life span and will eventually die. Most of the cells in the body divide and duplicate throughout life. In some tissues the cells either don't replenish them selves or do so in such small numbers that they cannot replace themselves fast enough to combat disease. Two examples are the brain and the heart. In heart and nervous-system diseases like stroke, heart attack and Parkinson's the death of cells is larger than the body's ability to replace them, and may cause partial or complete organ failure.

2.1.2. Cellular development

As the fertilized egg starts to divide, the first cells have the capacity to become all the cells of the body and placenta. They are called totipotent. After a few divisions among these cells, some are set apart to become the placenta, while others are set apart to become the embryo. As their developmental path is chosen, the cells “loose” their totipotent character. The cells that are developing into the embryo will at first have the capacity to become all the cells of the body, and are called pluripotent. As development continues, the cells choose different paths and specialize further into the three germ layers (mesoderm, endoderm and ectoderm). These give rise to the different tissues and organs (figure 1). The cells can now only become the types of cells that the layers will give rise to. They are multipotent. As the development continues, the cells differentiate and specialize to form organs; limbs and divers tissues (figure 1)[12].

2.1.3. Cellular research

Many cells can live and divide in cell cultures outside the body. Thus they can be studied independently of the organ or system they normally are a part of. The cultures contain medium and factors that mimic the normal environment of the cells.
Cells of one type that grow in a culture are called a cell line. In clonal cell lines all the cells are derived from one mother cell, and are identical.

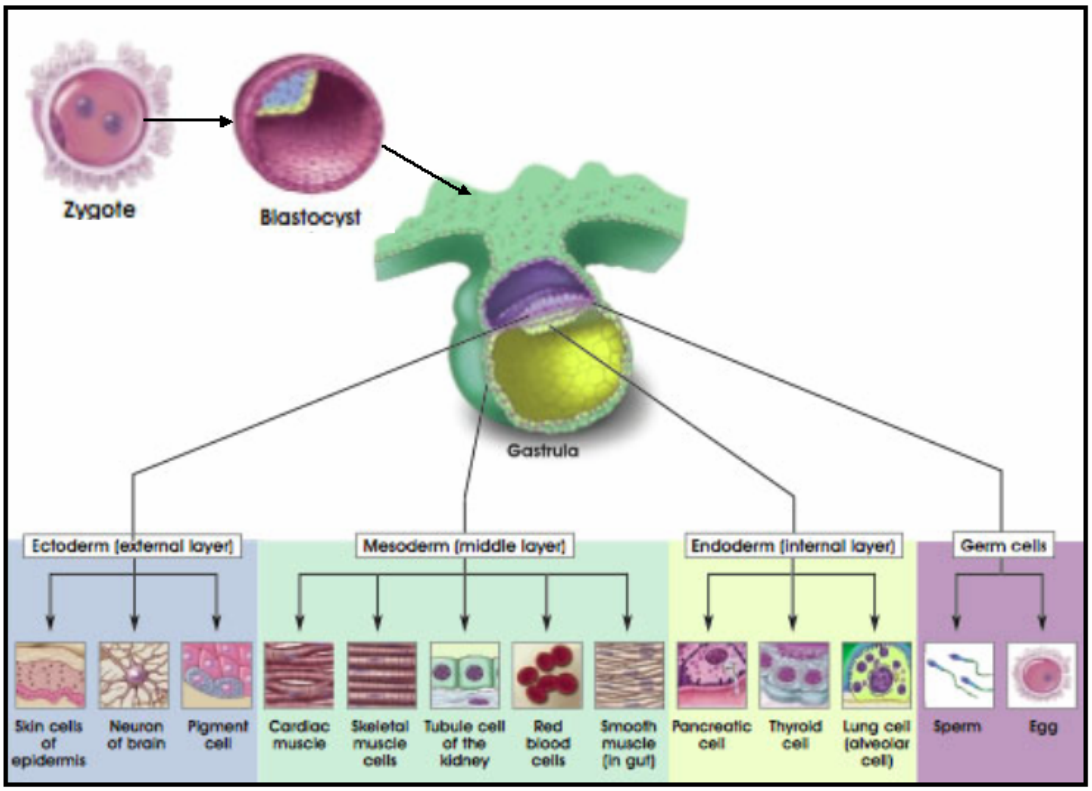


Figure 1: Cellular development
(source: medical litterature)

2.1.4. Stem cells

Stem cells can be broadly divided into two major types, the adult stem cells and the embryonic stem cells. These have different characteristics and potential. Embryonic stem cells are the pluripotent (or totipotent) cells that are found in the embryo in the early stages of development.

The adult body contains a special kind of cell that has two characteristics; first they are able to divide and maintain their own number in the grown body throughout life, and secondly they are able to maintain the number of specialized cells in the tissue by differentiating into specialized cells[36] (figure 2)[12]. They are the adult stem cells. They often have multipotent characteristics, like the early cells in the embryo. They can give rise to closely related families of cells within the tissues. They maintain the normal turnover of cells in the tissue[24], and replace damaged or dead tissue after an injury or disease[59]. Little is known about where stem cells arise in the development of the body.

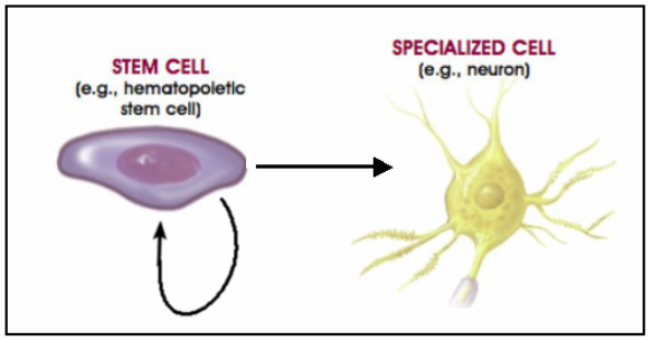


Figure 2: Stem cell characteristics.
(source: medical litterature)

Stem cells can generate intermediate cell types, before they differentiate to the fully specialized state. The intermediate cells are called precursor or progenitor cells. They are partially differentiated and give rise to new progenitor cells and specialized cells (figure 3)[12].

Adult stem cells have been identified in the bone marrow[4,22,66], peripheral blood[3], brain[63,69], liver[14], skeletal muscle[55], epithelia of skin and digestive tract[24] and pancreas[76].

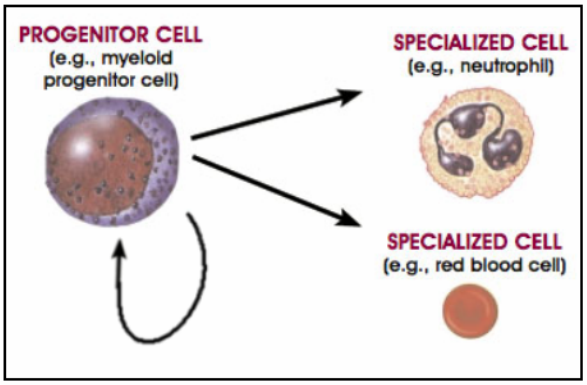


Figure 3: Progenitor cells
(source: medical litterature)

Researchers find that some stem cells can differentiate into specialized cells not typical of their tissue[19,50,64], if they are cultured under the right conditions. This characteristic, called plasticity has become a new and interesting field of study.

2.1.5. Stem cell therapy

For many years researchers have worked to find a way of using stem cells to replace missing or damaged cells or tissues. Stem cell therapy is the use of stem cells in therapy of diseases. Candidate diseases are heart attack, Alzheimer's, Parkinson's, diabetes, multiple sclerosis, rheumatoid arthritis and several cases of cancer. The increasing need of organ and tissue donors may some day be met by engineering these tissues from stem cells. The hope of being able to use stem cells to treat patients has given the stem cell research a lot of attention and publicity the last years.

2.1.6. Stem cell research - challenges and questions

Although stem cells may be found among the specialized cells of a tissue they are rare. Identifying, isolating, and culturing stem cells are a challenge. It is hard to determine if the stem cells you isolate are a homogenous population, containing one type of stem cells, or a heterogeneous population containing more than one type of stem cells. The experienced plasticity could be derived from stem cells with different potential of specialization that are isolated together. Most isolated adult stem cells are unable to proliferate in an unspecialized state for a long period of time, and this limits the number of cells one can study or use in therapy. Many of the current results in stem cell research are based on studies in mice (here mice act as a mammalian model organism) but there are differences between human and mice stem cells that must be further explored for researchers to know how comparable they are.

There are many unanswered questions about stem cells, including the origin, the different types and where they are found, what they can differentiate into, and plasticity. Little is known about the signals that govern the differentiation of the stem cells into specialized cells. Our group has focused on cellular stress and inflammation signals and the effect they have upon mesenchymal stem cells.

2.1.7. Bone marrow stem cells

The stem cells from bone marrow are the most studied stem cells.
There are two mayor types; the hematopoietic stem cells (HSCs) that give rise to blood and immune cells, and the mesenchymal stem cells (MSCs) (also called bone marrow stromal cells) that give rise to bone, cartilage and fat. Bone marrow stem cells were the first isolated[4,66] and the first used in stem cell therapy[1,65].

2.1.8. Hematopoietic stem cells

The HSCs are the cells that form the blood[15] and the immune cells of the body, a process that involved the production of billions of new cells every day. They are found in the bone marrow, (1 in10.000-15.000 cells[68]) and in the blood (1 in 100.000 cells).

2.1.9. Mesenchymal stem cells and hMSC-TERT

Non-hematopoietic stem cells were isolated from the bone marrow for the first time in 1970 by Friedenstein et al[21]and were called bone marrow stromal cells. The earliest method of isolation was based on adherence to plastic surfaces, a property that distinguishes MSCs from HSCs. MSCs are rare compared to the rest of the cells in the bone marrow (1 in 10,000 cells).

The morphology of MSCs is similar to that of fibroblasts, and MSCs form colonies in cell cultures. MSCs have been shown to differentiate into different mesenchymal derived cell types including; bone forming osteoblasts[29], fat storing adipocytes[28] and cartilage forming chondrocytes[26]. MSC have also shown some plasticity by differentiating into typical non-mesenchymal cell types as neuron-like[74] and endothelium-like cells[11,25].

MSCs may proliferate for up to 35 population doublings (PD)[6]. The cells will experience an age-associated growth inhibition when they reach their PD limit. Teleomer shortening is a part of this process[32].

To avoid age-associated growth inhibition seen in stem cells human MSCs have been transduced using a retroviral vector containing the human telomerase reverse transcriptase (hTERT) gene[57]. It was shown that the transduced cell line, called hMSC-TERT, has telomerase activity and that the transfection is stable[57].

The cell line shows no age-associated growth inhibition, and can in theory undergo unlimited PD. Research has shown that this cell line has the same characteristics as normal human MSCs, including the ability to differentiate into the same cell types, and expressing the same cellular markers[57].

MSCs have been used in stem cell therapy of myocardial infarcts, in both animal models and in clinical trials in humans[30,48,75]. During a heart attack, lack of O2 (ischemia) in tissue causes cellular stress, death, and inflammation. Unidentified signals affect stem cells that are injected into the blood or in the proximity of the damaged tissue. It has been shown that the stem cells can home to the site of damage, and that the function of the damage partially is restored by the entry of stem cells[48]. A question that arises is whether the signal that affects the stem cells is given from the damaged cells, from the remaining healthy cells or from the immune cells at the site of the inflamed and damaged tissue. It might well be that it is a combination of signals. What the signals are, and what morphological and differential changes they promote in the stem cells are not fully understood. Whether the stem cells actually differentiate into myocardium themselves, fuse with and repair damaged cells or promote other cells to become myocardium is still a question of controversy[46,48].

Our group is studying hMSC-TERT and the signals/signal pathways that govern the morphology, differentiation and movement of these cells. Our focus is on cellular stress, inflammatory signals and the signal pathways they induce. We also study the inhibiting effect the hMSC-TERT cells have on adaptive immunity.

2.2. Cell signaling pathways

To understand the stem cells, in our case the mesenchymal stem cells, the signals that govern their homeostasis and changes in the body must be studied. It is important to distinguish between extracellular signal molecules, ligands, which are ”sent” from one cell to another, and the intracellular signal molecules that relay a received signal into an effect inside the cell. The conversion of an extracellular signal, into a change inside the cell is a mechanism called signal transduction. It could be called “passing on a message” in simplified language.

Our objective is to establish a test system that can detect many of the parameters that act in signal transduction simultaneously in a multiparameter array. Such an array can detect analytes (in our case signal transducing molecules) from many types of signaling pathways at once. Here I will describe the general principles of signal transduction, and as an example I will describe a common signal pathway (I will not attempt to give a full picture of the diverse signal pathways that exist).

2.2.1. Signal transduction

Signal transduction is any process by which a cell converts a signal or stimulus into a cellular change/effect. Very often, this involves the binding of a signal substance (ligand) to a receptor on the outside of the cellular membrane or inside of the cell. The transduction of the signal involves a sequence of biochemical reactions inside the cell, which are carried out by signaling proteins, enzymes, adaptor proteins and small signal molecules called second messengers (figure 4). As a cell receives a signal the transduction occurs fast, from milliseconds to a few seconds (sometimes minutes). Cells are often able to give a substantial response even to very few signal molecules. The cell can do this by amplifying the few signals it receives by a chain of steps called a signaling cascade.

The amplification is due to the ability of enzymes to mediate many reactions quickly. One receptor that binds a signal molecule can activate many enzymes, and each of these enzymes can in turn activate a number of new target enzymes. Some enzymes in signal pathways mediate the formation of second messengers that bind and activate enzymes in the cell. With such a series of activation, one signal can be amplified quickly giving a high number of signaling units within the cell. The signal response in the cells can include events like gene activation, change in metabolism, change in cellular movement, altered secretion, and altered morphology by change in cytoskeleton.

Many receptors and ligand families have been described. They elicit a large number of different responses in the cells. The receptors a given cell expresses will depend on its differentiation and function.

The receptors act on many signal relaying enzymes within the cell, thus activating different pathways. Some receptors activate signal pathways that act in opposite ways inside the cell, while others add or modify each others effects. Synergy is a phenomenon where two signals act together to create an effect which is greater than the sum of the effects each is able to create independently. The opposite of synergy is antagonism, the phenomenon where two agents in combination have an overall effect which is less than the sum of their individual effects.

2.2.2 Signals - ligands

Extracellular factors, such as hormones or neurotransmitters, are common ligands that bind receptors on target cells. There are many ways a soluble signal may reach the receptor of a cell.

Endocrine signals (hormones) are produced by endocrine cells and travel through the blood to reach all parts of the body, affecting target cells in many tissues. Paracrine signals target only cells in the vicinity of the emitting cell. Autocrine signals only affect cells that are of the same type as the emitting cell. They also target the cell that is

emitting them. (There are also signal molecules that are not soluble, but are membrane bound. In this case the signal is given between two cells lying next to each other.)

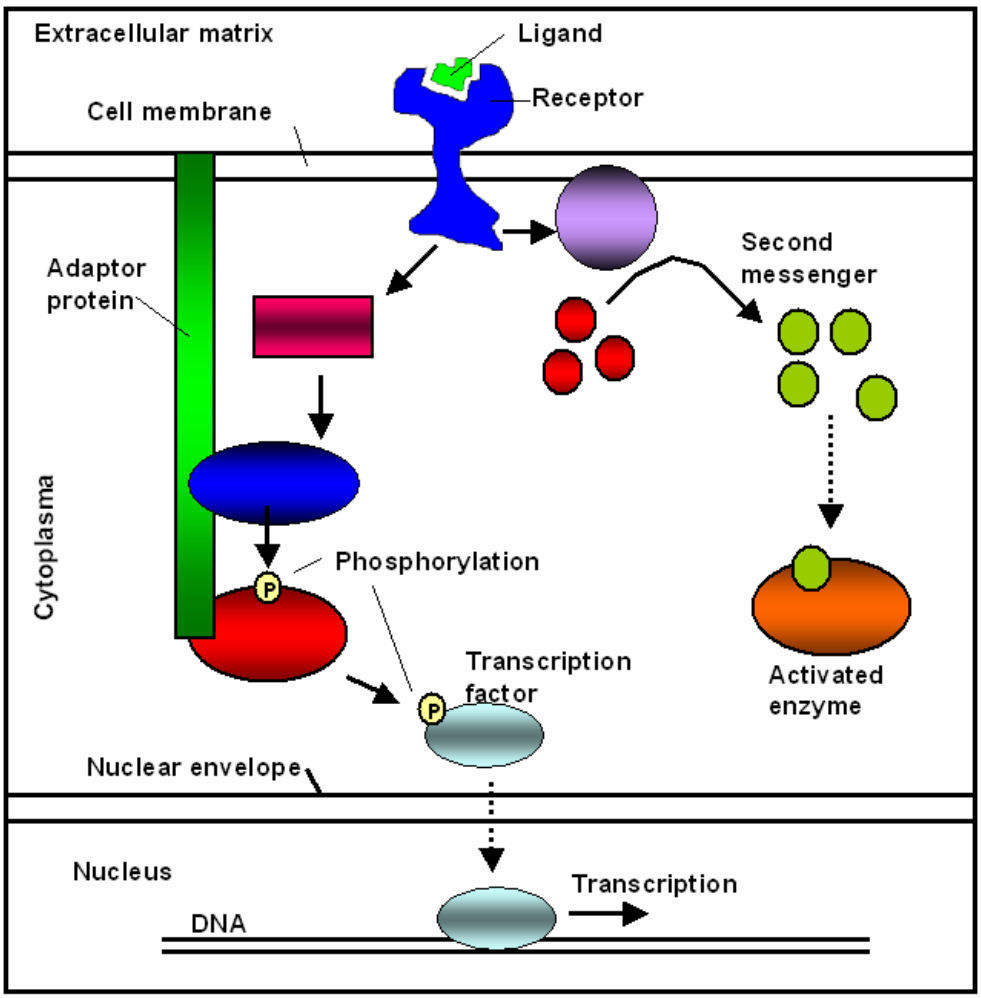


Figure 4. Common mechanisms in signal transduction

2.2.3. Receptors

A receptor can either be found in the cell membrane, looking like an antenna from the outside of the cell, or inside the cell. The membrane-bound receptors include G protein-coupled receptors, tyrosine kinase receptors and ion-channel receptors. These receptors transverse the membrane, and connect the outside with the inside of the cell. The function of the receptor involves the binding of a signal molecule (ligand) to a specific site on the

receptor (outside the cell), often causing a change in the conformation (shape) of the receptor. This change makes the receptor able to relay a signal inside the cell, by activating enzymes or mediating the formation of second messengers. The receptors have strong affinity for their specific ligand. Many extracellular ligands are water-soluble. Hydrophobic ligands can pass the cell membrane and bind receptors inside the cell. Such signaling molecules include steroids, and other fat soluble signal molecules.

