CHAPTER II

INTRODUCTION TO THE TANNOUS LAB

an integrated approach of bioluminescence imaging and gene therapy

1. GENE THERAPY

Gene therapy (GT) is based on the idea to use nucleic acid (DNA or RNA) as a pharmaceutical agent to treat disease.[1,2] This idea came to life as soon as Watson and Clark unraveled the mysteries of DNA in the 1950's. In 1972 Friedman and Roblin published a paper in Science titled "Gene Therapy for Human Genetic Disease?" formulating the first concrete concept of the field.[6] It would take another 20 years before the FDA approved the use of gene therapy in the United States, but then in 1992 Ashanti da Silva, a four years old girl, became the first person in history to receive gene therapy.[7] She suffered from X-linked SCID, a single genetic defect resulting in immune deficiencies. Treatment was successful, however the effects were temporally. Since then, over 2000 clinical trials with gene therapy have been conducted (www.clinicaltrials.gov) and now this field seems to hold one of the promises of modern medicine.

In contrast to drug-based therapies, that are administered exogenously and repeatedly, gene therapy aims to heal from within. DNA recombination techniques allow pieces of DNA (or RNA) to be created from scratch, resulting in genes with desirable characteristics. These newly created genes can be incorporated in the genome of the cells of choice, where they'll start producing the tools for the intended intervention. Since integration in the host genome will theoretically result in long-term expression of the therapeutic gene, there's no need for repeated administration. Virtually any type of intervention can be programmed into DNA, allowing the cell's original genes to be inactivated or upregulated, nonfunctioning genes to be replaced, and genes with desirable characteristics to be introduced.

1.1 Viral vectors

Viral vectors are the vehicles of choice for transgene delivery in the gene therapy field. Viruses are characterized by their ability to introduce their genetic material into host cells as part of their replication cycle, while to a certain extent avoiding immuno-surveillance. When the host cells are attacked by the virus, introduction of the viral genetic material takes place. As soon as this newly introduced information is translated, hijacking of the host replication machinery is initiated. The host cell will

carry out the instructions of the viral genetic material, creating more and more virus to invade surrounding cells. This way, viruses are capable of surrendering large populations of cells.

In gene therapy several types of viruses are redesigned in such a manner that, instead of their own genetic material, they carry therapeutic genes into the host cells. Each type of virus has its own characteristics (Table 1), making it suited for different appliances. For the projects in this thesis, we mainly worked with adeno-associated viruses (AAV) and Lentivirus.

Adeno-associated virus (AAV) is the virus of choice for the treatment of many neurological disorders. Several serotypes exists (2,4,5,7,8), with 2 being the one most often used in the brain, due to its preference for neuronal cells.[8] AAV is capable of infecting both dividing and non-dividing cells and long time stable gene expression is achieved. Wild type AAV is known to integrate its DNA on a specific locus on chromosome 19, but this specificity is lost with the different serotypes, which usually maintain their DNA in a linear configuration in the cytoplasm (episomaly).[9] AAV is associated with very low toxicity and does not seem to evoke an immune response. Due to all these features, it is currently used in clinical trials for neurological disorders. A disadvantage of AAV is its low genomic capacity. A maximum of 5 kb can be packaged into AAV vectors, which makes it often impossible to deliver both a transgene and its reporter.[10]

Lentivirus (HIV-based) is a subgroup of the retroviridae family. It can deliver significant amounts of DNA and is capable of integrating its DNA permanently into the host genome, resulting in long-term stable gene expression.[11] This might lead to a risk of oncogenic mutagenesis when integration happens at the site of e.g. a tumor suppressor gene.[12] This is the reason that lentiviruses are primarily used for in vitro systems or in animal models after ex vivo infection. Some clinical trials are testing lentivirus safety in humans.[13]

		Adenovirus	Adeno-associated virus	Alphavirus	Herpesvirus	Retrovirus / Lentivirus	Vaccinia virus
Particle characteristics	Genome	dsDNA	ssDNA	ssRNA (+)	dsDNA	ssRNA (+)	dsDNA
	Capsid	Icosahedral	Icosahedral	Icosahedral	Icosahedral	Icosahedral	Complex
	Coat	Naked	Naked	Enveloped	Enveloped	Enveloped	Enveloped
	Virion polymerase	Negative	Negative	Negative	Negative	Positive	Positive
	Virion diameter	70 - 90 nm	18 - 26 nm	60 - 70 nm	150 - 200nm	80 - 130 nm	170 - 200 X 300 - 450nm
	Genome size	39 - 38 kb	5 kb	12 kb	120 - 200 kb	3 - 9 kb	130 - 280 kb
							