2.2.4. Regulations of enzymes by phosphorylation

Many of the enzymes and proteins involved in cell signaling pathways have a special way of being activated, turned on, or inactivated, turned off. This activation and inactivation involves adding or removing a phosphate group from certain amino acids in the enzyme/protein. Binding a charged phosphate on a protein will cause new ionic bonds to form and this will change the 3-D structure (shape) of the protein, thus affecting its activity. Two kinds of enzymes are involved in this process. Kinases are enzymes that bind phosphate to a protein. Phosphatases remove phosphate from proteins. The site where the phosphate group is added or removed from is called a phosphorylation site. Many of the kinases and phosphatases have phosphorylation sites that control their own activity. Many of them also act on other kinases and phosphatases. Phosphorylation may cause enzymes to become active or inactive, depending upon what phosphorylation sites the enzyme has, and what changes the phosphorylation will cause in the enzyme. Some enzymes have both activating and inactivating phosphorylation sites. Three types of amino acids can act as phosphorylation sites, tyrosine (Tyr), serine (Ser) and threonine (Thr). Some phosphorylation sites act as “docking” sites for proteins that contain special domains that can bind phosphorylated phosphorylation sites.

Phosphorylated proteins in these pathways are potential targets in a multiparameter array. Detecting the amount of a signaling protein in the cell will not give information on activity in the pathway the protein is a part of. If phosphorylation of the protein activates it, detection of the phosphorylated variant of the protein will indicate if the pathway is

active or not. The mitogen activated protein kinase (MAPK) signaling pathway is one of many common signaling pathways that mediate signals inside the cell.

2.2.5. The MAPK pathways

Mitogen-activated protein kinases (MAP kinases) are protein kinases that respond to various extracellular stimuli (mitogens) and cellular stress via different receptors. The pathways regulate various cellular activities such as gene expression, differentiation, mitosis, and cell survival/apoptosis[34]. Thus they have a profound effect upon cell physiology[23,45]. Inflammation and cellular stress are important inducers of MAPK pathways[33]. MAPK signaling conveys a signal in the form of phosphorylation events, and involves a basic three step activation pattern that is common for all the MAPK signal transduction pathways[34,71] (figure5).

Extracellular stimuli lead to a signaling cascade composed of MAP kinase kinase kinases (MAP3K), MAP kinase kinase (MAPKK), and MAP kinase (MAPK). A MAP3K is activated by extracellular stimuli and phosphorylates a MAPKK on its serine and threonine residues, and then this MAPKK activates a MAPK through phosphorylation on its serine and tyrosine residues.
The MAPK signaling pathways is found in organisms from yeast to humans, and is highly conserved. Four distinct groups of MAPKs have been characterized in mammals: (1) extracellular signal-regulated kinases (ERKs), (2) c-Jun N-terminal kinases (JNKs), (3)

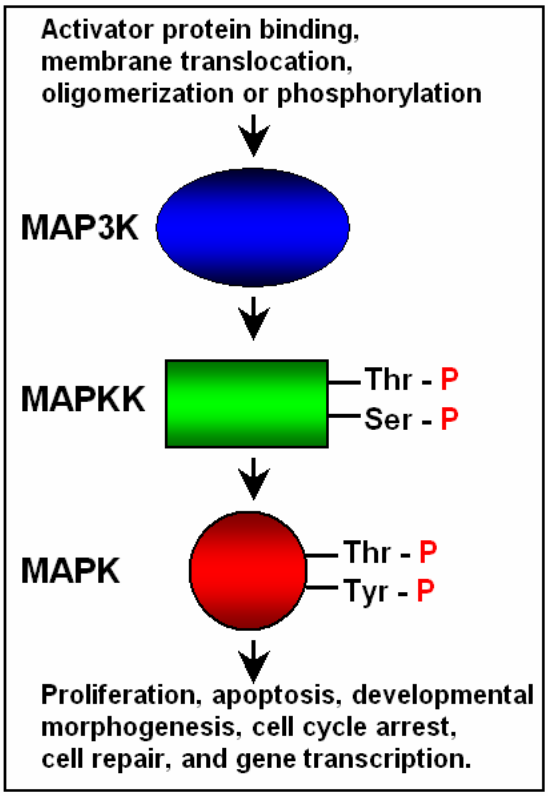


Figure 5. MAPK basic signaling module

p38 isoforms, and (4) ERK5. The ERK (also known as classical MAPK) signaling pathway is activated in response to hormones, growth factors and phorbol ester (a tumor promoter), and regulates cell proliferation and cell differentiation. The JNKs (also known as stress-activated protein kinases; SAPKs) and p38 signaling pathways respond to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation and apoptosis. ERK5 is activated both by growth factors and by stress stimuli, and it participates in cell proliferation.

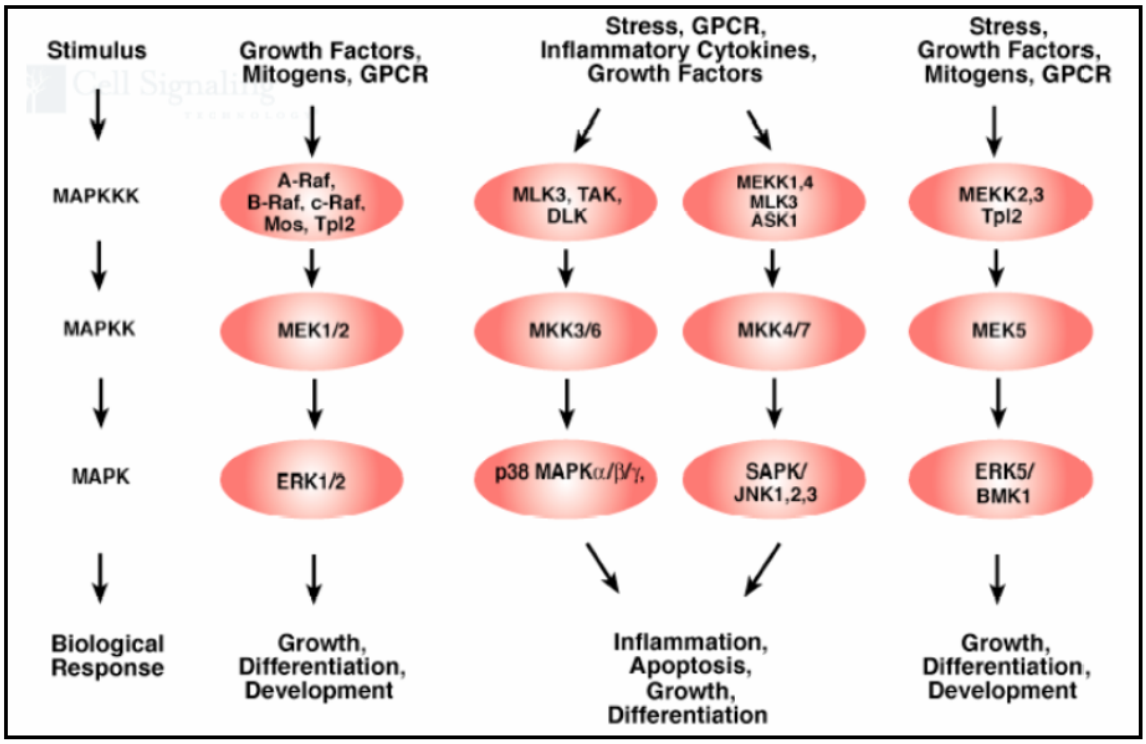


Figure 6. MAPK signaling pathways

2.2.6. Challenges in the study of signaling pathways and interactions

When studying signal transduction pathways one obtains data on what signals lead to activation of a pathway in the cell. An interesting feature of signal pathways is that some pathway components are found in more than one pathway in the same cell. They can act as bridges between pathways. In the normal body-environment a given cell receives many signals and must be able to integrate many signaling pathways, using scaffolding

proteins and cellular compartments to help direct the signals[70]. These networks of signal pathways are important to understand.

2.3. Multiplex analysis versus conventional assays

2.3.1. Conventional immunoassays

Conventional immunoassays include assays like Western blotting and enzyme-linked immunosorbent assay (ELISA). Here one analyte is measured in a series of samples, giving semiquantitaive or quantitative measurements. The techniques are laborious compared to the amount of data obtained.

2.3.2. Multiplex protein arrays

In study of cell signaling by protein phosphorylation many still use the conventional immunoassays. Some multi parameter or multiplex systems have been developed and commercialized, but usually it requires special and expensive equipment on top of the costly kits that follow. Multiparameter assays give large amounts of data, much faster, and more quantitatively than conventional techniques. They provide powerful tools in studying cell signaling.

An example of a recently developed multiplex is the Luminex® based Bioplex delivered by Bio-Rad (http://www.bio-rad.com). This system is based on a Luminex® instrument (http://www.luminexcorp.com) where two lasers are used to detect beads and analytes bound to the beads. The beads are swollen in solutions containing different dilutions of two fluorochromes, to make 100 populations of differently colored beads (figure 7). One laser is used to detect the color of the passing beads, while the other excites the detection antibody in the target antibody sandwich on the bead to quantitatively measure amount of bound target (figure 8).

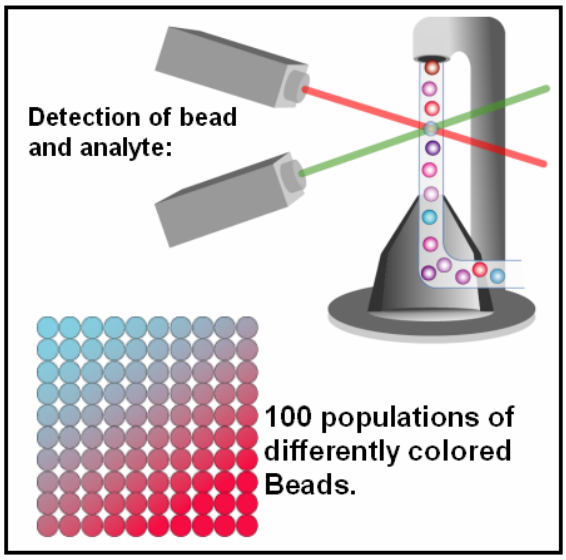


Figure 7. Fluorescence-based multiplex I.

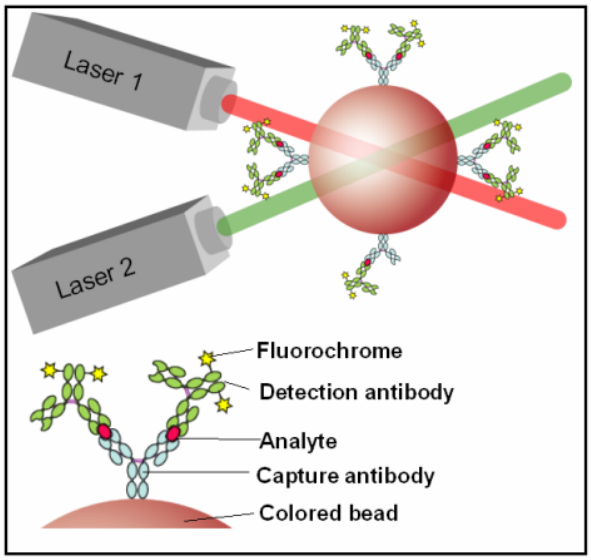


Figure 8. Fluorescence-based multiplex II.

As mentioned earlier, the objective of my master is to establish a multi parameter system like this that can be used in a common flow cytometer, thus avoiding the need for new expensive equipment.

2.4. Flow Cytometry

Flow cytometry[9] is a technique for quantitatively measuring properties of single cells or particles at a high rate of speed (I'll use the term particle for cells, beads, and any particular material that can be measured in a flow cytometer). By doing measurements on one particle at a time, one can gain a measurement of each individual particle from a much larger population of particles. We believe the flow cytometer is a powerful tool, that be used to detect signal proteins in multiparameter assays based on an array of differently colored particles.

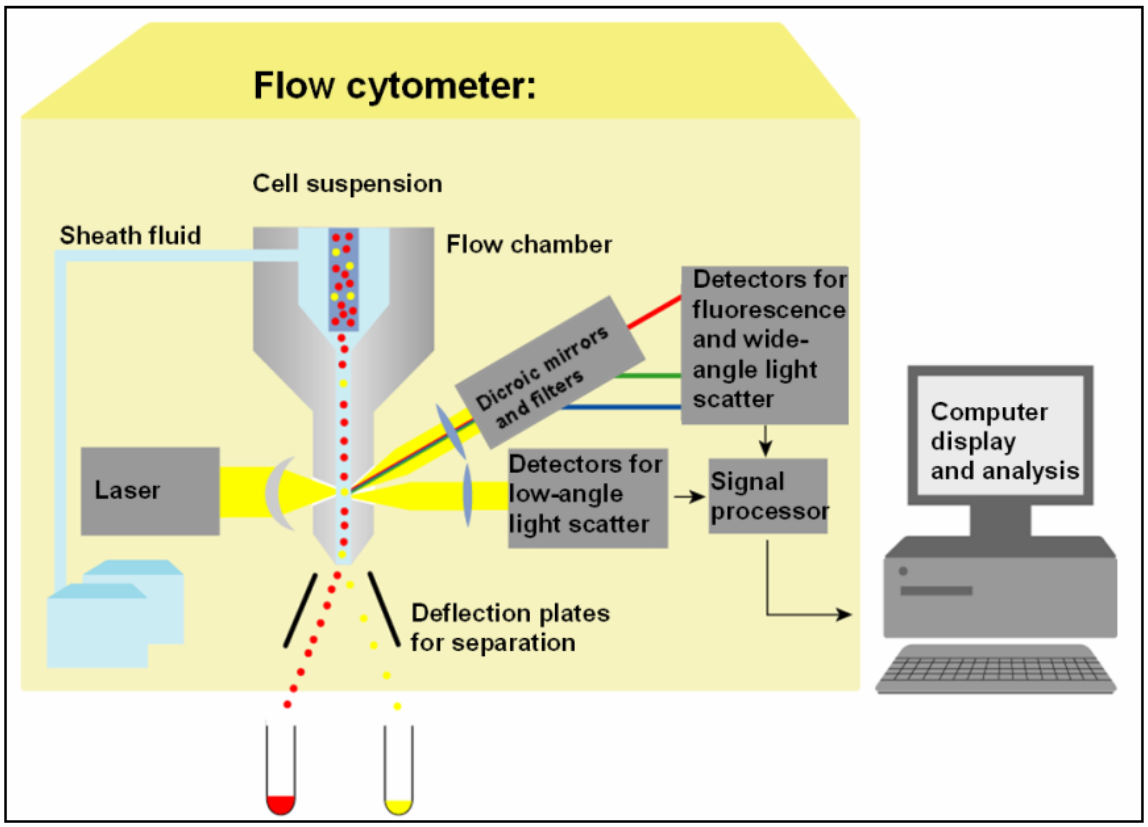


Figure 9: Basic flow cytometer instrumentation

2.4.1. Instrumentation

The flow cytometer consists of a flow chamber (where the separation of particles is achieved using principles of liquid flow), lasers, optical detection units, signal conversion and amplification, data display and analysis (figure 9).

2.4.2. The flow chamber

The concept of flow cytometry may be summarized in a simple figure of the flow chamber (figure 10). The flow chamber consists of an injector needle in the center of a larger circular tube, with the tube narrowing to a constricted region just beneath the needle. The constricted region contains the laser and measuring optical units. The continuing sheath flow centers and concentrates the sample stream distributed by the injector needle. By adjusting the sheath pressure and the sample pressure, one can vary

the diameter of the sample stream to fit the size of particles to be separated. Thus a stream where the particles come as beads on a string, one after the other, can be acquired. Effective measurements of up to 10,000 particles/second may be achieved[9].

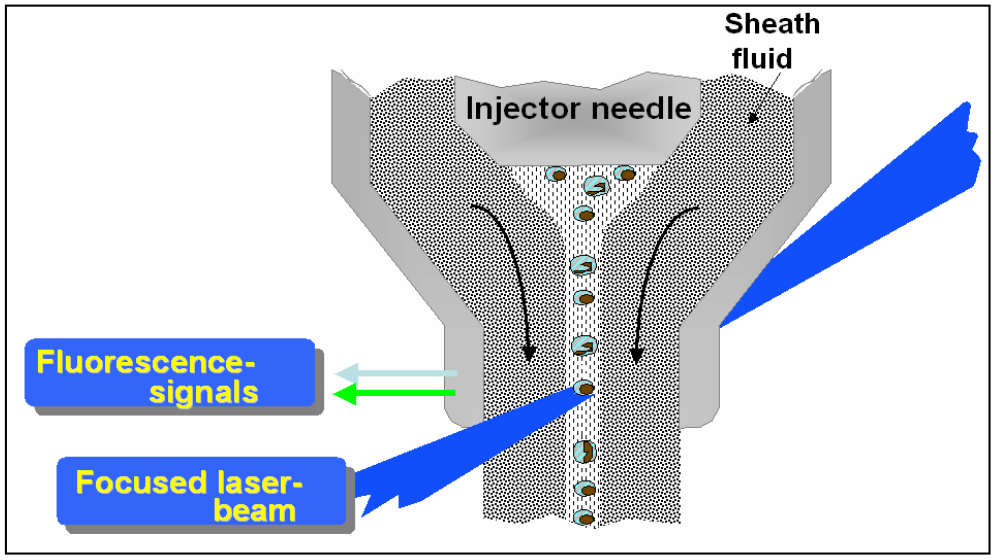


Figure 10: The principle of flow cytometry.

2.4.3. Fluorochromes, lasers and optical detection

Fluorochromes are substances that can absorb and emit light of different wavelengths. The excitation and emission properties of some common fluorochromes are shown in figure 11. A large number of fluorochromes with specificity towards different cellular components have been developed. Thus they can stain a specific property, component or antigen on (or in) a particle. The presence and amount of fluorochrome staining on each particle can then be detected.

As single particles enter the constricted region in the flow chamber they cross a laser beam. The flow cytometers usually have one or more lasers that excite the fluorochromes in the sample to be measured. Some common laser wavelengths are shown in figure 11.

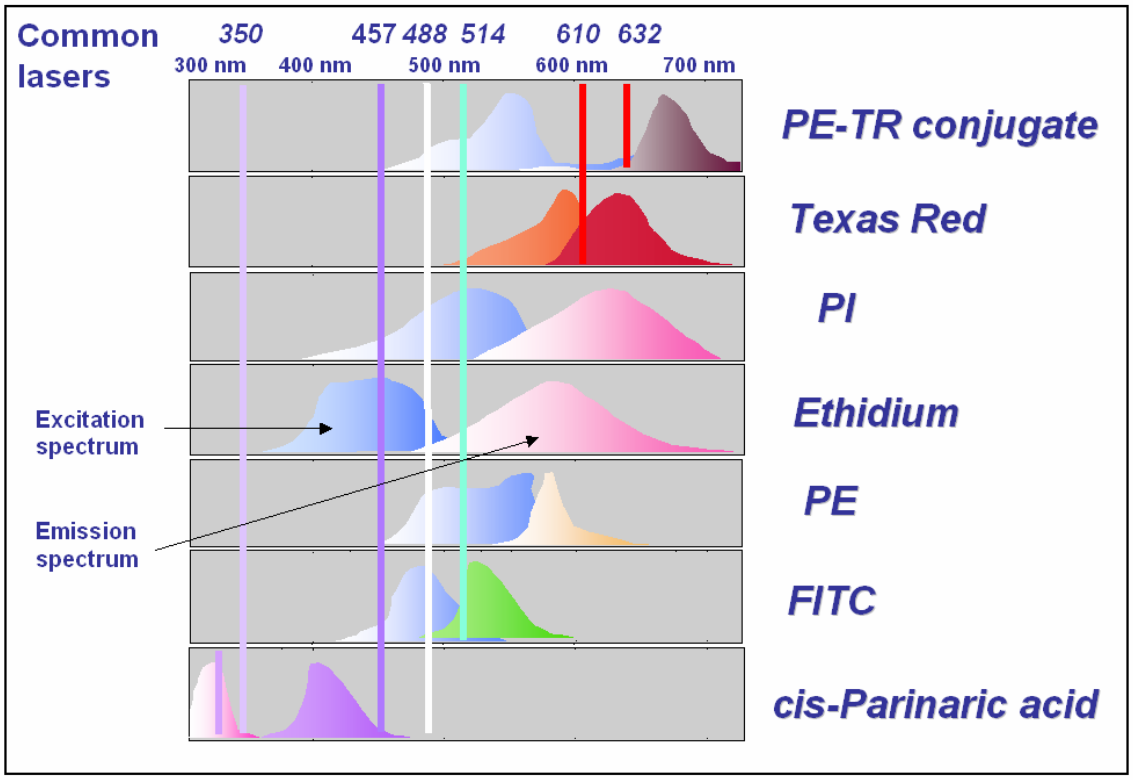


Figure 11: Common laser wavelengths and common fluorochromes with their excitation and emission spectrums.

The light scatter properties and fluorescence emission of the particles are measured by optical detectors. The low angle light scatter, called forward scatter (FSC), is measured by an optical detector that is placed opposite to the laser beam (figure 12). Wide angle scatter, called side scatter (SSC), and fluorescence emissions is measured in an optical channel that is placed at right angles to the intersection of laser beam and sample flow (figure 12). The fluorescence parameters measured is the total and maximum intensity, polarization and lifetime. The light scattering properties of a particle is dependent upon particle size and surface characteristics, and indicates the granularity of the particle. In the optical channel the side scattered light and the fluorochrome-emitted light is separated into different wavelengths using dichroic mirrors and filters, before the signals are detected in the optic detector.

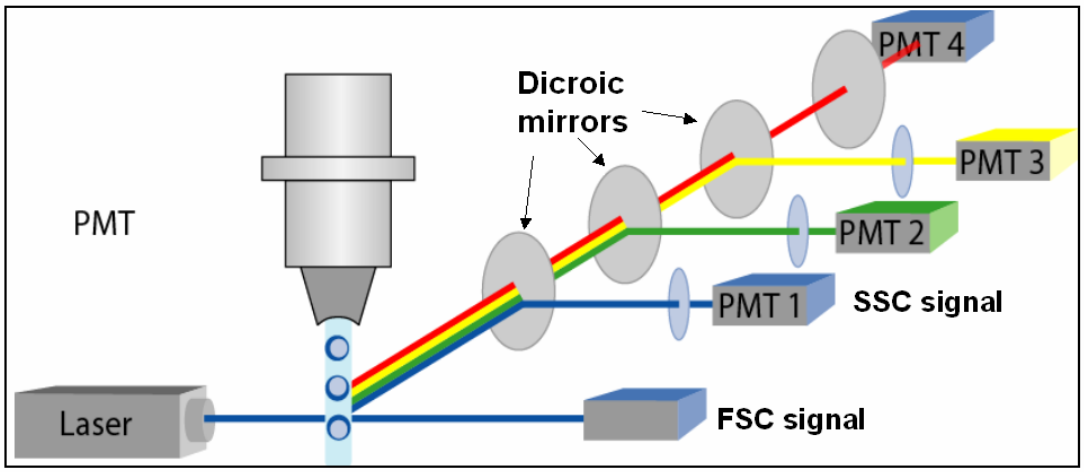


Figure 12: Optical detection.

2.4.4. Signal conversion and display

The signals from the optical detectors are converted to electronic signals and amplified in a signal processor using either linear or logarithmic amplification. The choice between the two modes of amplification depends on the dynamic range of the signal measured, with linear amplification for limited ranges, and logarithmic amplification for broad dynamic ranges. The data obtained by measuring a sample is thus converted, amplified and stored in a computer for later analysis. There are many modes of graphic displays with statistical and parametric tools to picture, evaluate and analyze the data.

2.4.5. Use of flow cytometers

Flow cytometry has a large number of applications and are used by researchers in a wide range of fields[7,9]. The use varies from routine measurements like the nucleic acid content or enzymatic activity of cells, to studies of blood cell populations[7], cell growth/apoptosis and intracellular cytokines[2]/phosphoproteins[13,31]. Researchers have managed simultaneous measurement of multiple active kinases[49], showing the potential of the flow cytometer to measure many parameters at once.

With two or more measurements made on each individual particle, one can distinguish subpopulations from the total pool of particles. This is providing that the measurements

can discriminate properties that are unique to the subpopulation. This can be achieved by adding a fluorochrome that only can bind the "unique property", thus marking the subpopulation with a measurable tag. A subpopulation that only differs a little from the overall population will fail to be detected by measuring the average of the population, as is usual in many conventional tests.

With the high sensitivity, speed and the possibility of measuring up to four or five parameters at a time, the technique and use of flow cytometry demands excellent sample quality. To keep the flow uniform and to avoid turbulence that might disturb the readout, the sample must be homogenous, containing single particles and minimal debris. The choice of fluorochromes must be considered carefully to avoid spectral emission overlap and energy transfer. Any overlap or energy transfer must be compensated for electronically while establishing the right parameters at the start of an analysis.
We have seen the advantage of using flow cytometry to rapidly detect many parameters on single particles, and have chosen it as a base of the multiparameter test system that we are developing. Some bead-based assays are already available for use in the flow cytometer (http://www.spherotech.com/flow_cyt_multiplex_par.htm) (http://www.bdbiosciences.com/pharmingen/products/display_product.php?keyID=9).

Methods for precipitation of analytes from a cell lysate with particle-bound antibodies, and detection particle-bound analytes in a flow cytometer have been established[44]. Combining these findings with the ability of the flow cytometer to measure multiple parameters, we have found that the flow cytometer has a great potential for making a particle based phosphoprotein array.

2.5. Antibodies

To establish a multiparameter array antibodies are used to capture and detect analytes that take part in cell signaling. Antibody structure is summarized in figure 13.

2.5.1. Biochemical applications of antibodies

All proteins have amino acid sequences and structural shapes that are unique to their type of protein. By creating antibodies against structures or sequences that are unique to different proteins, one gets the means of targeting these different proteins with antibodies that bind them specifically. Given almost any substance, it is possible to create antibodies that specifically bind it. The antibodies can then serve to detect, quantify or purify the analyte they are specific for.

Many proteins have similar structures (domains) that give them common epitopes (recognizable structures that can be bound by an antibody), especially within families of proteins. Native proteins and denatured proteins can have different epitopes. Antibodies may be made against surface epitopes of a native protein, or against sequence epitopes of a denatured protein. For the antibodies to have optimal reactivity it is important to know if the antibody is directed against a structural conformation or a sequence. With sequence directed antibodies it may be necessary to denature the analytes for optimal binding.

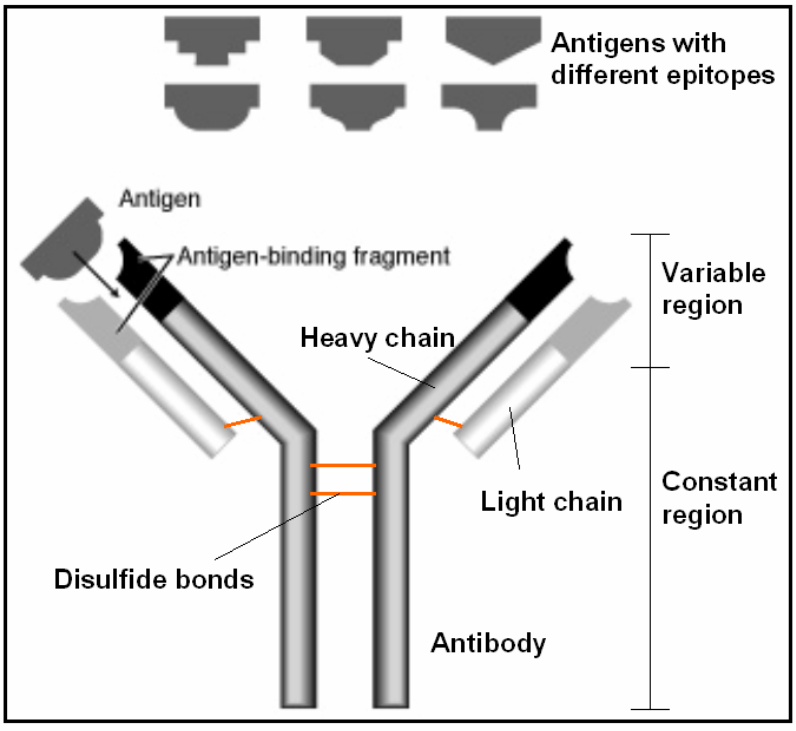


Figure 13. Antibody structure

2.5.2. Specificity and cross reactivity

When using antibodies it is important to determine their reactivity and properties. I will use the term property for the total reactivity of the antibody including specificity, cross reactivity, binding strength (avidity and affinity), and antibody stability. An antibody with specificity towards a certain epitope might still be able to bind a different epitope to some degree. This weak interaction with partially similar epitopes is called cross reactivity. While using antibodies in a highly sensitive analysis, it is important to avoid cross reactivity that can alter the true result. By adding substances that hinder partial binding, but not direct binding, one can block some of the cross reactivity. Binding an antibody to a solid faze may alter its properties. When developing a particle-based immuno array the property of each antibody must be assessed.

The production of new monoclonal and polyclonal antibodies has grown tremendously the last years. Companies verify the properties of the antibodies using their own conditions. Antibodies are typically tested for their properties in common immuno assays and techniques like Western blotting, immunoprecipitation, and ELISA.
In our test system the optimal conditions may be different from the conditions used in these assays. As we want to use many antibodies together (that might have been developed by different companies), we need to test the properties of each antibody under our conditions. The high sensitivity of a flow cytometer demands high quality antibodies that have little cross reactivity. Especially in a system where many antibodies are used together, and the background from each separate antibody adds up to a higher total background.

The testing of antibody properties will be an important part of developing a functional and dependable multiparameter assay. We will use both flow cytometry and Western blotting to test the properties of our antibodies.

2.6. Establishment of a multiplex assay

With the need of a good multiparameter assay system we have recognized the quality and potential of flow cytometry as an exceptional tool. By developing a flow cytometer based multiparameter system; the need to buy new specialized and expensive equipment will be surpassed by many laboratories, as flow cytometers are widely in use in research laboratories and medical facilities.

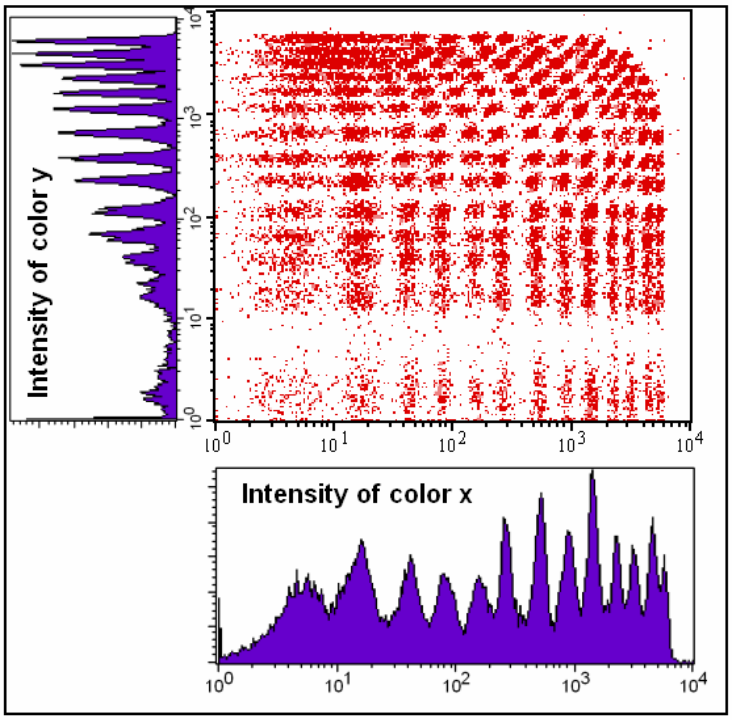
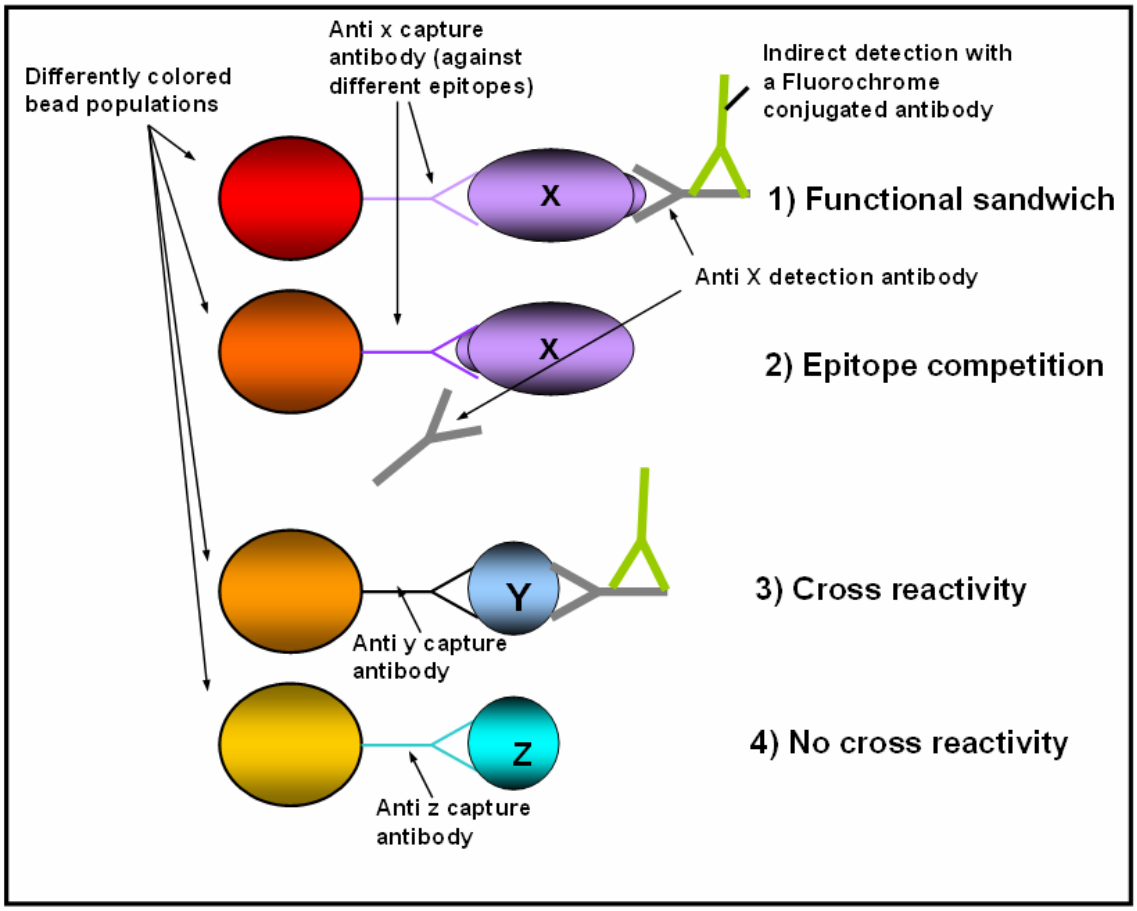


Figure 14. Dual color based multiplex bead assay

Recent studies and techniques[44] have shown that cell signaling pathways may be studied using antibody coupled particles in the flow cytometer, leading to a project in our institute to develop a multiparameter test system.