	Family	*Adenoviridae*	*Parvoviridae*	*Togaviridae*	*Herpesviridae*	*Retroviridae*	*Poxviridae*
Gene Therapy Properties	Infection / tropism	Dividing and non-diving cells	Dividing and non-diving cells	Dividing and non-diving cells	Dividing and non-diving cells	Dividing cells*	Dividing and non-diving cells
	Host genome interaction	Non-integrating	Non-integrating*	Non-integrating	Non-integrating	Integrating	Non-integrating
	Transgene expression	Transient	Potential long lasting	Transient	Potential long lasting	Long lasting	Transient
	Packaging capacity	7.5 kb	4.5 kb	7.5 kb	> 30 kb	8 kb	25 kb

Table 1 Characteristics of the most commonly used viral vectors for gene therapy. Image www.genetherapy.net1.3 Gene therapy for GBM

1.2 Gene therapy and cancer

Gene therapy against cancer is based on the delivery of cytotoxic genes to the tumor cells to achieve cell death directly, or by delivering “replacement genes” to overcome the resistance to therapeutics that is seen in many cancer types.[1,14] The most studied direct cytotoxic transgene in GBM is the Herpes Simplex Type 1 Thymidine Kinase (TK).[15] This gene enables the conversion of the prodrug ganciclovir into the highly toxic deoxyguanosine triphosphate, resulting in early chain termination of nascent DNA strands. The advantage of this approach is that only cells expressing the TK gene will convert ganciclovir (intracellular) into its lethal counterpart, while regular cells will remain unharmed. A modest increase in survival is reported in several studies. Another commonly used transgene if the E. Coli cytosine deaminase (CD) which converts 5-fluorocytine (5-FC) into its toxic variant 5-fluorouracil (5-FU).[16]

After successful introduction of the gene and administration of 5-FC, extensive cell death could be observed. Unlike TK, the CD therapeutic strategy has the advantage of excellent bystander effect since the 5-FU can travel from cell-to-cell via gap junctions.

Instead of introducing drug-activating genes into cells, it is also possible to deliver genes coding for immunotoxins. An example of such an approach is an AAV encoding transgenes for PE (pseudomonas exotoxin).[17] Expression of the PE gene leads to disruption of the cellular protein translation and causes cell death. To prevent elimination of normal non-tumor cells, the PE toxin is linked to human IL 13Rα2, a variant of the IL13 receptor that is overexpressed by 50-80% of GBM cells, but not by normal cells. This approach is still in an experimental phase[18], but tumor regression and long-term survival was observed in ~70% of the animals.

Despite some success stories, gene therapy is not living up to its full promise yet. It appears to be very difficult to achieve a high enough percentage of transduced cells, limiting the effectiveness of the therapy.[19] GBM cells prove to be particular difficult to transduce, due to the characteristic intra-tumoral heterogeneity and high intracranial pressure. Bystander effect of the transgene, in which the neighboring non-transduced cells are also killed, is needed to ensure effective therapy. Another problem is caused by the short-lived nature of this approach. Whereas theoretically integration of DNA in the host genome will result in long-term expression, this seems to be problematic to achieve in real life. After initial transduction, loss of transgene expression can be observed over time. Due to problems with integration of viral vector DNA into the genome and rapidly dividing cells, no permanent expression can be acquired so that patients require multiple rounds of gene therapy. Other concerns for viral therapy include toxicity of the gene or its vector to normal tissue, the host immune-response, tissue targeting, gene control, virus safety and the chance of inducing tumors by insertional mutagenesis. As of today, it is still impossible to exactly pinpoint the spot in the DNA where the new gene is to be integrated. Chances are the new gene integrates at the wrong place along the DNA strand, could disrupt a tumor suppressor gene and there slowly activates the process of cancer.