A technique for stably coloring latex particles with two different fluorochromes have been developed by researchers in our institute as well as methods for coupling antibodies to the fluorochrome colored beads. I have participated in some of this work, laying a foundation for my further studies, but since these techniques are in the process of patenting, they will not be discussed here in detail, and I will only briefly present it as theory that I have based my studies on. In short we obtain stable populations of differently colored particles and can bind one type of capture antibody to each of these populations. These populations can be identified and measured in a flow cytometer (figure14). The latex particles in this array (figure 14) are of uniform size, and only two

of the optical channels of the flow cytometer (FL1 and FL4) are in use to detect the fluorescent dyes. The particle fluorochrome emissions transverse 4 logs of magnitude. Most of the particle population can be seen as separate regions. Arrays with more than 100 distinguishable particle regions can be made using this technique. Particle coloring and capture antibody coupling stability have been tested.



Using a small version of this kind of array, I will investigate the potential of our test system to distinguish and measure closely related analytes. In this process I will test the reactivity of selected antibodies that are directed against closely related signaling analytes. The principle of detection in our array is built like a common fluorescence based immuno detection assay with capture and detection antibodies that bind the analyte in a sandwich (figure 15). The capture antibodies are in our case coupled to latex particles. The immuno sandwiches are detected by adding a fluorochrome-conjugated

anti-species-IgG antibody (figure 15). We will test antibodies from different companies that are directed against analytes from closely related signaling protein families. Some of them are phospho-specific, and others are isoform specific. We will test which of the antibodies that will function together in a sandwich, and look at epitope compatibility and cross reactivity (figure 15).

Western blotting is reckoned as the golden standard in immuno analysis, and to verify the results we get from the array we will test the same antibodies using Western analysis.

2.7. Introductory studies of signal transduction in hMSC-TERT

We believe that a multiparameter assay designed for studies of signal pathways will be a powerful tool. Through introductory studies we will lay the foundation for the preparation of an array that can be used in studies of signal pathways.

Our group has focused on cellular stress, inflammatory signals and the hMSC-TERT cell line. Studies have suggested that MSCs have an inhibitory effect upon adaptive immunity[37,51,52,67]. Allogeneic MSCs are not rejected by recipients[41]. Allogeneic transplantation will normally lead to inflammation and tissue rejection. Studies have shown that transplantation of MSCs from a third party, along with an allogeneic transplant inhibits the rejection process[38,40]. This is a very important discovery, and it suggests that MSCs have unique properties that may be used to prevent allogenic rejection[39]. The mechanisms behind this immunosuppressive effect are an important field of study.

Our group has shown that normal MSCs and hMSC-TERT produce the prostaglandin PGE2 constitutively (unpublished data), and PGE2 have been shown to inhibit T cell activation51. To study the effect of hMSC-TERT on activated T-cell proliferation in a co-culture the hMSC-TERT cells were irradiated to inhibit proliferation in these cells (that would interfere with the measured T-cell proliferation). The gamma irradiation increased both the PGE2 production and the inhibiting effect upon activated T-cells. This suggests

that irradiation has some effect upon PGE2 production. As the immunosuppressive effect of MSCs may be partially conducted by PGE2, any mechanisms PGE2 induction in these cells will be important to study.

A part of my master's objective is to begin introductory studies of signal pathways in hMSC-TERT with a focus on the cellular response to gamma irradiation induced cellular stress. Having first used Western blotting to investigate the gamma irradiation induced effect I will proceed to establish a small array with the same antibodies used in the introductory studies.

2.7.1. Cyclooxygenase enzymes and prostaglandin production

PGE2 and prostaglandin production are catalyzed by the cyclooxygenase (COX) enzymes[47,56]. The COX enzymes have important functions in the cardiovascular, neuronal, renal, immune, gastrointestinal and reproductive systems. This they do by catalyzing the formation of important biological mediators called prostanoids; including prostaglandins, thromboxane and levuloglandins.

2.7.2. Prostaglandins and PGE2

Prostaglandins (PG) are signal molecules that are involved in cell signaling via their binding to G-protein coupled receptors, and their signals mediate a wide array of functions in the tissues of the body. They are autocrine and paracrine lipid mediators which act upon many cell types. Pharmacological inhibition of the COX enzymes can provide relief from the symptoms of inflammation and pain. This is the method of action of well-known drugs such as aspirin and ibuprofen.

The COX enzymes convert arachidonic acid to prostaglandin H2 (PGH2), the precursor of all prostanoids. The enzyme contains two active sites; a heme with peroxidase activity, responsible for the reduction of PGG2 to PGH2, and a cyclooxygenase site, where arachidonic acid is converted into the hydroperoxy endoperoxide prostaglandin G2 (PGG2). PGE2 synthesis is mediated by the COX 2 enzyme.

2.7.3. The COX enzymes

The production of prostaglandins is mediated by the COX enzymes[20]. COX 1 and COX 2 are the most common and most studied isosymes. Different tissues express varying levels of COX-1 and COX-2. COX-1 is considered a constitutive enzyme, being found in most mammalian cells. COX-2, on the other hand, is undetectable in most normal tissues. It is an inducible enzyme, becoming abundant in activated macrophages and other cells at sites of inflammation. Thus PGE2 production is only located to sites of inflammation and sites where stimulus induces the COX 2 enzyme. COX-1 and COX-2 are of similar molecular weight (67 and 72 kDa respectively), and they have 65% amino acid sequence homology and near-identical catalytic sites.

2.7.4. Induction and regulation of COX 2

The promoter-region of the COX 2 gene contains sites where transcription factors bind, including cAMP (cyclic adenosine mono phosphate) response elements[72], C/EBP beta[58] and NF-kB[10] sites, and these act together for maximal induction of the COX 2 gene. DNA elements where protein factors that enhance transcription bind have also been identified[60].

Studies have suggested that mRNA stability is an important level of regulation in COX 2 transcription[17,53]. Dexamethasone have been shown to destabilize cytokine induced COX 2 mRNA in synovial fibroblasts[54]. It has been shown that p38 stress-activated pathway is critical for cytokine induced COX 2 mRNA stability[8,61]. The p38 pathway has also been proposed to be a part of a positive feedback loop leading to increased COX 2 mRNA stability[18]. COX 2 have been found to be up regulated in irradiated prostate cancer cells, where it was seen that pretreatment with NAC (N-Acetyl-l-cysteine) for 24 hours inhibited this effect, suggesting that the effect is mediated by reactive oxygen species[42]. COX 2 is often seen expressed in high levels in many types of cancer[5,27], including colorectal cancer[16,73]. In addition it has been shown that the tumor suppressor p53, which is activated by DNA damage and cellular stress, has an inhibiting effect upon the COX 2 gene[62].

3. Materials and methods

3.1. Buffers and solution

Lysisbuffer: 10mM Tris-HCL (Sigma Chemical Co., USA), pH 7.4, 0.15 M NaCl (Merck, Germany), 1 mM EDTA (Sigma Chemical Co.), 1 % (v/v) NP-40 (Nonidet P-40 from Calbiochem-Novabiochem Co. USA), 1mM Na3VO4 (Sigma Chemical Co.) and 50 mM NaF (Merck).

Protein transfer buffer: 25 mM Trizma base pH 10, 192 mM Glycine (both from Sigma Chemical Co.) and 20 % (v/v) Methanol (Prolabo, Merck).

Tris-buffered saline (TBS) (blot washing solution): 20mM Tris-HCL pH 7.6, 0.9 % (w/v) NaCl.

TBS-Tween (TBS-T): TBS with 0.1 % (v/v) Tween 20 (Polyoksyetylen sorbitan monolaurat) (Sigma Chemical Co.)

D-PBS (Dulbeccos phosphate buffered saline) (Gibco, Invitrogen Co., Great Britain); contains 0.9 % (w/v) NaCl and 10mM sodiumphosphate.

Trypsin: 0.25 % (w/v) trypsin in D-PBS (from PAA laboratories, Austria)

Dry milk blocking solution: D-PBS with 5 % (w/v) Molico dry milk (without fat) (NESTLE Switzerland and Norway) and 0.1 % Tween 20.

BSA blocking solution: TBS-T with 5 % (w/v) BSA (Bovine serum albumin) (Bio-Rad)

3.2. Antibody reagents

Anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) and anti-phospho-SAPK/JNK (Thr183/Tyr185), are both monoclonal mouse IgG from Cell Signaling Technology, USA.

Anti-pan ERK mouse monoclonal antibody (from Transduction laboratories, BD Biosciences, CA, USA).

Anti-actin (20-33) polyclonal rabbit IgG (20-33) (Sigma Chemical Co.).

Anti-phospho-p53 (Ser15), anti-phospho-NF-kB (Ser529), anti-phospho-ERK1/2 (Thr185/Tyr187), and anti-phospho-JNK 1/2 (Thr185/Tyr187), all polyclonal rabbit IgG from Biosource International, CA, USA.

Goat anti-rabbit IgG (H+L)-HRP and goat anti-mouse IgG (H+L)-HRP (Southern Biotechnology associates, Inc. USA)

Peroxidase conjugated affinity purified F(ab')2 Fragment donkey anti-mouse IgG (H +L), peroxidase conjugated affinity purified F(ab')2 Fragment donkey anti-rabbit IgG (H +L), peroxidase conjugated affinity purified F(ab')2 Fragment donkey anti-goat IgG (H +L), polyclonal IgG antibody from non-immunized mice and anti-rabbit IgG PE conjugate (mouse adsorbed) (anti-rabbit-PE), all from Jackson ImmunoResearch Laboratories, Inc., Pennsylvania, USA.

The following antibody reagents are from Santa Cruz Biotechnology (Sc/sc) (CA, USA): Sc-93 anti-ERK 1, sc-94 anti-ERK 1, sc-153 anti-ERK 2, sc-154 anti-ERK 2, sc-571 anti-JNK 1, sc-572 anti-JNK 2, sc-474 anti-JNK 1, sc-827 anti-JNK 2, all rabbit polyclonal IgG. Sc-7976 anti-pERK (Tyr204) and sc-1747 anti-Cox-2, both goat polyclonal IgG. Sc-1647 anti-ERK 2, sc-1648 anti-JNK 1, sc-7345 anti-JNK 2, sc-6254 anti-pJNK (Thr183/Tyr185), sc-7383 anti-pERK, all mouse monoclonal IgG.

3.3. Cell work methods

3.3.1. Culturing medium

RPMI 1640 (+L-glutamine) with 10% (v/v) inactivated fetal calf serum (FCS), 1 % (v/v) Sodium Pyruvate, 1 % (v/v) Non essential amino acids (all from Gibco), 50 µL/L 1 M monothioglycerol (Sigma Chemical Co.), Garamycin ® (40mg/mL) (Schering-Plough, Belgium).

3.3.2. Cells

hMSC-TERT[57]
K562[43]
Jurcat, human T-cell line.
EBV transformed human B-cell line.
Human mononuclear cells isolated from blood (provided by Ellen Karlstrøm, IMMI, Rikshospitalet).

3.3.3. Procedure for freezing cells

Freezing medium (FM), RPMI 1640 with 20% FCS and 20% dimethylsulfoxid (DMSO) (from Merck), were prepared by putting 50mL RPMI with 20% FCS on ice. 10 mL of DMSO were added drop by drop, while stirring the solution. (Dissolving DMSO will produce heat that may denature proteins.) Cells were centrifuged at 400 rcf for 8 min (using a Megafuge 1.0 from Heraeus Instruments). The supernatant was discarded, and the cells resuspended in RPMI with 20% FCS to a concentration of 5-20 mill/mL, and put on ice. After cooling the sample, a 1:1 volume of FM was added drop by drop while gently vortexing the tube and keeping it cold. The cell suspension was then added to Cryotube ™ vials (from NUNC A/S, Rosenkilde, Denmark) and incubated on ice for 10 min. Then the tubes were placed in a -70˚C freezer for 4 hours before placing it for long time storage in liquid nitrogen.

3.3.4. Procedure for thawing cells from a nitrogen tank

The sample tube was transferred directly from the nitrogen tank to a 37˚C water bath, and incubated until almost all the ice had thawed. The tube was then transferred to ice, to keep the temperature low. The thawed cell suspension were transferred to a 15 mL centrifugation tube (from Corning Incorporated, Corning NY, USA) and diluted with cold RPMI 1640 medium containing 5% FCS. The tube was centrifuged at 1300 rcf for 10 minutes. The supernatant were then discarded without disturbing the pellet, and to wash away DMSO the pellet were resuspended carefully in 1 mL cold D-PBS. Cold D-PBS was added to a total of 10mL. The tube was centrifuged at 1300 rcf for 10 minutes and the washing steps with D-PBS were repeated again. After washing twice the cells were resuspended in 1 mL medium (RPMI 1640 with 10% FCS), and counted using the cell counting procedure.

3.3.5. Cell counting procedure

The instrument (Coulter Z1 from Coulter Electronics Ltd., England) was maintained according to the manufacturer's instructions, and the recommended buffers were used. To count cells, 20 µL of cell suspension were added to 10 mL 0.9 % (w/v) NaCl in dH2O, and mixed well. The cells were then counted (cells between 5 and 50 µm), and results were displayed in 1000 = 10^6cells/mL.

3.3.6. Culture techniques

Cells were cultured in cell culture flasks (Nunclon ™ Surface, from NUNC), and 24 well/6 well plates (Corning Incorporated), covered in culturing medium. Cells were cultured up to 80-90% confluence. They were split and given new culturing medium regularly to maintain a stable environment. The cell incubator (Water Jacketed Incubator from Forma Scientific) holds a constant of 37 ˚C, 100 % moisture and 5.1 % CO2.

3.3.7. Trypsination

To remove adhesive cells from the culture plates, the medium was removed and the cells washed in D-PBS, witch was discarded. Trypsin was added (3-5 mL for flasks and 300-500 µL for wells) and the plate was incubated for 5 minutes at 37˚C or until cells loosened from the plastic surface. Medium was added to wash out the cells and the suspension was transferred to a centrifugation tube, and centrifuged at 1300 rcf for 10 min. The supernatant was discarded and the cells resuspended in fresh medium and counted according to the procedure above. The cells were then re-cultured in new flasks or stimulated/lysed.

3.3.8. Cell viability testing protocol

100x viability test stock solution were prepared by dissolving 50 mg etidium bromide and 15 mg acridin orange in 1mL 96% ethanol (all from Sigma Chemical Co.). 49 mL of dH-2O were added and the stock solution was stored in the freezer. Fresh solution was prepared by 100x dilution in D-PBS, and stored in the refrigerator.
To test the viability of a cell sample (cells thawed from freezer or stimulated cells), a drop of cells and a drop of the viability test solution were mixed on an object glass and were studied in a Leica fluorescence microscope (Leitz Wetzlar, Germany), with a FITC filter and a Xenon lamp. Green fluorescent, healthy cells, and red fluorescent damaged/dead cells were counted and the viability determined as a ratio of healthy versus dead cells.

3.3.9. Cell lysis protocol

Lysisbuffer were prepared and a mixture of protease inhibitors, Leupeptin, Antipain, Pepstatin A and Chymostatin, all used at 10 µg/mL (all from Sigma Chemical Co.) were added fresh. Cells in suspension were centrifuged at 200 rcf for 2 minutes at 4˚C. The supernatant was discarded, ice-cold lysisbuffer added, and the cells resuspended gently. For 0.5 -1.0 million cells 100 µL of lysisbuffer were added, while 200-500 µL of lysisbuffer were added to 5-10 million cells. (The exact amount of buffer was adjusted

according to the amount of cells in the sample, and anti-actin blots were produced to control the gel-loading concentration of all samples). After resuspending the cells in the lysisbuffer the samples were left on ice for 30 minutes. The suspension was then centrifuged at 1300 rcf for 10 minutes at 4˚C. The supernatant was transferred to a tube (1,5 mL micro tubes from Sarstedt, Numbrecht, Germany), and either frozen and stored directly in a -20˚C freezer, or heated at 95˚C for 5 minutes after adding 1:1 of 2X-SDS loading sample buffer.
(The storage of the lysates is a lot more stable after the SDS treatment, so the lysates without SDS treatment were not thawed and refrozen more than a couple of times.)

3.3.10. Lysate concentration test

To determine lysate concentration a RC DC protein assay from Bio-Rad were used following the protocol given by Bio-Rad.

3.3.11. Cell stimulations

Pervanadate (Pv) (a tyrosine phosphatase inhibitor) was prepared freshly by adding 3 % (v/v) H2O2 (Norsk Medisinaldepot, Norway) to 10 mM sodium orthovanadate (Sigma Chemical Co.). Pv stimulation was preformed using Pv to a final concentration of 0.2 mM in the medium. The cells were incubated for 5-10 minutes or longer at 37˚C before lysis.

Phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co.) was stored as a 1mg/mL stock in DMSO, and used at 50 ng/mL for PMA stimulations in overnight culture.

Dexamethasone (dexA) (Sigma Chemical Co.) was used at a final concentration of 2 mM in the culture medium where cells were treated with dexA.

5 mM N-Acetyl-L-cysteine (NAC) (pH adjusted to around pH 7 when dissolving) in culture medium were used as overnight pretreatment and overnight treatment of cells.

Gamma radiation treatment was done using a Gammacell® 3000 Elan MDS Nordion. The Gammacell® was certified 01.10.1998 with a dose speed of 5.34 Gy/min. Dose speed was calculated to be 4.55 Gy/min 01.10.2005 and 4.52 Gy/min 10.10.2006. 2 million hMSC TERT were suspended in 500 µL culturing medium in a tube after trypsination /counting. The samples, in tubes, to be irradiated were placed inside a 50 mL centrifugation tube (Corning Incorporated). This tube was then placed in the Gammacell® and irradiated for the desired dose. After irradiation the cells were either incubated at 37 °C for the desired time and lysed, or re-cultured for 24 hours, before lysis and western analysis.

For overnight incubations, $2 \cdot 10^6$ cells were used per sample, in 2 mL medium.
For 5-30 minute incubations, $0.5 \cdot 10^6$ cells were incubated in 500 µL medium.

3.4. SDS-PAGE and Western blotting

SDS-PAGE was preformed using the Laemmli[35] standard buffer and gel system. The prepared lysates were separated at 20 mA by SDS-PAGE gel electrophoresis using a Bio-Rad electrophoresis system. Each gel was run with 4 µL protein standard in a control well (Precision Plus ProteinTM Standards, Dual Color, from Bio-Rad). After running the gel it was prepared for Western blotting by incubation in for 10 min in protein transfer buffer.

The proteins were transferred (blotted) onto a PVDF membrane (polyvinyliden difluorid, Amersham Biosciences, UK) using a Hoefer Semiphor semi-dry transfer unit with 65 mA pr. gel for 1 hour. After blotting the membrane was removed and incubated in blocking buffer for 1 hour at room temperature (RT) with gentle rocking. Alternatively the blocking was carried out overnight at 4 ˚C with gentle shaking. Primary incubation was carried out overnight at 4 ˚C on a gentle shaker, or at RT for 1 hour, using the desired dilution of antibody in the desired blocking buffer.

After the primary incubation the membrane was washed 5x10 min in T-TBS. Secondary incubation was done for 1 hour at RT with gentle rocking, using the desired HRP conjugated second step antibody at the desired dilution. After the secondary incubation the membrane was washed 5x10 min in T-TBS. The membrane was incubated in 5 mL HRP - substrate for 5 minutes (SuperSignal® West Pico Chemiluminescent Substrate, Pierce, USA). The membrane was visualized by exposing the blot to a photo film, and the film was developed using standard procedures in the dark room.

For the slot blotting Western analysis 400 ng of pervanadate treated K562 lysate was run for the slot blots. After running broad well gels, and blotting the proteins as described in the blotting protocol above, and blocking the membrane in the desired blocking buffer, the membrane was placed in a Mini-protean®II multi screen unit (from Bio-Rad) (slot blot), and incubated with up to 20 primary antibodies overnight in the cold room. 600 µL (antibodies diluted in blocking buffer) were used in each well of the slot blot. After incubation the antibody solutions were removed and the wells washed three times in TBS-T. The blot was then removed and washed 2x10 min in TBS-T. Secondary incubation, washing and photo detection was then preformed as described above.

Some membranes were re-incubated with anti-actin control antibody, dried or stripped to re-incubate with another antibody. The membrane was placed in pre-warmed stripping buffer and incubated for 30 minutes at 60 °C. It was washed three times with T-TBS and blocked again for 1 hour before new antibody incubations.

3.5. Immuno precipitation procedure

Immuno precipitations (IPs) for western blotting analysis were made using 10 µL protein G sepharose (from Amersham Biosciences) pr. sample tube. The protein G sepharose was washed in 1 mL D-PBS with 0.1% (v/v) Tween 20 and 1.5 mM EDTA (Sigma Chemical Co) (PBS-TE), and spun down using a micro centrifuge. The supernatant was discarded and 10 µL of antibody solution were added to each tube and incubated for 1 hour at RT with occasional shaking. The protein G sepharose was then washed with 1 mL PBS-TE,

spun down and the supernatant discarded. Lysate from Pv treated K562 cells were denatured by adding 1% (w/v) SDS (Sigma Chemical Co.), and heating at 95 °C for 5 minutes. The SDS was removed on a biospin column (micro Bio-Spin® chromatography column from Bio-Rad) with Sephadex® G-25 Sepharose (from Pharmacia Biotech, Sweden) in PBS-TE. 100 µL denatured lysate were added on the biospin column, and 20 µL PBS-TE were added before spinning the tube at 1500 rcf for 5 minutes. 20 µL of the buffer-exchanged lysate were added to each tube and immuno precipitated over night at 4°C with gentle shaking. The protein G Sepharose IPs was then washed in 1 mL PBS-TE tree times, discarding the supernatants after spinning down the sepharose. The IP's were heated for 5 minutes in 20 µL of 2X SDS loading sample buffer, and the samples were analyzed using western protocol as describes above.

3.6. Assay preparation and flow cytometry

Nine fluorochrome-colored latex bead populations were coupled (procedures cannot be described here due to patenting) to selected monoclonal anti MAPK antibodies (1 µg IgG per 10^6 particles) (see figure 17) and mixed together to form a nine-bead assay. The beads were blocked in PBS-TE with 10 % (v/v) mouse serum (Jackson ImmunoResearch) for one hour.

Pv-treated K562 cells were lysed and denatured at 95 °C with 1% (w/v) SDS added to the lysate. SDS was removed using buffer-exchange on a biospin column with G-25 Sepharose in PBS-TE.

The mixed assay beads were divided into a number of sample tubes and each tube was incubated with the denatured cell lysate over night at 4 °C.
The beads were then washed with PBS-TE, incubated with 2 µg of indicated detection rabbit anti-MAPK antibody (see 4.2. Flow cytometry bead assay results) in PBS-TE. They were washed again (as above) and then incubated with 10 µL of anti-rabbit-PE (mouse adsorbed) diluted 1:100 in PBS-TE with 5% human albumin (Octapharma AG, Ziegelbrücke, Switzerland).

Flow cytometer instrument is FACSCalibur coupled to a MAC computer with CellQuestTM software program (both from Becton Dickinson, USA)
This flow cytometers readout channels include FSC (forward scatter), SSC (side scatter, below 500 nm), FL-1 green, FL-2 yellow (PE), FL-3 red and FL-4 red.

3.7. Lysate biotinylation procedure

For lysate biotinylation 100 µg/mL biotin (Molecular Probes, Oregon, USA) was added to 2 mg/mL lysate, and incubated for 10 minutes. The free biotin was removed on a biospin column with G-25 Sepharose in PBS-TE.

4. Results

The main objective was to establish a multiparameter test system for flow cytometric detection of signaling molecules. Fluorochrome conjugation, antibody coupling to particles, properties of different types of particles, stability of stained particles and antibody conjugation and optimal array conditions were tested.

Table 1 – Antibodies tested in our test system and Western analysis

Antibody and species:	Company:	Epitope: (from datasheets distributed by the company)	Antibody dilution in Western:
Sc-93 anti-ERK 1	Santa Cruz Biotechnology	C-16, C-terminus	1:1500
Sc-94 anti-ERK 1	Santa Cruz Biotechnology	K-32, subdomain XI	1:3000
Sc-153 anti-ERK 2	Santa Cruz Biotechnology	K-32, subdomain XI	1:3000
Sc-154 anti-ERK 2	Santa Cruz Biotechnology	C-14, C-terminus	1:3000
Sc-571 anti-JNK 1	Santa Cruz Biotechnology	FL	1:1500
Sc-572 anti-JNK 2	Santa Cruz Biotechnology	FL	1:300
Sc-474 anti-JNK 1	Santa Cruz Biotechnology	C-17, C terminus	1:300
Sc-827 anti-JNK 2	Santa Cruz Biotechnology	N-18, N terminus	1:300
Anti-pERK	Biosource	pThr185/pTyr187	1:3000
Anti-pJNK	Biosource	pThr183/pTyr185	1:3000
Polyclonal goat IgG:			
Sc-7976 anti-pERK	Santa Cruz Biotechnology	pTyr204	1:1500
Sc-1747 anti-COX 2	Santa Cruz Biotechnology	M-19, C-terminus	1:2000
Monoclonal mouse IgG:			
Sc-1647 anti-ERK 2	Santa Cruz Biotechnology	D-2, C-terminus	1:3000
Sc-1648 anti-JNK 1	Santa Cruz Biotechnology	F-3, FL	1:300
Sc-7345 anti-JNK 2	Santa Cruz Biotechnology	D-2, FL	1:1000
Sc-6254 anti-pJNK	Santa Cruz Biotechnology	G-7, pThr183/pTyr185	1:300
Sc-7383 anti-pERK	Santa Cruz Biotechnology	E-4, pTyr204	1:1500
Anti-p44/42 MAP kinase, anti-pERK	Cell Signaling Technology	pThr202/pTyr204	1:3000
Anti-pSAPK/JNK	Cell Signaling Technology	pThr183/pTyr185	1:500
Anti-pan ERK	Transduction laboratories BD		-
Anti-pp38/SAPK	Transduction laboratories BD	pThr180/pTyr182	1:1500

Having optimized methods for producing fluorochrome coupled particles that can be sufficiently distinguished in a flow cytometer; we proceeded to establish the test system.

If the test system can detect and discriminate between closely related analytes, it may be useful for multiparameter analysis. Distinguishing between analytes is the main challenge when detecting multiple analytes simultaneously. To be able to distinguish related analytes, it is critical to have antibodies with the desired properties. The properties of both the capture antibody and the detection antibodies will be important in each immuno-sandwich.

Antibodies directed against the ERK and JNK signaling protein families were selected as a model system. The antibody panel (Table 1) included monoclonal and polyclonal antibodies, from different companies, against epitopes on these two protein families (ERK and JNK). The antibodies were phospho-specific or isoform specific.
Monoclonal antibodies were used as capture antibodies on the particles, and polyclonal antibodies as detection antibodies. PE conjugated anti-rabbit IgG (anti-rabbit-PE) was used to detect the immuno sandwiches formed (figure 15).

4.1. Making a bead array

For trial purposes we needed a small array. Nine differently colored particle populations were prepared. Eight of the populations were coupled with mouse monoclonal anti-ERK and anti-JNK antibodies (figure 18). The last particle population was coupled with non-immune polyclonal mouse IgG as a negative control (isotype) (figure 18). Finally the particles were mixed. An array sample was run in a flow cytometer, to ascertain separation of particle populations.
The forward and side scatter of the particles is displayed in figure 16. The nine separate populations of fluorochrome stained particles are shown by fluorescence levels in the two colors, measured by the FL1 and FL4 channels of the flow cytometer (figure 17) (only

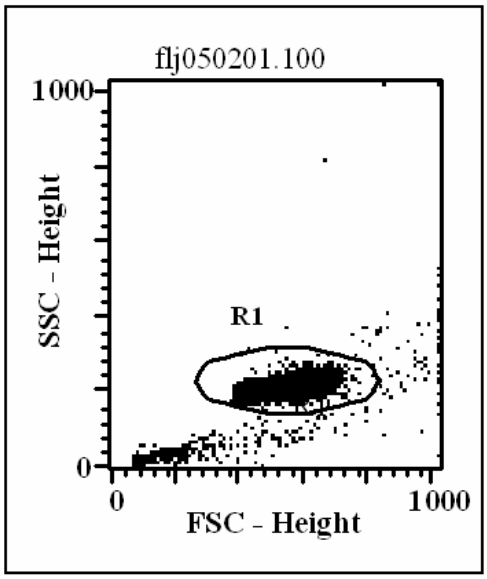


Figure 16. Forward and side scatter

particles confined to the R1 region are displayed here). The particle populations measured in one sample have been split into two regions; R2 and R3, and these regions will be displayed separately in the subsequent flow cytometer figures.

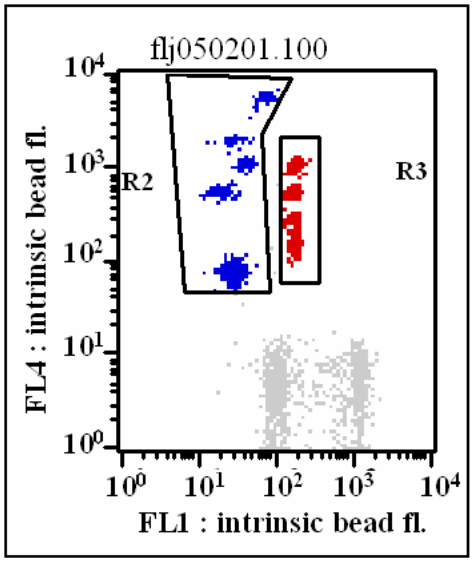


Figure 17. Bead regions by bead fluorescence

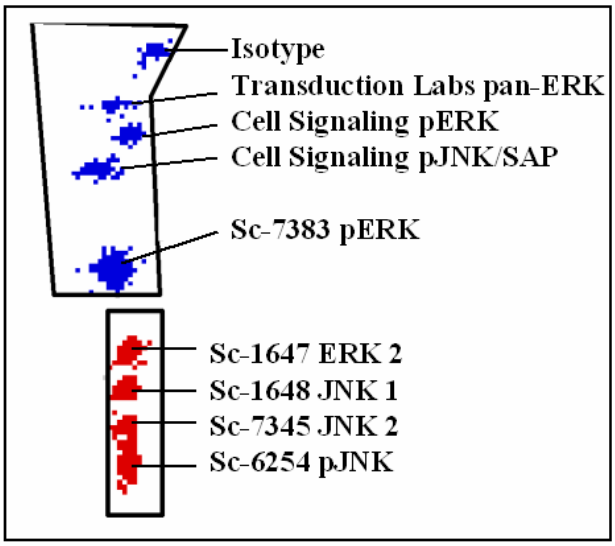


Figure 18. Monoclonal Ab. on the beads

4.2. Flow cytometry bead array results

The particle array was split into several samples, and incubated with SDS-denatured lysate from pervanadate treated K562 cells. Denaturing was chosen since the array antibodies were directed against sequential epitopes. Pervanadate treatment was performed as phosphospecific antibodies were included in the array. After lysate incubation one detection antibody were added to each sample. Since the polyclonal detection antibodies were unlabeled species-specific anti-rabbit IgG antibodies coupled with PE were added on top. This was done to test if the capture and detection antibodies could bind their specific analytes.

The samples were then run in the flow cytometer, and the results are listed one by one below. The results from the detection of the reactivity and property of the different antibodies will indicate if the test system can detect and discriminate related analytes.

4.2.1. Sc-94 anti-ERK 1 array detection

The reactivity of the sc-94 anti-ERK 1 detection antibody was investigated.

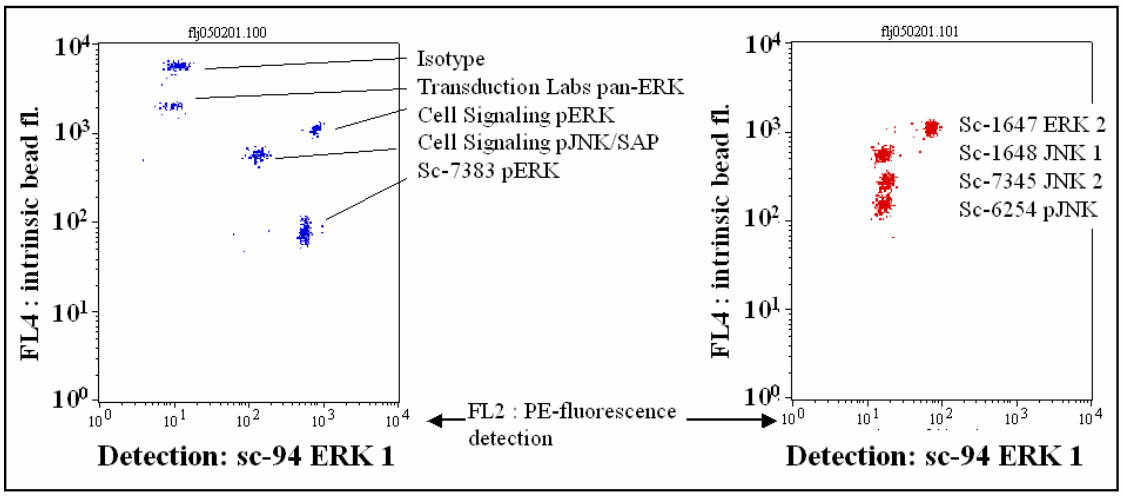


Figure 19: Array with indicated capture antibodies was incubated with lysate and the sc-94 detection antibody was added. Anti-rabbit-PE was used to detect binding of the detection antibody. The x-axis displays the PE -fluorescence level measured on each particle population. The isotype control shows low background. No analytes captured with the Transduction Labs pan-ERK can be detected by sc-94. The sc-94 ERK 1 detection antibody binds analytes on both the Cell Signaling pERK and the sc-7383 pERK, resulting in a signal increase of nearly two logs. Sc-94 has some cross reactivity to analytes on the sc-1647 ERK 2 coupled particles, resulting in a signal increase under one log. The sc-94 ERK 1 also gives one log signal increase with binding to the cell signaling pJNK/SAPK antibody bound analyte, but not with any of the other JNK-capture antibodies.

Figure 19 show that the sc-94 anti-ERK 1 antibody gives good signals in sandwich with the pERK antibodies. It cannot bind the analytes captured by pan-ERK. The cross reactivity seen with analytes bound by the ERK 2-capture antibody is either due to ERK 2 capturing ERK 1 that is detected by sc-94, or due to sc-94 cross reacting with ERK 2. The cross reactivity seen with analytes bound by the pJNK-capture antibody is either due to the pJNK antibody capturing ERK 1 that is detected by sc-94, or due to sc-94 cross reacting with JNK.

4.2.2. Sc-153 anti-ERK 2 array detection

The reactivity of sc-153 anti-ERK 2 was investigated.

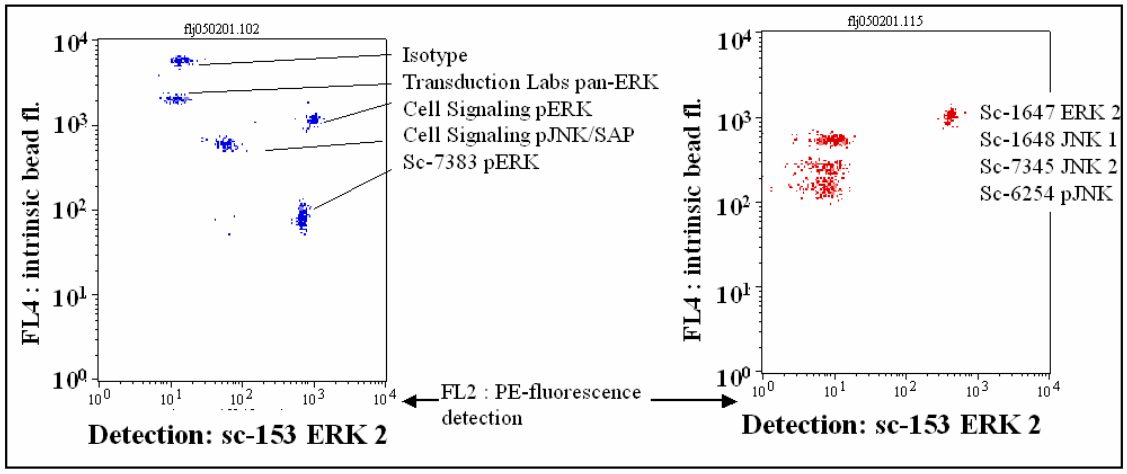


Figure 20: Sc-153 ERK 2 detects analytes bound to both the cell signaling pERK and the sc-7383 pERK, resulting in a signal increase of nearly two logs. It detects the sc-1647 ERK 2 bound analyte with a signal increase of about one and a half logs. It gives about one log signal increase with binding to the Cell Signaling pJNK/SAPK bound analytes, but does not detect any of the other JNK target analytes. It does not bind the analytes on the Transduction Labs pan-ERK.