As mentioned above one of the challenges of using gene therapy in GBM is the achievement of a high enough yield of viral expression. Most approaches are focused on direct intratumoral injection of the virus to achieve expression of the therapeutic protein by the tumor cells themselves. Besides the fact that it is very difficult to establish consistent transduction of this heterogeneous cell population, another problem arises. Once the virus delivers its therapeutic gene to the tumor cells, production of the desired anti tumor proteins begins. Anti tumor proteins are designed to be highly lethal in order to be as effective in eliminating tumor growth as possible. However, since the tumor cells express the drug themselves, the therapeutic reservoir is rapidly depleted once the drug gets effective and the cells start dying, allowing for the escape of residual cells. To overcome this problem, Maguire et al designed a different approach in which not the tumor cells but the surrounding healthy brain parenchyma was engineered to express and anti-cancer agent, creating a 'zone of resistance.'[20] Using an AAV8 vector expressing interferon beta (INFβ) to transduce the normal brain, tumor growth in orthotopic xenograft models was completely prevented, even in the contralateral hemisphere and complete eradication of established tumors was achieved. These results open new possibilities for a more effective treatment of GBM with gene therapy.

All the above strategies are aimed at the elimination of tumor cells. This is however not the only way gene therapy can be of use in GBM. A very important role is reserved for its attribution to the knowledge of GBM tumor biology. Combining gene therapy and molecular imaging has lead to major discoveries in tumor signaling pathways, behavior, response to therapy and the role of the microenvironment, allowing the development of more effective treatment options.

1.3 An alternative approach: Liposomes

To enhance the diagnostics and treatment of many disorders, including cancer, one should think of disease-specific-targeting strategies. Most conventional chemotherapeutics lack the ability to deliver the therapeutic content specifically to the target tissues, which results in toxicity to healthy structures and often inadequate levels of therapeutic agents at the targeted tissue. Furthermore, gene therapy by use

of viral vectors is not yet at the level where we can 100% specifically target a specific group of cells. Liposomes might provide an interesting alternative for selective target delivery of therapeutics. Since many liposomes are currently widely used for pharmaceutical therapy and thereby approved for clinical purposes, they are of great interest for further exploration and applications.

About 45 years ago, Alec Bangham observed that phospholipids form closed bilayered structures when put in aqueous solutions, which were named liposomes. In short, liposomes are spherical, self-closed structures formed by one or several concentric lipid bilayers with an aqueous phase inside and between the lipid bilayers.[21] A real breakthrough occurred around 20 years ago when several liposomal drugs were approved and thereby many biomedical products and technologies were developed. The ideal liposome has 1. a size of around 80-120 nm to minimalize phagocytosis of the liposome particles in the blood [22], 2. a coat of for example PEG to slow down liposome recognition by the immune system and the resulting clearance of liposomes [21], and 3. target specificity such as immunoliposomes, foliate-mediated targeted liposomes, transferrin-mediated liposomes or biotin-streptavidin liposomes**.** Furthermore, liposomes are often PH-sensitive for efficient release of their contents inside the acidic endosome of the cell or the liposomes can be thermo-sensitive for ultra sound release of their therapeutic content. [23,24]

Besides the use of liposomes for therapy, they are also suitable for the delivery of imaging agents, which can be detected with most of the imaging modalities described in the next paragraph. For gamma-scintigraphy and MRI, the liposomes require respectively a sufficient quantity of radionuclide or paramagnetic metal. CT-contrast agents also can be incorporated into either the inner water compartment of the liposomes or in the liposomal membrane, as well as the incorporation of gas bubbles, which are sound reflectors, for the use of ultra sound. [21,25] The use of MRI for liposomal therapy and delivery of imaging agents has a great advantage due to its non-invasive character, the lack of radiation and its high spatial resolution. On the other hand, the sensitivity of MRI can often be disappointing, which fortunately can be overcome by a good contrast agent. Liposomes are known to be useful carriers of these contrast agents.

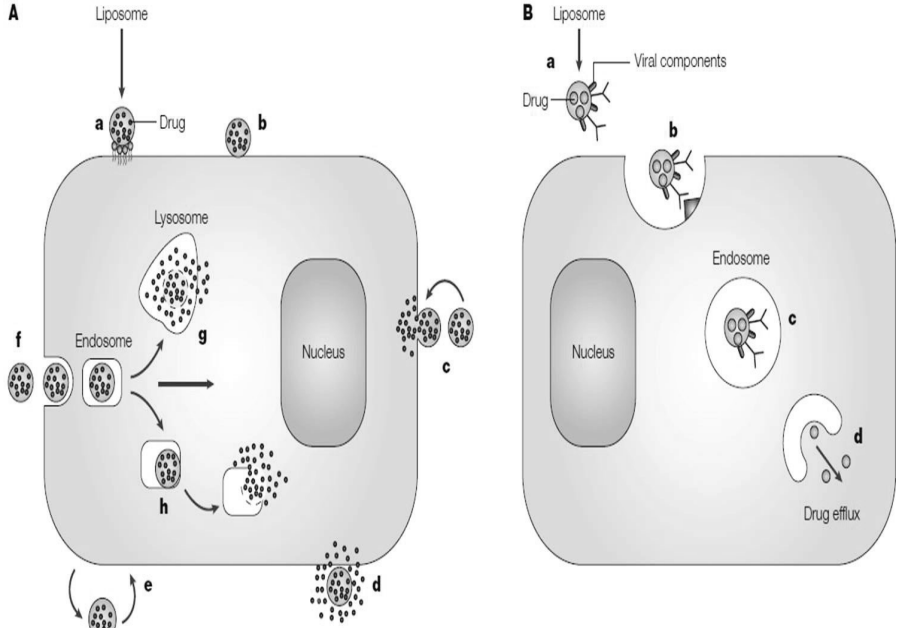

Figure 1: Liposome cell interaction. A) Drug loaded liposomes can specifically (a) or non-specifically (b) absorb onto the cell surface. Liposomes can also fuse with the membrane (c) and release their contents into the cytoplasm, or can be destabilized by certain cell membrane components when absorbed on the surface (d) so that the released drug can enter the cell via micropinocytosis. Liposomes can undergo the direct or transfer-protein mediated exchange of lipid components with the cell membrane (e) or be subjected to specific or non-specific endocytosis (f). in case of endocytosis, a liposome can be delivered by the endosome into the lysosome (g), or, en route to the lysosome the liposome can provoke endosome destabilization (h), which results in drug liberation into the cell cytoplasm. B) Liposome modified with viral components (a) and loaded with a drug can specifically interact with cells (b), provoke endocytosis, and via the interaction of viral components with the inner membrane of the endosome (c), allow for drug efflux into the cell cytoplasm (d). [21]

2. MOLECULAR IMAGING

Molecular Imaging (MI), defined by the Center of Molecular Imaging Innovation and Translation as 'the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in humans and other living systems,' was developed in the early 21st century and has since rapidly evolved as a useful tool in the biomedical research field.[26] MI has provided the opportunity to visualize

and monitor biological processes at both cellular and subcellular level without disturbing the living organism itself, thereby allowing the *in vivo* monitoring of specific molecular and cellular processes as gene expression, multiple simultaneous molecular events, progression or regression of cancer, and drug- and gene therapy.[27]

Currently disease is seen as the development of anatomic changes or physiologic changes, but unquestionably molecular changes underlie these developments. Direct imaging of these molecular changes will allow for detection of the disease in a much earlier stage. Further, the effects of the chosen treatment can be monitored.[28] Molecular Imaging is especially getting more and more essential in the cancer field. Our assessment of tumor type and diagnosis, prognostic markers, gene expressions, behavioral predictions, location, infiltration and response to therapy, e.g. our complete anti-tumor toolbox almost entirely relies on the (combined) use of various imaging modalities. Since Molecular Imaging allows for real time in vivo monitoring, understanding of the complicated and dynamic intra-tumoral processes becomes possible. Biological processes can be studied in their own physiologically authentic environment, instead of in laboratory-designed models. This is in huge contrast with the *ex vivo* techniques such as histology that we had to rely on before. Only a single static point in time could be measured, showing the end effects of molecular alterations, but not how and why those alterations took place. This 'how' and 'why' can now be visualized with molecular imaging and this knowledge can be directly applied to new treatment strategies.

In order to successfully visualize these biological processes *in vivo*, specific and sensitive molecular imaging probes are needed. These probes are molecules such as radiolabeled ligands, substrates, antibodies or cytokines, which can be used to differentiate between the different molecular events. Often it is useful to create inactive probes that require a substrate-enzyme interaction or the unquenching of a fluorophor to become active. Molecular Imaging consists of a wide array of these probes to produce imaging signals. Nuclear medicine relies on the decay of radioactive molecules (tracers), while sound (ultrasound), magnetism (MRI) and light (optical techniques; fluorescence and bioluminescence) are other key players (Table 2).[29] The required common characteristics of all these techniques is the availability of

a stable, non toxic high affinity probe or reporter, their ability to overcome physiological barriers (blood-brain-barrier), the use of amplification techniques to increase the signal to noise ratio, and the availability of high resolution fast imaging modalities.[28]