Figure 20 show that the sc-153 anti-ERK 2 antibodies give strong signals in sandwich with the pERK antibodies and the sc-1647 ERK 2 antibody. Sc-153 has cross reactivity towards analytes bound by the Cell Signaling pJNK.

4.2.3. Sc-154 anti-ERK 2 array detection

The reactivity of sc-154 anti-ERK 2 was investigated.

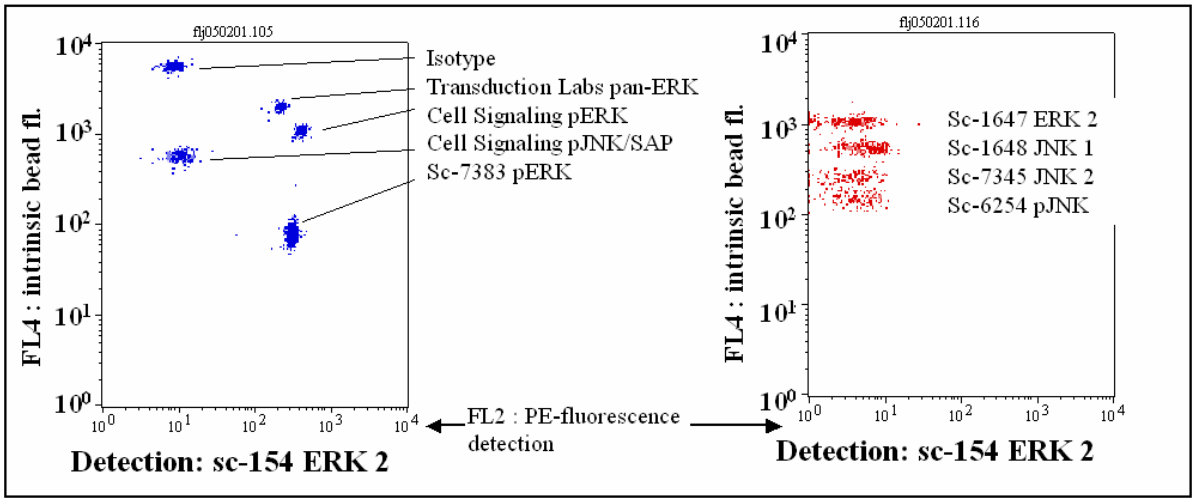


Figure 21: The Transduction Lab pan-ERK bound analytes give a signal increase of one and a half logs with sc-154 detection. The sc-1647 ERK 2 bound analyte is not at all detected with sc-154. The two pERK antibody target analytes are detected with a signal increase of two logs. None of the JNK capture antibodies have bound analytes that are detected with sc-154.

From figure 21 we see that the sc-154 anti-ERK 2 antibodies give strong signals in sandwich with the pERK and the pan-ERK bound analytes. It cannot bind analytes on the sc-1647 particles.

4.2.4. Sc-474 anti-JNK 1 array detection

The reactivity of sc-474 anti-JNK 1 was investigated.

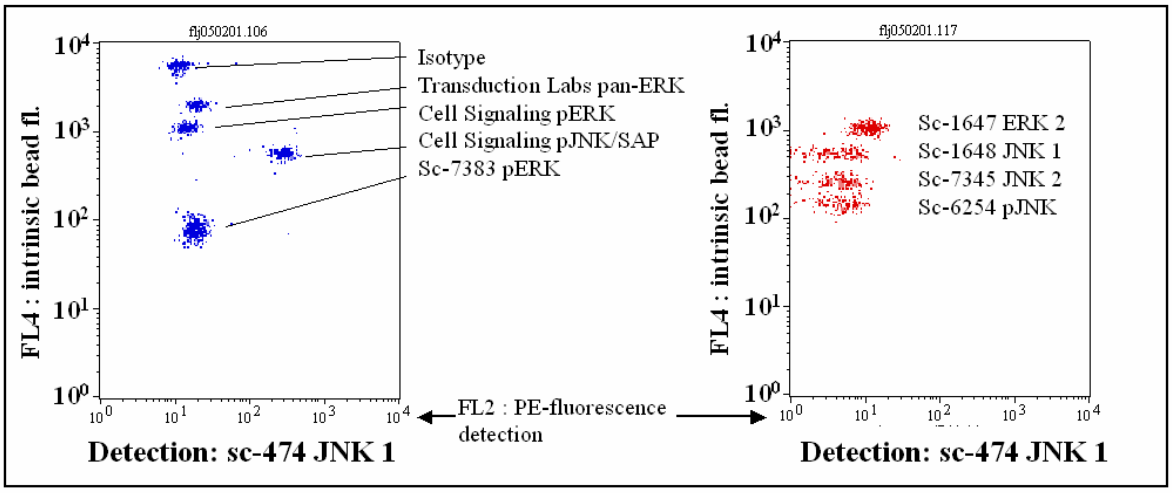


Figure 22: The sc-474 detects the Cell Signaling pJNK antibody bound analyte. The sc-1647 ERK 2 bound analytes give a weak signal with sc-474 detection.

From figure 22 it's seen that sc-474 only detects analytes on the Cell Signaling pJNK, and that sc-474 cross-reacts with analytes bound by sc-1647 ERK 2. This is either due to ERK 2 binding JNK analytes or the JNK antibody binding ERK 2.

4.2.5. Sc-571 anti-JNK 1 array detection

The reactivity of the sc-571 anti-JNK 1 detection antibody was investigated.

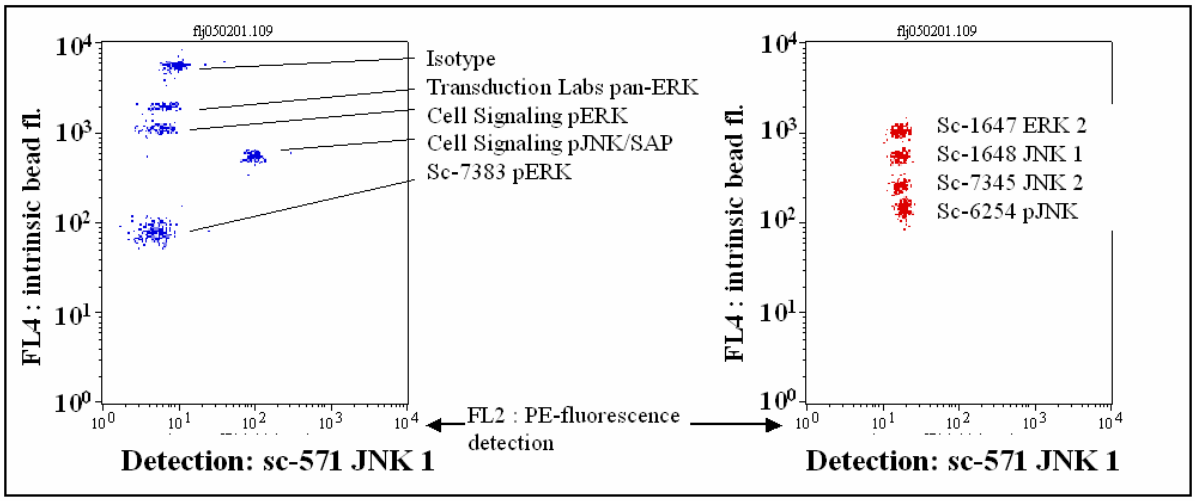


Figure 23: The sc-571 detection gives approx. one log signal increase with the Cell Signaling pJNK/SAPK bound analytes. No other reactivity is seen.

Figure 23 shows that this antibody only binds analytes captured by the Cell Signaling pJNK/SAPK antibody, and that it has no cross reactivity to analytes bound in the array.

4.2.6. Sc-572 anti-JNK 2 array detection

The reactivity of sc-572 anti-JNK 2 was detected.

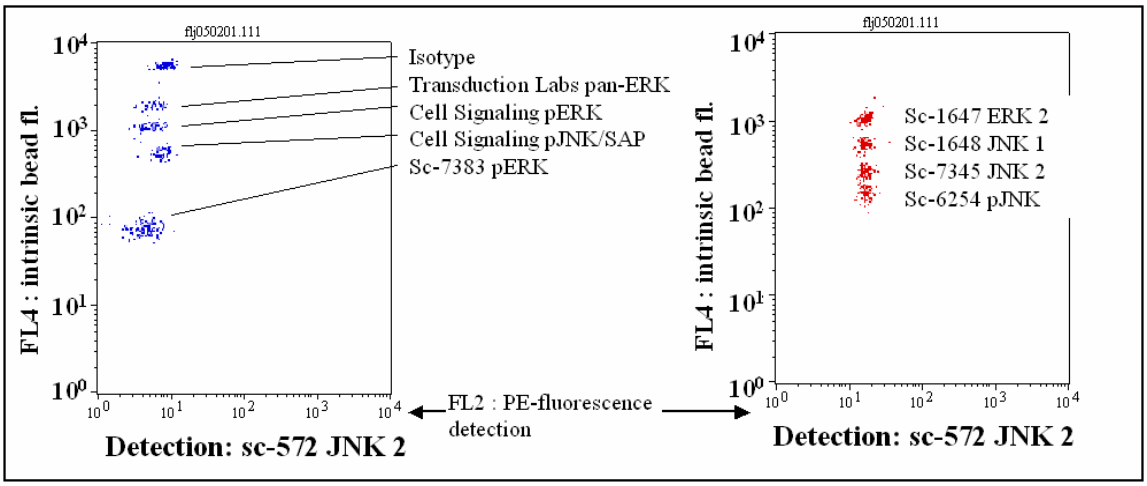


Figure 24: No signal is detected in the array with sc-572 JNK 2.

From figure 24 it's seen that this antibody does not detect any analytes in this array.

4.2.7. Sc-827 anti-JNK 2 array detection

The reactivity of sc-827 anti-JNK 2 was tested.

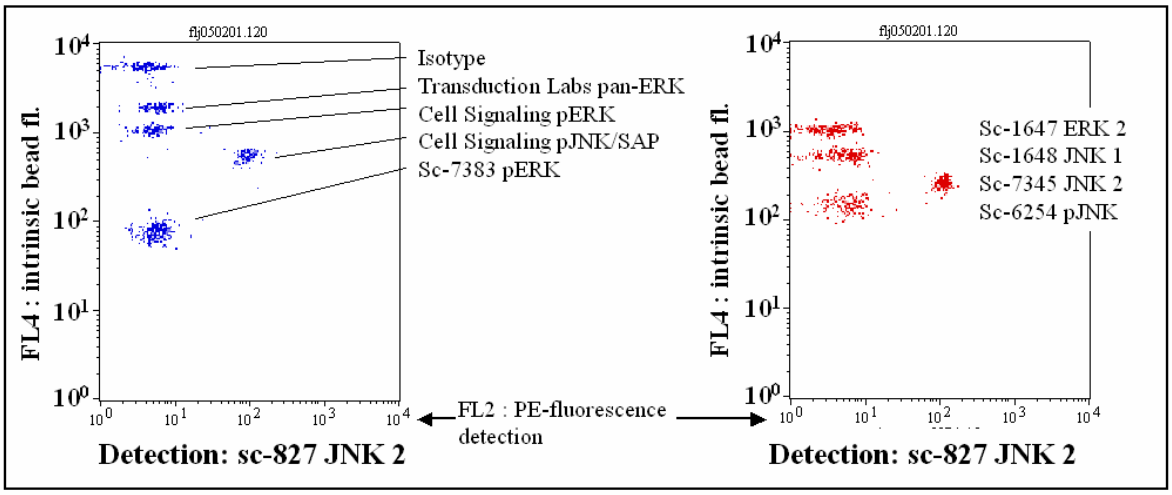


Figure 25: The Cell Signaling pJNK/SAPK and the sc-7543 JNK 2 bound analytes are detected with approx. one log increase of signal when using sc-827 JNK 2. No other signals are seen.

Figure 25 shows that the sc-827 antibody detects two of anti-JNK bound analytes, and no cross reactivity is seen.

Some of the tested antibodies have cross reactivity, while others only bind their specific analytes. Some of the antibodies do not work in the array at all.

4.3. Western results

Having investigated the panel of antibodies in the array test system, the same antibodies were tested in Western blotting analysis. Western analysis was chosen as it is counted as the Golden standard of immunoassays. Western analysis gives a separation of proteins according to molecular weights and was used to verify that the sandwiched analytes in the array are proteins of the right molecular weight. The Western results would also indicate if the antibodies had the same level of reactivity compared to each other as in the test system.

4.3.1. Western detection with polyclonal anti-ERK and anti-JNK

The reactivity of the polyclonal anti-JNK and anti-ERK antibodies were tested in Western analysis (some anti-ERK and anti-JNK that were included that were not tested in the array). The panel antibodies were titrated before use in these experiments. Results have indicated that the lysates contain more ERK than JNK proteins.

ERK 1 and ERK 2 have molecular weights of 44 kilo-Dalton (kDa) and 42 kDa respectively. For JNK 1 two isoforms exist, of 44 kDa and 48 kDa molecular weight. The JNK 2 isoforms have 44 kDa and 48 kDa molecular weights. JNK 3 also has two isoforms that have 48 kDa and 52 kDa molecular weights. As ERK 1 and the 44 kDa JNK isoforms have the same molecular weight, it will be difficult to verify cross reactivity in this region. In addition phosphorylation of the proteins may alter the exact area where the proteins separate. A 15% gel was used to achieve separation in the 37-50 kDa region. The blot was developed and is displayed in figure 26. Antibody dilutions are listed in Table 1.

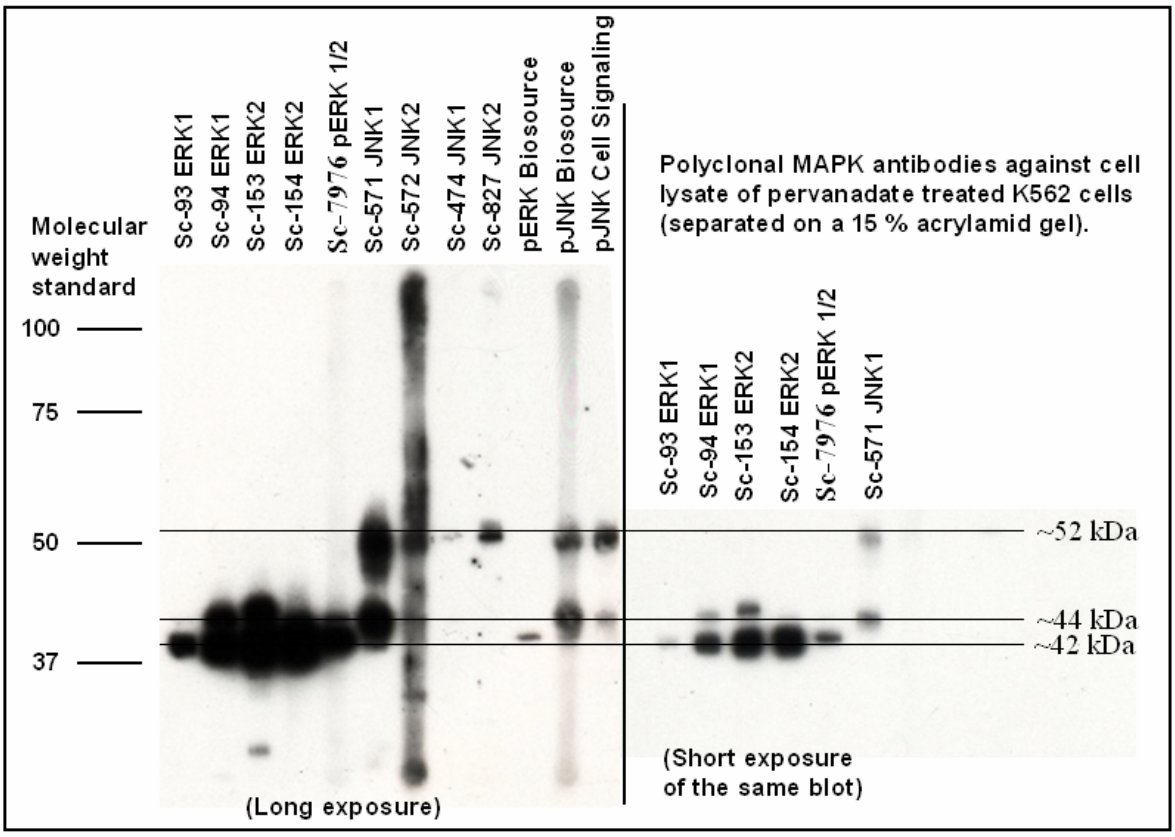


Figure 26: Detection of polyclonal anti-MAPK antibodies.
Sc-93 ERK 1 binds a ~42 kDa analyte, which most likely is ERK 2, while the antibody is directed against ERK 1. Sc-94 ERK 1 gives ~42 kDa and ~44 kDa bands, which are probably ERK 1 and ERK 2. Sc-94 cross-reacts strongly with ERK 2. The ~44 kDa band can contain crossing with 44 kDa JNK. Sc-153 ERK 2 binds ~42 kDa ERK 2 but cross-reacts with an analyte at ~46 kDa. In the long exposure we see that sc-153 also has cross reactivity towards a ~25 kDa analyte. Sc-154 ERK 2 binds ~42 kDa ERK 2. The sc-7976 pERK and the Biosource pERK give bands at ~42 kDa, and the sc-7976 antibody gives the strongest signal. In the long exposure for sc-7976, we see both 44 kDa and 44 kDa bands. Sc-571JNK 1 give strong signals around 44 kDa, which is where JNK 1 separates, and 52 kDa where JNK 3 separates. In the long exposure it has some signal in the 42 kDa region which can be cross reactivity to ERK 2, and the 48 kDa region where JNK 1 isoforms may separate. The sc-572 JNK 2 gives a lot of background, and seems to have a band in the 52 kDa region, but bands of cross reactivity is also seen at other molecular weights. In the short exposure the rest of the JNK antibodies do not give any signal but signals come up when exposing the blot longer. The sc-474 JNK 1 gives what looks like a weak signal around 52 kDa while the sc-827 JNK 2 gives a stronger signal in the same area, suggesting they cross react with JNK 3. The Biosource and Cell Signaling pJNK antibodies bind targets in the 44 kDa and 52 kDa regions.