Whereas techniques such as positron emission tomography (PET), single photon emission computed tomography (SPECT), computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound are widely used in the clinical setting, they all have different shortcoming for pre-clinical small animal imaging. On the other hand, optical imaging including bioluminescence imaging (BLI) and fluorescence imaging (FLI) are valuable for pre-clinical small animal imaging.[30] Application of multimodality imaging using probes which can be imaged with a combination of either one of the above techniques results in fast validation in animal models (e.g. BLI) and translation into the clinic (e.g. MRI/PET).[31]

2.1 Overview of different imaging modalities

To gain insight into the different modalities used in molecular imaging and to obtain better understanding of the following chapters, a brief overview of the most commonly used imaging modalities will be provided.

Nuclear Imaging. Nuclear medicine gained recognition as a medical specialty in the 50's. [32,33] This specialism makes use of radiation to image biological processes for medical purposes. To explain the different modalities that are often used in nuclear medicine, one should first know the concept of a gamma camera. A **gamma camera**, also known **scintigraphy** or an Anger camera, counts the gamma photons absorbed by the crystal of the camera. When a gamma photon radiates from the patient (originating from an administered pharmaceutical) and reaches the camera, an electron is released from the iodine in the crystal of the camera, which upon finding its minimal energy state produces a faint flash of light. Thereby a 2-dimensional image is created. Both **PET** and **SPECT** scanners are based on the principle of a gamma camera. A PET scanner can detect gamma photons that are the result of a hit between a positron, emitted by the molecular probe administered, and an electron. The molecular probe consists of a decaying nucleotide such as

Carbon-11, Fluorine-18, Oxygen-15, or Iodine-124. A PET scanner can thereby reconstruct an image of the positron-emitting radionucleotide tagged to a specific molecule, which is recognized by enzymes or prone to binding to receptors, to visualize for example the expression of a therapeutic gene of interest. [34,35,36,37] The PET technique is widely used to visualize molecular processes and a majority of these are related to tumor cell growth.[38] Whereas PET uses the collision of positrons and electrons, SPECT tracks the position of gamma radiation directly by a rotation process around the body. Therefore, SPECT acquires information of the concentration of gamma emitting radionuclide, such as Technetium-99m, Indium-111 and Iodine-131, instead of a volumetric distribution that can be acquired by PET. Since PET uses this indirect radiation for a real-time image in time and space it is more sensitive than SPECT. SPECT on the other hand is significantly less expensive.[27] PET and SPECT are both used in the clinic and for small animal imaging.

X-ray and CT imaging. An **X-ray** is an image of electromagnetic radiation sent through the body and recorded on a film. Dense structures will appear as white on the image by blocking most of the radiation, whereas black displays the opposite namely air. X-ray images are commonly used for the evaluation of anatomical structures and are easy and relatively cheap to acquire.[39,40] **CT**, also known as CAT scanning (Computerized Axial Tomography) uses the same X-ray absorption as simple X-rays but it acquires different type of images by rotating the source and the detector around the body, resulting in multiple serial images and volumetric data.[27,41] CT is applied both in the clinical settings and for small animal research, although the utility of the CT technique for molecular imaging remains controversial mainly because of the difficulty in designing adequate contrast agents and probes for this modality.[28,42]

Ultrasound (US). This technique of high-frequency sound waves transmitted through tissues and then reflected back and detected, is often used to observe perfusion and anatomical characteristics.
Currently, contrast agents detectable by US and useful for molecular imaging are tested in animals. These contrast agents consists of microbubbles to which proteins and antibodies can be attached. When these microbubbles are linked to a

therapeutic agent the ultrasound can be used to release this therapeutic agent at the specific disease site, leading to therapy. [43,44,26]

Magnetic resonance imaging. MRI stands for high resolution imaging without ionizing radiation. When a temporary radiofrequency pulse is given to the hydrogen atoms in the body, whose spins are aligned because of a strong magnetic field, they temporarily change their alignment of spinning and get into an excited state, creating a pulse of radio wave the moment they switch back to ground state. These radio waves are detected and quantified. The major advantage of MRI is that it is suitable for high-resolution tissue images and contrast agents that can even enhance the signal, such as paramagnetic gadolinium, are available making MRI an important imaging modality. [27,45] Nevertheless, certain drawbacks remain for the use of MRI including longer image acquisition time, often large amounts of contrast agent [46], difficulty in delivery of those contrast agents, and poor sensitivity due to lack of accumulation of the contrast agent at the targeted site. [45] In order to make MRI more suited for molecular imaging, it is unnecessary to explain the importance of exploring new contrast techniques with higher sensitivity and specificity for targeted imaging and treatment.

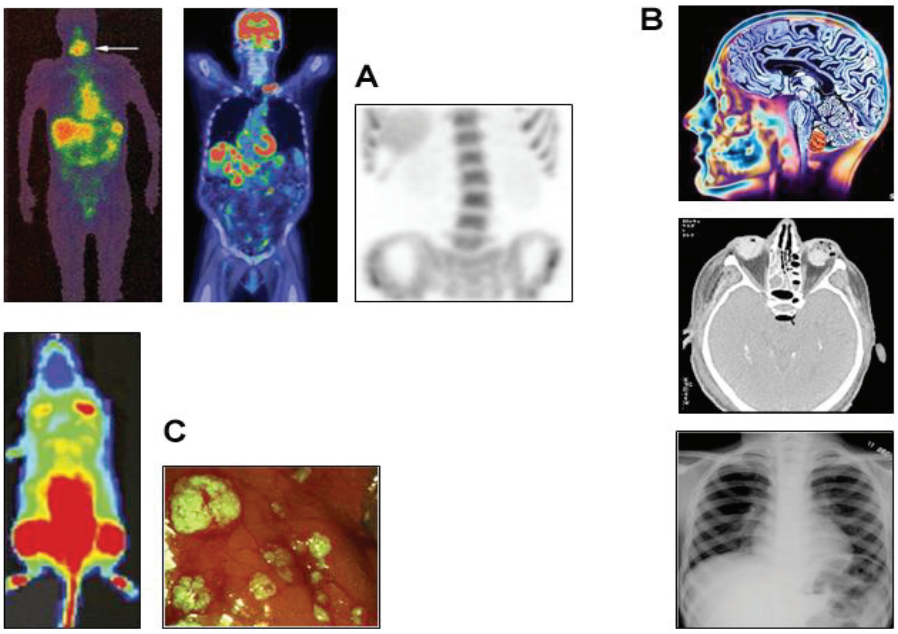

Figure 2: Imaging Modalities, adapted from [47,48,49,50,51,52,53]. A) Nuclear medicine, from left to right: Gamma scintigraphy, PET and SPECT scan. B) From top to bottom: MRI, CT and X-ray. C) Optical imaging, from left to right: BLI in a mouse and FLI during surgery.

Imaging Technique	EM Radiation Spectrum Used In Image Generation	Advantages	Disadvantages
Positron emission tomography (PET)	High energy gamma rays	High sensitivity; isotopes can substitute for naturally occurring atoms; quantitative; translational research	PET cyclotron or generator needed; relatively low spatial resolution; radiation of subject
Single photon emission computed tomography (SPECT)	Lower energy gamma rays	Many molecular probes available; can image multiple probes simultaneously; may be adapted to clinical imaging systems	Relatively low spatial resolution; radiation
Optical bioluminescence imaging	Visible light	Highest sensitivity; quick, easy, low cost, and relatively high throughput	Low spatial resolution; current 2-D imaging only; relatively surface-weighted; limited translational research
Optical fluorescence imaging	Visible light or near-infrared	High sensitivity; detects fluorochrome in live and dead cells	Relatively low spatial resolution; relatively surface-weighted
Magnetic resonance imaging (MRI)	Radio waves	Highest spatial resolution; combines morphologic and functional imaging	Relatively low sensitivity; long scan and postprocessing time; mass quantity of probe may be needed
Computed tomography (CT)	X-rays	Bone and tumor imaging; anatomic imaging	Limited 'molecular' applications; limited soft tissue resolution; radiation
Ultrasound	High-frequency sound	Real time; low cost	Limited spatial resolution; mostly morphologic although targeted microbubbles under development

Table 2 Characteristics of the main techniques used in Molecular Imaging. Copyright Center for Molecular Imaging Innovation and Translation

2.2 Optical Imaging

Optical imaging is specific type of molecular imaging. The basis of optical imaging techniques consists of photons travelling through tissue and interacting with tissue components.[29] Genes encoding fluorescent proteins or luciferases can be engineered, transferred into host cells and/or living animals and their light output can be measured with sensitive cameras. Optical imaging is highly sensitive for contrast agents and reporter molecules *in vivo,* meaning that even small signals can be detected. Further, it proved to be an excellent tool for use *in vitro* and in small animal models. It is inexpensive, highly sensitive, and the procedure is not very time consuming. The only disadvantage of optical imaging is the limited transmission of light through animal tissues, resulting in a decreased signal quantitation. Fluorescence imaging and Bioluminescence imaging are the two optical imaging techniques used in this thesis.