From figure 26 it's clear that the ERK antibodies generally give strong signals but many of them cross react with ERK isoforms they are not directed against. The sc-153 ERK 2 antibody cross-reacts with an analyte in the 25 kDa region. The JNK antibodies also experience some cross reactivity to other JNK isoforms. The pJNK antibodies work well. A couple of the JNK antibodies react poorly, either giving background or very weak signals.

4.3.2. Western detection of monoclonal anti-ERK and anti-JNK

The reactivity of the monoclonal anti-JNK and anti-ERK antibodies were tested in Western analysis (an antibody against the MAPK p38 protein was included). Antibody dilutions are listed in table 1.

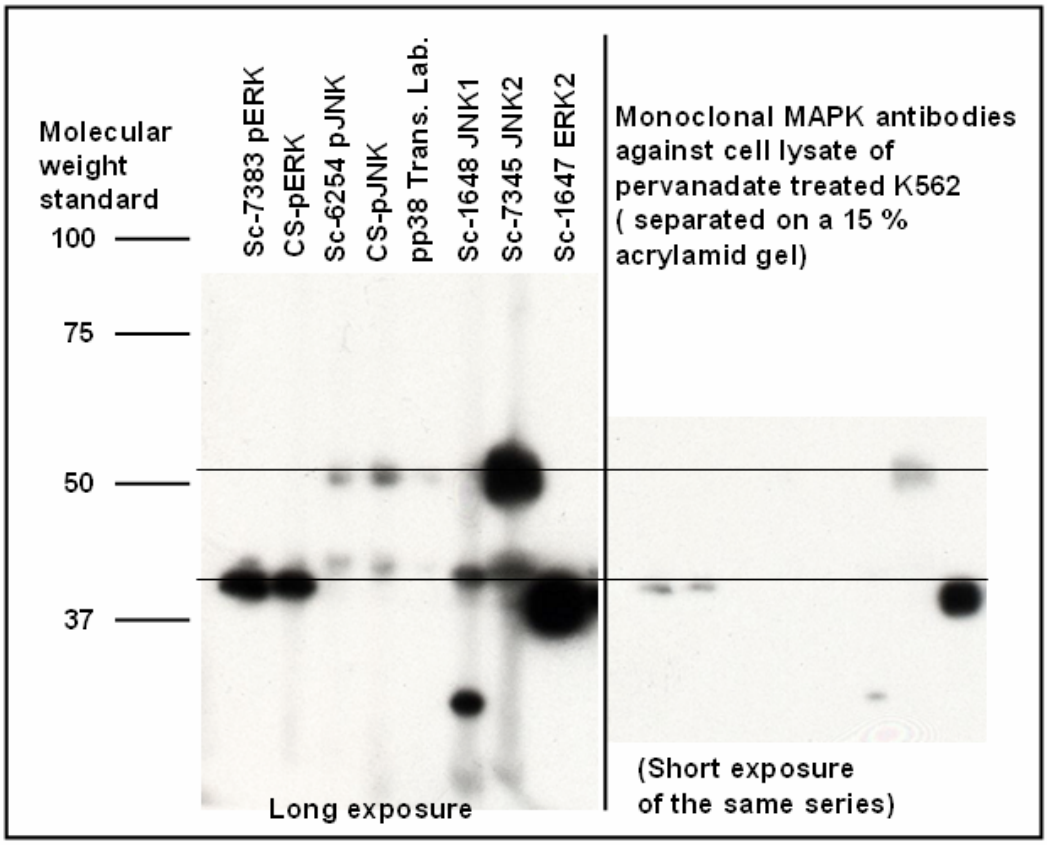


Figure 27: The pERK antibodies have very similar reactivity, binding 42 kDa ERK 2 and 44 kDa ERK 1. The pJNK antibodies bind 44 kDa JNK ½ and 52 kDa JNK 3. The pp38 antibody has a weak cross reactivity to JNK and no band can be seen around 38 kDa. The sc-1648 JNK 1 antibody gives a signal at 44 kDa that is probably JNK 1, but it also cross reacts with an analyte at 25 kDa. The sc-7345 JNK 2 antibody gives a signal at 44 kDa that is JNK 1 or JNK 2 and also a very strong signal from cross reactivity with JNK 3 at 52 kDa. The sc-1647 ERK 2 antibody gives a very strong signal with ERK 2 at 42 kDa.

From figure 27 it's clear that the phospho-specific antibodies give good signals at the right molecular weights. The sc-1648 JNK 1 cross-reacts with a 25 kDa analyte. The JNK 2 cross-reacts with JNK 3. The ERK 2 antibody binds ERK 2.

4.3.3. Immunoprecipitation and Western detection with anti-ERK and anti-JNK

When separating proteins from lysate by SDS-PAGE electrophoresis and detecting by Western blotting, we se whether the antibodies bind their right targets. Detection of proteins on a membrane, and detection of proteins that are captured by an antibody coupled to a bead are done under different conditions, and the reactivity and properties of the antibodies may be slightly different. To better simulate the array conditions while detecting the results in Western analysis, immunoprecipitation was performed and analyzed in Western. Two anti-ERK 2 and three anti-JNK antibodies (all polyclonal) were coupled to protein G Sepharose. These were incubated in the same lysate as used in the experiments above. The immuno-precipitated proteins were denatured and the five samples were run as two series on two 10 % SDS-PAGE gels. One of the two resulting blots was detected with a monoclonal anti-ERK 2 antibody, to detect whether the ERK and JNK antibodies would capture ERK targets in the IP. The other blot was detected with a monoclonal anti-JNK antibody, to detect whether the ERK and JNK antibodies would capture JNK targets in the IP. The binding of monoclonal antibody was detected with an anti-mouse IgG HRP conjugate, and the blots were developed. After being developed, both of the blots were incubated with an anti-rabbit IgG HRP conjugate to detect heavy and light chains that come from the capture antibody in the IP. Thus the analyte and heavy/light chain staining can be discriminated if the conjugate cross reacts. The ERK-detection of the polyclonal IP series is shown in figure 28.

From figure 28 we see that sc-153 and sc-154 can precipitate 42 kDa ERK 2 and that the sc-1647 ERK 2 detects the ERK 2 giving strong signals.

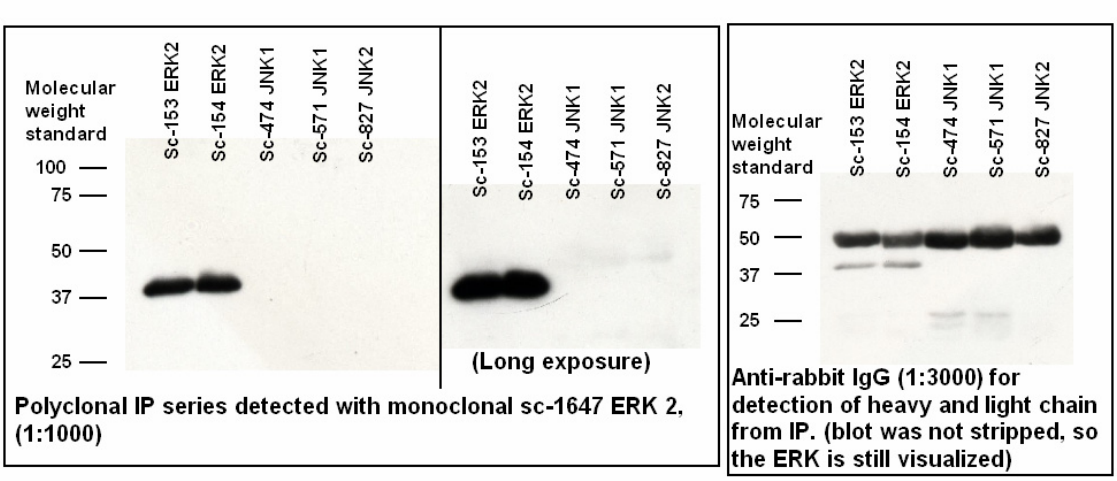


Figure 28: The sc-153 ERK 2 and sc-154 ERK 2 both immunoprecipitated ERK 2 that were detected by sc-1647 ERK 2, giving signals at 42 kDa. In the long exposure a very weak signal can be seen in the sc-571 and sc-827 samples. The detected heavy chains are seen around 50 kDa, above the ERK signals.

The JNK detection is shown in figure 29.

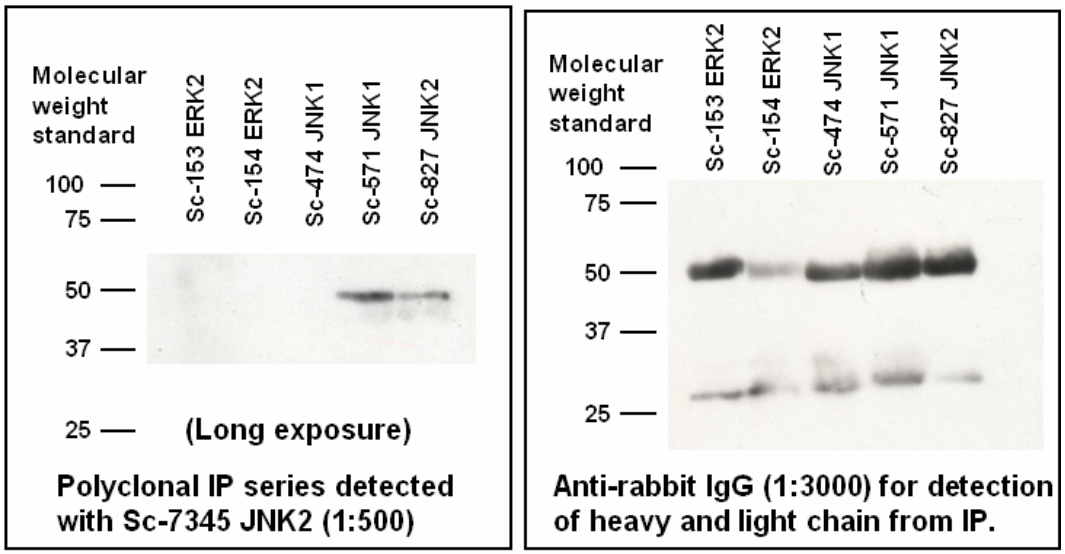


Figure 29: The ERK 2 targets give no signals when detected with sc-7345 JNK 2. The sc-571 JNK 1 IP and sc-827 JNK 2 IP both give signals around 50 kDa, which is not very strong (long exposure) and very weak signals around 45 kDa that can be JNK 1 or 2. The 50 kDa band is either cross-reaction to JNK 3 that has been IP'd, and cross-reacting detection by the JNK 2 antibody, or crossing of the anti-mouse IgG with the capture antibody heavy chains. The heavy chains give signals in the 50 kDa regions.

From figure 29 it's seen that the JNK antibodies precipitate analytes that separate around 45 kDa, giving very weak signals. The 50 kDa bands is either cross reaction of JNK 1 and 2 with JNK 3, or cross reaction of anti-mouse IgG with rabbit IgG.

The results demonstrate that the test system can distinguish and detect closely related signaling proteins, if antibodies with the right reactivity's are selected. Many of the antibodies tested are not of this quality, and have cross reactivity to isoforms in the same family or to closely related families.

Having investigated the potential of the test system, introductory studies were performed as a basis for the construction of a new array. These introductory studies were focused on gamma irradiation induced stress and mechanism for PGE2 induction. Thus we can obtain an array for our test system that can be used to study stress related signal pathways in stem cells.

To construct a multiparameter array for cell signaling studies, antibodies were selected and tested in Western blotting for their reactivity. The selection of antibodies was based on introductory studies of the gamma-induced effect.

4.4. Introductory studies of hMSC-TERT and gamma radiation

The immunosuppressive effect of MSCs is most likely mediated by PGE2. Our group has shown that normal MSCs and hMSC-TERT constitutively produce PGE2. In co-culturing experiments with activated T-cells, MSCs inhibited proliferation of activated T-cells. In an experiment this kind of it was discovered that gamma radiation increased the PGE2 production and the proliferation inhibiting effect on activated T-cells. Due to this discovery the question whether gamma radiation could induce COX 2, an enzyme that mediates PGE2 synthesis was investigated. After introductory studies in this effect the object was to construct an array directed against the signal molecules involved, thus obtaining a tool for studies of gamma irradiation induced signaling.

4.4.1. Gamma radiation and NAC with COX 2 detection

To investigate if Gamma irradiation could induce COX 2 in hMSC-TERT and if NAC (N-Acetyl-L-cysteine) would inhibit this induction, hMSC-TERT was stimulated with

gamma irradiation with and without 5 mM NAC present. If reactive oxygen species mediated the gamma induced COX 2 expression, the antioxidant NAC would inhibit this effect. Anti-COX 2 was used to detect if this enzyme was induced in the cells. Unstimulated and PMA stimulated hMSC-TERT were included as controls.

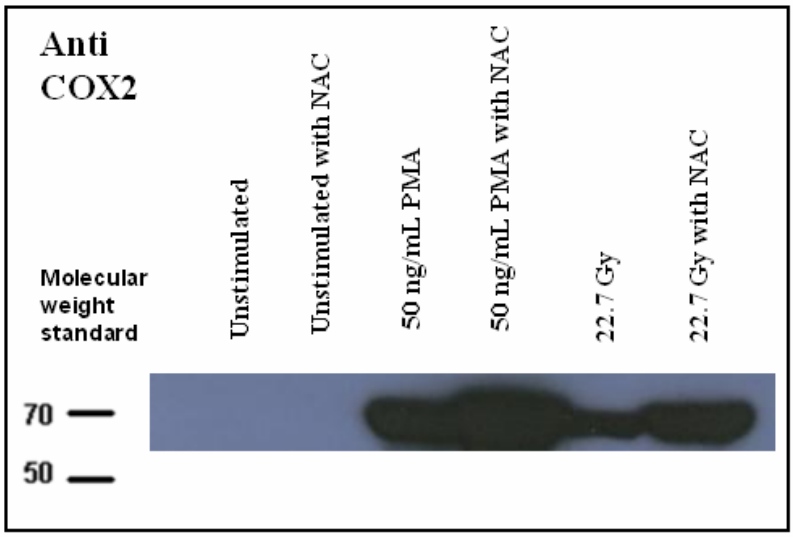


Figure 30: Induction of COX 2 expression mediated by gamma irradiation and PMA. NAC does not inhibit, but increases the PMA and gamma irradiation induced COX 2 expression. hMSC-TERT was cultured for 24 hours with and without 5mM NAC present. The NAC pretreated and non-pretreated hMSC-TERT cells were then stimulated with PMA and 22.7 Gy gamma irradiation and incubated overnight. The NAC pretreated cells were cultured with 5mM NAC present after the stimulations. Lysates were prepared and analyzed by Western blot with anti-COX 2 at 1:2000.

From figure 30 it is clear that gamma irradiation induces COX 2, and that NAC does not inhibit, but in fact increases the induced COX 2 expression. The induction of COX 2 in these cells is not mediated by oxygen radicals.

Results in our lab have shown that COX 2 is detectable in our cells about 4-5 hours after radiation, with the levels increasing to the strength of the signal seen after overnight incubation in the results above. We have not been able to detect this gamma-induced effect in other cell types (Jurcat a human T-cell line, EBV transformed B-cells and Human mononuclear cells) tested in similar experiments, suggesting this effect is cell-specific for the hMSC-TERT cell line.

4.4.2. The effect of dexamethasone upon gamma induced COX 2

Dexamethasone has been shown to affect stability of COX 2 mRNA and to inhibit NF-kB activation. This experiment was performed to investigate if dexamethasone could inhibit the gamma radiation- induced COX 2 expression, and to investigate the relationship between gamma radiation dose and COX 2 expression.

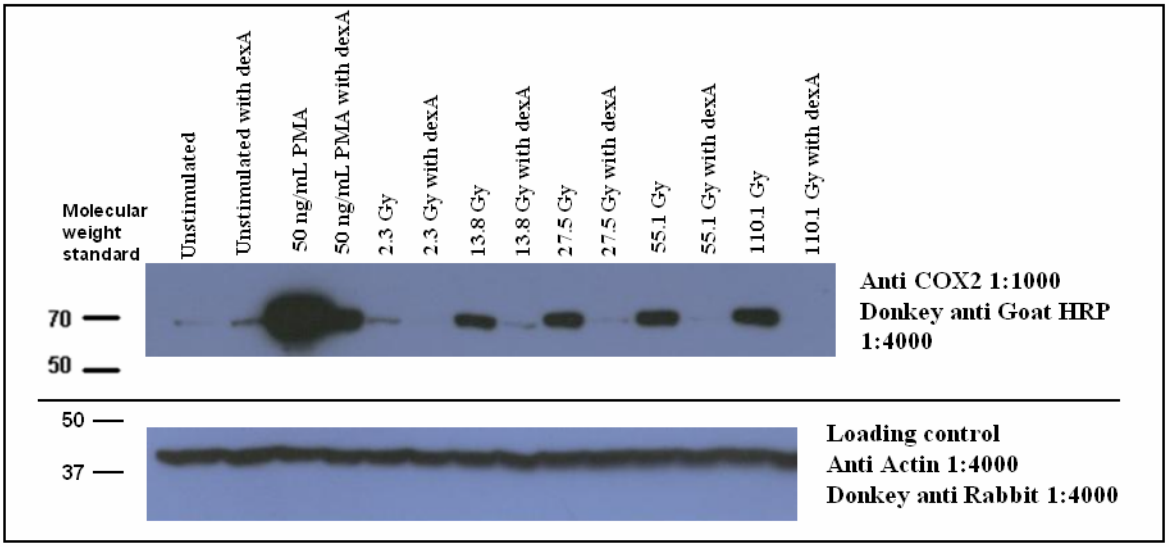


Figure 31: Inhibition of gamma irradiation induced COX 2 expression by dexamethasone (dexA), and gamma irradiation dose response. Dexamethasone inhibits the PMA and gamma irradiation induced COX 2 expression. The induced COX 2 expression increases with increasing Gy radiation dose. hMSC TERT were stimulated with increasing doses of gamma irradiation with or without dexamethasone present, and PMA and unstimulated controls were included.