2.2.1 Fluorescence Imaging

Fluorescence refers to the property of certain molecules to absorb light at a particular wavelength and to emit light of a longer wavelength after a brief interval known as the fluorescence lifetime.[29] The most commonly used fluorescent reporter is the green fluorescent protein (GFP). GFP is a 27 kDa protein derived from the jellyfish Aquorea Victoria and it emits green light upon illumination with ultraviolet light.[54] The GFP gene is easily introduced to virtually any cell type and is extensively used as a reporter of expression or as a biosensor. When fused to other proteins, it becomes possible to monitor specific cell compartments, protein trafficking and all kind of cellular dynamic processes.[55] Since GFP expression is not harmful to cells, it has become a very powerful tool for fluorescence microscopy to observe cellular processes over time. Using mutagenesis at the chromophore region of the GFP gene, several variants were engineered.[56] With the creation of BFP (blue), YFP (yellow) and CFP (cyan), each using a different excitation and emission wavelength, simultaneous measurements became possible. In 2008 the discovery of GFP was awarded the Nobel Prize for Chemistry.

So far, the use of fluorescence has mostly been in in vitro setting due to its emission peak in the 500 nm wavelength (green light) leading to poor tissue penetration. *In vivo*, only 1-2 mm depth can be detected, and often surgical exposure is needed before visualization of the reporter is possible. Further, an external light source is needed for excitation, leading to high background signals due to tissue auto-fluorescence. Current research focuses on overcoming these limitations. Proteins that emit light in the far-red region of the spectrum or that show brighter light emission might improve the *in vivo* use of fluorescence. Due to a spectrum shift towards a wavelength longer than 600-610 nm, signals from red fluorescent (RFP) can be detected for several millimeters to centimeters in tissues, without the attenuation that occurs with other fluorescent proteins.[57,58,59,60,61,62] Recently new imaging technologies have made it possible to acquire 3D fluorescence images, resulting in further improvement of sensitivity.[63] This and several other developments in the fluorescence imaging field cleared the road for the use of this technique in the clinic. At the moment FLI is near to serving as a highly useful tool for breast imaging as well as for intraoperative guidance.[45,64]

2.2.2 Bioluminescence imaging

Bioluminescence Imaging (BLI) is a technique based on converting chemical energy into visible light in living animals. For centuries seamen and fishermen had seen lights in the waters and realized that these lights were emitted by organisms living in the water; this phenomenon was called **bioluminescence**. Only about 45 years ago, these organisms began to be characterized.[65,66,67] It was discovered that their light reaction depends on a luciferase (an enzyme), and its substrate (called a luciferin); coupling of enzyme and substrate causes a chemical conversion of the substrate resulting in light emission as is depicted in figure 3.[68] All known luciferases use molecular oxygen to catalyze this reaction, and some luciferases require the presence of co-factors such as ATP and Mg2+ for activity. Since no external light source is needed, BLI has virtually no background. Luciferases comprise a wide range of enzymes; usually they are found in lower organisms such as insects, fungi, bacteria and marine crustaceans, but no shared sequence homology exists.[68] Luciferase have proved to be very useful reporters in mammalian cells, since they are easily introduced in virtually all cell types by means of viral vectors, and they can provide real time, non-invasive measurements of in situ biological events, thereby giving a complete picture of the kinetics of an entire process.[68-70] With the current techniques, as few as a 10 cells expressing a luciferase can be detected in deep tissue of some animal models using a cooled charge-coupled device (CCD) camera.