From figure 31 we see that dexamethasone inhibits COX 2 induction, and that increasing doses of gamma irradiation gives increasing induction of COX 2.

4.4.3. Gamma irradiation induced DNA damage response

Gamma irradiation induces DNA damage, leading to signaling events in the cells that affect DNA repair and cell cycle arrest. The induction of COX 2 expression in our cells in response to gamma irradiation might be induced by DNA damage signaling events. p53 is a important protein in DNA damage response signaling, and it regulates the cell cycle in response to DNA damage and cellular stress. To investigate the level of signaling

in response to DNA damage we investigated the level of activating phosphorylation in p53 after irradiation.

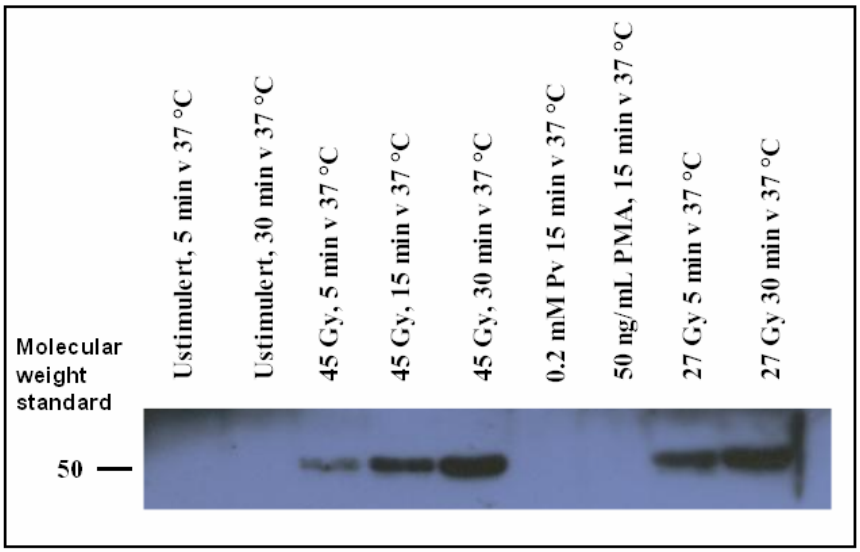


Figure 32: p53 activation in response to gamma irradiation induced DNA damage. Gamma irradiation gives activating phosphorylation of p53 within minutes of irradiation.
hMSC-TERT was irradiated with two different doses and incubated at 37˚C for different intervals of time. Unstimulated, pervanadate treated and PMA stimulated hMSC-TERT were included as negative controls. An antibody against a phospho-serine on activated p53 was used.

From figure 32 we see that gamma irradiation gives induction of p53 within minutes after radiation. There is no activating phosphorylation in the negative controls. We have investigated the levels of activating phosphorylation in NF-kB and NF-kB translocation to the nucleus, but have not been able to draw a conclusion on the effect of gamma irradiation upon NF-kB. Our results (not shown) indicate that NF-kB is activated in some degree in unstimulated proliferating hMSC-TERT cells, and that gamma irradiation gives some increase in NF-kB translocation to the nucleus.
Having done some introductory studies into the effect of gamma irradiation, some candidate antibodies for an array had been tested.

4.5. Particle array development – COX 2 coupled particles

Having done these introductory studies, the next objective was to construct an array to study these effects. However, time did not allow the development of a whole array. The polyclonal COX 2 antibody was coupled to a population of uncolored particles. With no

other COX 2 antibody available, the lysate proteins were biotinylated so that PE conjugated streptavidin could be used to detect binding of COX 2 to the particles.

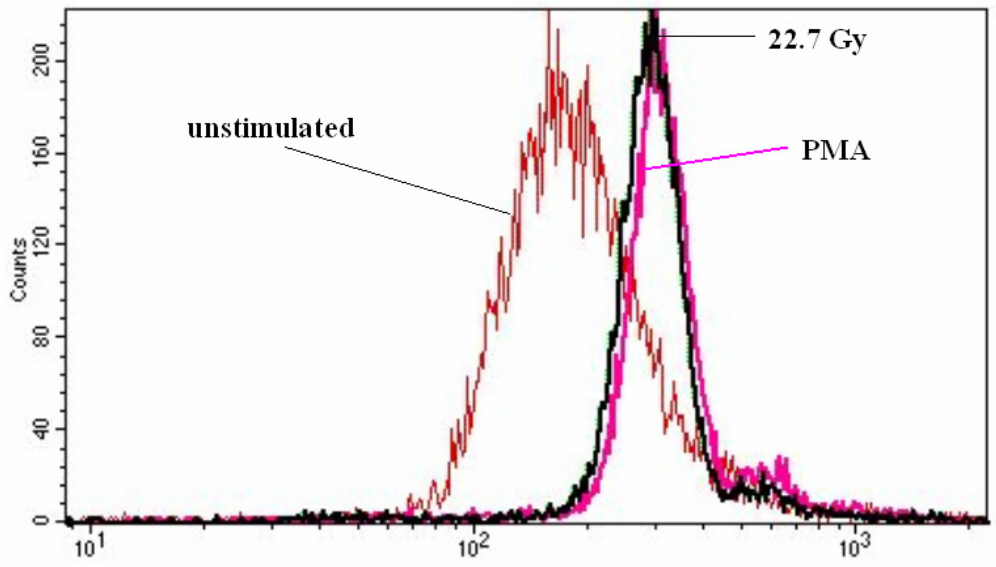


Figure 33: Particles with anti-COX 2 as capture antibody and biotinylated lysate containing biotinylated analytes detected with a SA-PE conjugate. Gamma radiation and the PMA stimulated positive control give an increase of PE signal compared to the unstimulated control. The background in the unstimulated sample is high, but the stimulated samples have a clearly visual increase of signal.
COX 2 coupled particles were incubated with biotinylated lysate from unstimulated, PMA stimulated and gamma radiation stimulated hMSC-TERT, were incubated with the streptavidin-PE conjugate and run in the flow cytometer. The three results are shown together in one graph.

The PMA stimulated and gamma irradiated cells have an increased signal compared to the unstimulated background. The lysates were not denatured in this experiment, and the background was quite high.

5. Discussion

The model array is based on optimized procedures for fluorochrome staining and antibody conjugation. When constructing an array it is important that a large batch can be made and stored in a stable manner, to ascertain the reproducibility of the array. The particle staining is very stable. After antibody coupling to the particles the antibodies should retain their reactivity upon storage. Freeze and thaw experiments have not revealed a large fall in reactivity. However, since the system includes many antibodies the stability must be assessed for each antibody included.

The fluorochrome staining of particles result in particle populations that are clearly distinguishable in the flow cytometer. This has been demonstrated repeatedly in our lab.

The main objective was to test whether antibodies directed against closely related analytes could be used and that the analytes were detected specifically, without cross reactivity. The multiparameter detection should be comparable with results from Western blotting analysis. It was shown in both the array and Western analysis that the lysates contained substantial amounts of the analytes detected. In general ERK detection has given stronger signals than JNK detection, indicating that there is more ERK analytes in the lysates.

The multiparameter test system is based on immuno-sandwiches with indirect detection. This system contains capture antibodies, detection antibodies and the indirect detection fluorochrome-conjugated antibodies. In the testing of this system a few possible scenarios may arise and these will be discussed here with examples from the results.

First, if a capture antibody and a detection antibody directed against the same analyte can bind their epitopes without hindrance, a functional sandwich can form. This sandwich can give a detectable signal when the indirect detection antibody is added. The signal measured is derived from the specific analyte. The sc-153 anti-ERK 2 detection of analytes bound by sc-1647 anti-ERK 2 in figure 20 is a good example of this case. Here

two antibodies directed against different epitopes of ERK 2 make a sandwich that is detected with strong signals. Sc-1647 anti-ERK 2 only binds ERK 2 in the Western blot (figure 27), thus verifying that ERK 2 is the analyte detected here.

Secondly, if two antibodies directed against an analyte have the same, or closely located, epitopes, they cannot make a functional sandwich. The capture antibodies will block the binding of the detection antibody to the analyte and no sandwich will form that can be detected. A good example of this case is seen in figure 19 and 20, where sc-94 anti-ERK 1 and sc-153 anti-ERK 2 fail to detect analytes bound by the pan ERK antibody from Transduction Labs. The Transduction Labs pan ERK binds ERK well, as seen in the strong detection with sc-154 anti-ERK 2 (figure 21). Both sc-94 and s-153 give strong signals in sandwich with other ERK capture antibodies (figure 19 and 20). The lack of signal seen with Transduction Labs pan ERK can only be described by epitope blocking (competition). This fits well with the fact that sc-94 and sc-153 are both directed against the same epitope.

The third case is where a capture or detection antibody has cross reactivity to analytes bound by other antibodies in the array. This will lead to detection of sandwiches on particle populations where the capture antibody and detection antibody is directed against different analytes or different isoforms of the same analyte. Both of these have been seen in the array testing. A good example of isoform cross reaction is the sc-94 anti-ERK 1 detection of sc-1647 anti ERK 2 in figure 19. It is difficult to determine which of the antibodies that is cross-reacting in this case based on the array results alone. The Western blots (figure 26 and 27) show that sc1647 only binds ERK 2 but sc-94 binds both ERK 1 and ERK 2, indicating that it is sc-94 that is responsible for the cross reaction in this case. A good example of cross reaction to different analytes (though closely related) is the cross reaction seen with sc-94 anti-ERK 1 and Cell Signaling anti-pSAPK/JNK in figure 19. Here it is also difficult to determine which antibody that cross-reacts. As JNK 1 and ERK 1 both can separate at ~44 kDa, the Western blots shed no light in this case.

A fourth case is when antibodies that should be able to form functional sandwiches give no signal. We have two cases like this in our array. The sc-6254 anti-pJNK is not detected by any of the JNK antibodies, even though many of these detect analytes bound by the Cell Signaling anti-pSAPK/JNK. Sc-6254 gives signals in the Western blot (figure27) that are only slightly weaker that the Cell Signaling pSAPK/JNK signals. The lack of reactivity seen in the array is either due to a failure in the coupling procedure, or due to an assay parameter or array preparation handling that has affected the reactivity of the antibody. There are many parameters that can affect a monoclonal antibody. In immunoassay techniques where antibodies are coupled to a solid face some monoclonal antibodies loose their reactivity. By using polyclonal antibodies this effect is avoided. Some of the antibody clones may be affected, but if many of them are not the reactivity of the antibody will still be seen. Without further analysis of the properties of this antibody no conclusions can be drawn as to the cause to the lack of reactivity.
Another antibody that had no reactivity in the array testing is the sc-572 anti-JNK 2 detection antibody (figure 24). In the Western blot (figure 26) this antibody has a very high background, and the JNK signal at 52 kDa is hard to distinguish. This antibody proved difficult to titrate during the preparatory studies, and have generally shown very low reactivity both in the test system and Western.

The panel of antibodies tested in this multiparameter system has demonstrated that this array system can discriminate closely related analytes. This is provided that antibodies with the right properties and reactivity are used in combinations that give functional sandwiches and no cross reactivity. Antibodies that fulfill these requirements in a small array might not do so if more antibodies are added to the array, giving new bead bound analytes that the detection antibodies might cross with. Due to the sensitivity in this system the reactivity of each antibody in an array must be verified in combination with the other antibodies.

Western analysis of the antibody reactivity's is essential in determining that the analytes the antibodies bind are of the right molecular weights.

The Western results have backed the results obtained from the array. The ERK antibodies in both systems have reacted with stronger signals than the JNK antibodies. Western analysis showed that many of the isoform directed antibodies cross to other isoforms of the same protein family. An example is sc-94 in figure 19. The manufacturers of this antibody sell it as an anti-ERK 1 antibody, but their datasheet shows what the Western results have shown; that the antibody binds ERK 2 just as well as ERK 1. As described above this was also detected in the array tests. The correlation between the Western results and the array results is a good indication that the multiparameter test system is reliable. However, a lot more work must be done to establish fully reliable arrays for multiparameter detection.

The IP and western analysis of ERK 2 (figure 28) showed again that sc-1647 anti ERK 2 antibody reacts with ERK 2 alone. Due to the JNK signals being at the same molecular weight as the heavy chains it is hard to determine if the signals seen is due to immunoprecipitated JNK or cross reactivity to heavy chains.

The negative control particles in the array are coupled to non-immune mouse IgG, while the other bead populations have different monoclonal mouse IgG. The control will have a variety of multiple clones, while the other beads only have similar antibodies. Assay parameters that cannot affect the general population of mouse IgG on the control bead might still affect monoclonal antibodies. However, most of the monoclonal capture antibodies worked well, while the negative control showed the same low background in each experiment.

We used denatured lysates as the antibodies were directed against peptides. However, the possibility of using antibodies directed against conformational epitopes should be assessed. Even though excess SDS was removed after lysate denaturing, residual SDS could represent a parameter that can affect antibody reactivity in the array. Many of the antibodies work quite well in our array, indicating that assay parameters are not critically affecting antibody reactivity. The Western results have verified some of the lack of

reactivity. It is important however to remember that antibodies can have different properties.

To develop reliable arrays using this system, more antibodies must be tested, and good capture and detection antibodies must be selected. It is also important to see what background effects will come from joining all the detection antibodies together in array analysis. We started looking at the possibility of using a common detection antibody for the whole assay, such as a tyrosine phosphospecific antibody. Thus we can use capture antibodies directed against different analytes, and detect which of the analytes that are tyrosine phosphorylated. Arrays with up to 100 separate regions have been made several times. If arrays of clearly distinguishable particles are made using particles of slightly different sizes, this should theoretically give rise to a third dimension of particle populations. Thus one can theoretically create array with the possibility of measuring many hundred different parameters simultaneously. This would however demand large numbers of antibodies of excellent quality, and the testing of so many antibodies will be a very large undertaking.

One objective of this master assignment was to construct an array for studies of stress and inflammation induced signaling in stem cells. To construct an array for such studies antibodies had to be selected and tested. The selection of antibodies would be based on introductory studies in stem cells. Due to unpublished results in our lab that indicated gamma irradiation as an inducer of PGE2 production, the introductory studies were based on this effect.

Gamma irradiation clearly induces COX 2 expression in hMSC-TERT. As gamma irradiation is used to prevent proliferation in stimulator cells in many co-culturing test systems, other cell types were tested for the gamma irradiation induced effect. Jurkat, EBV transformed B-cells and mononuclear cells were tested using the same conditions as for hMSC-TERT, but no COX 2 expression was induced in these cell types, indicating that this is a cell-specific effect.

MSCs (and hMSC-TERT) have inhibitory effects upon T-cell activation. This has suggested to be mediated through PGE2 produced by MSCs. As gamma radiation induces COX 2, the question arises whether gamma radiation can be used to 'boost' the PGE2 synthesis and T-cell inhibition by hMSC-TERT.

It has previously been shown that gamma irradiation induce COX 2 in prostate cancer cells, and the effect was inhibited by the antioxidant NAC, suggesting the induction to depend upon the redox status in the cells[42]. NAC on the contrary were not able to inhibit this effect in our cells. Dexamethasone inhibited COX 2 induction in our cells. Others have reported that COX 2 mRNA stability is decreased with dexamethasone treatment[17,54]; this is probably true for hMSC-TERT.

p53 is activated in hMSC-TERT shortly after gamma irradiation. This indicates activity in signal pathways that respond to DNA damage or cellular stress. It is not clear to what degree p53 signaling or other signaling components that participate in DNA damage response affects or induces COX 2. In fact p53 have been shown to inhibit COX 2[62]. As NF-kB has been shown to be important in COX 2 induction[10] NF-kB has been studied. As measured by NF-kB phosphorylation, some NF-kB activation could be measured in unstimulated cells. A couple of experiments, with long exposures of blots, have shown weak COX 2 levels in unstimulated cells. This may indicate that there are low levels of COX 2 in hMSC-TERT before irradiation, and that irradiation increases these levels. NF-kB activation and translocation to the nucleus and the mechanisms behind the induction of COX 2 after gamma irradiation must be investigated further.

The high background in the experiment where anti COX 2 were coupled to the beads (figure 33) might be due to residual biotin contaminating the particles. As all proteins in the lysate are biotinylated, proteins that have some interaction with the beads can also lead to the higher background seen in this experiment. However, the results show a increased signal for gamma irradiated and PMA treated hMSC-TERT and this is the same result shown in Western (figure 33).

If the immunosuppressive effect of MSCs seen in allo-transplantation is due to PGE2 secretion, radiation may be a way of pre-treating cells for transplantation, making them even more effective as immunosuppressors. The possibility of using gamma radiation to induce COX 2 and thus PGE2 secretion in these cells may provide a way to increase the inhibiting effect these cells have upon adaptive immunity.

The objectives of my masters have been to establish a test system for multiplex analysis of intracellular signal pathways by the use of flow cytometry. This has included development of the test system, testing of the potential in this test system and introductory studies in stem cells as a basis of making a new array.

We have shown that the test system distinguishes between closely related signaling proteins if the right antibodies are used in combination. The array results correlate well with the data obtained with the same antibodies in Western blotting, indicating that the test system is reliable. However, more work has to be carried out to develop arrays that can be used in studies of signaling pathways. Still we have shown that the test system principle works.

As introductory studies of hMSC-TERT the effect of gamma irradiation on COX 2 expression was investigated. Gamma irradiation induces COX 2 in hMSC-TERT, but the mechanisms behind this induction must be investigated further. The development of a multiparameter array could be useful in this process.

Time did not allow the development of such an array, but we tested the anti-COX 2 antibody on our array beads, and obtained signals that resembled the Western results.

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