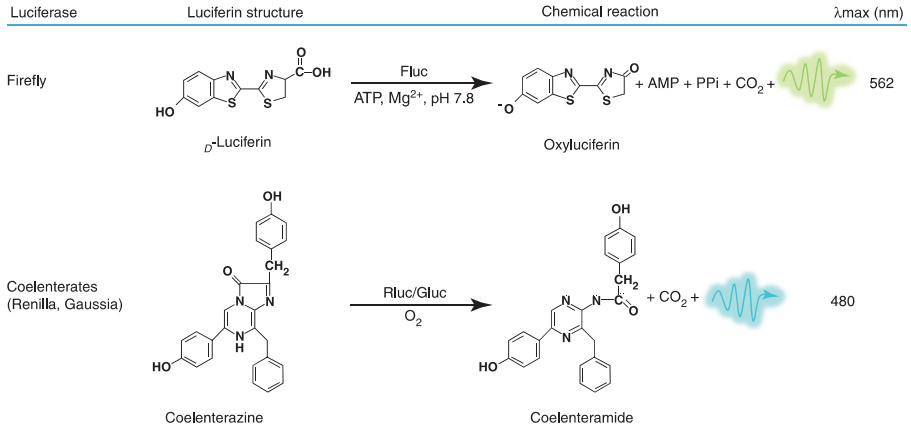

Figure 3. The Bioluminescent reactions of Firefly luciferase (Fluc), Renilla reniformis (Rluc) and Gaussia luciferase (Gluc). The chemical structure, the chemical reaction and the peak light emission (λ max) are depicted for each luciferase. Adapted from Badr and Tannous, Trends in Biotechnology, 2011.

American Firefly Luciferase, or Fluc, is derived from the light emitting organ of the *Phrotinus Pyralis* and is one the most commonly used luciferases. This 62 kDa protein has a very high quantum yield (>88%) and emits green light (562 nm peak with a broad shoulder) upon oxidation of its substrate $_D$-luciferin (Figure 4). The presence of ATP and Mg2+ is required for this reaction to take place. Fluc is expressed in the cytoplasm and therefore is cell-associated. Fluc displays a glow-type light emission kinetics with a half-life of 10 minutes, making it one of the best luciferases available to yield stable light output.[71-73]

The luciferase from the sea pansy *Renilla Reniformis* (Rluc) is another commonly used reporter. Rluc is a 34kDa protein, which uses coelenterazine as a substrate, and emits blue light with a peak at 480 nm (Figure 4). No ATP is needed for Rluc activity, however the enzymatic turnover and quantum yield of this reaction is only 6% making it not very suited as a reporter.[74]

The luciferase from the marine copepod *Gaussia Princeps* (Gluc) is the smallest luciferase (19.9 kDa) known. Similar to Rluc, Gluc uses coelenterazine for its chemical reaction, resulting in blue light emission with a peak at 480 nm. Gluc is over 2000-fold more sensitive than either Fluc or Rluc when expressed in mammalian

cells culture and therefore has become the reporter of choice.[68] While most luciferases are retained in the cell, *Gaussia* Luciferase is unique as its cDNA possesses a natural signal sequence and therefore is secreted upon expression in mammalian cells. While this causes a certain loss of signal at the site of origin, it does allow Gluc to report both from the cells themselves as well as their environment. Therefore, the Tannous Lab developed Gluc as a blood reporter. Since Gluc is naturally secreted, the level of Gluc in the blood correlates with the specific biological process (cell number, viral transduction, tumor volume, etc).[75,76] Despite it secretion, Gluc still emits over 200-fold higher signal than Rluc, and is comparable in signal intensity to Fluc when imaged *in vivo*[68]. A Gluc-variant that is expressed on the cell surface was recently designed and showed to be a sensitive reporter for tracking T-cells in vivo.[77] Gluc is currently the reporter of choice for monitoring of different biological phenomena and in different fields including gene expression, tumor volume, cell viability in high throughput screening for drug discovery, protein-protein interactions in BRET.[78-84]

The luciferase from the *Vargula hilgendorfi* (Vluc), a marine ostracod, is one of the few other naturally secreted luciferases[85]. In the presence of Vargulin, Vluc emits a blue light (462nm). However, the in-availability of the Vluc substrate limited its use. Recently, Vargulin became commercially available, so that use at a larger scale can be initiated.

Table 3: Different luciferases and their origin, substrate, wavelength and emission type.

Luciferase	Origin	Substrate	Wave length	Flash/Glow
Firefly (Fluc)	*Photinus pyralis* American Firefly	Beetle D-luciferin Mg2+, ATP	562nm	Flash
Renilla (Rluc)	*Renilla reniformis*	Coelenterazine	480nm	Flash
Gaussia (Gluc)	*Gaussia princeps*	Coelenterazine	480nm	Flash
***Vargula hilgendorfii* (Vluc)**	*Vargula hilgendorfii*	Vargullin	467nm	Glow
Luciola italica	*Luciola italica*	Beetle D-luciferin	550nm GliFluc	Flash

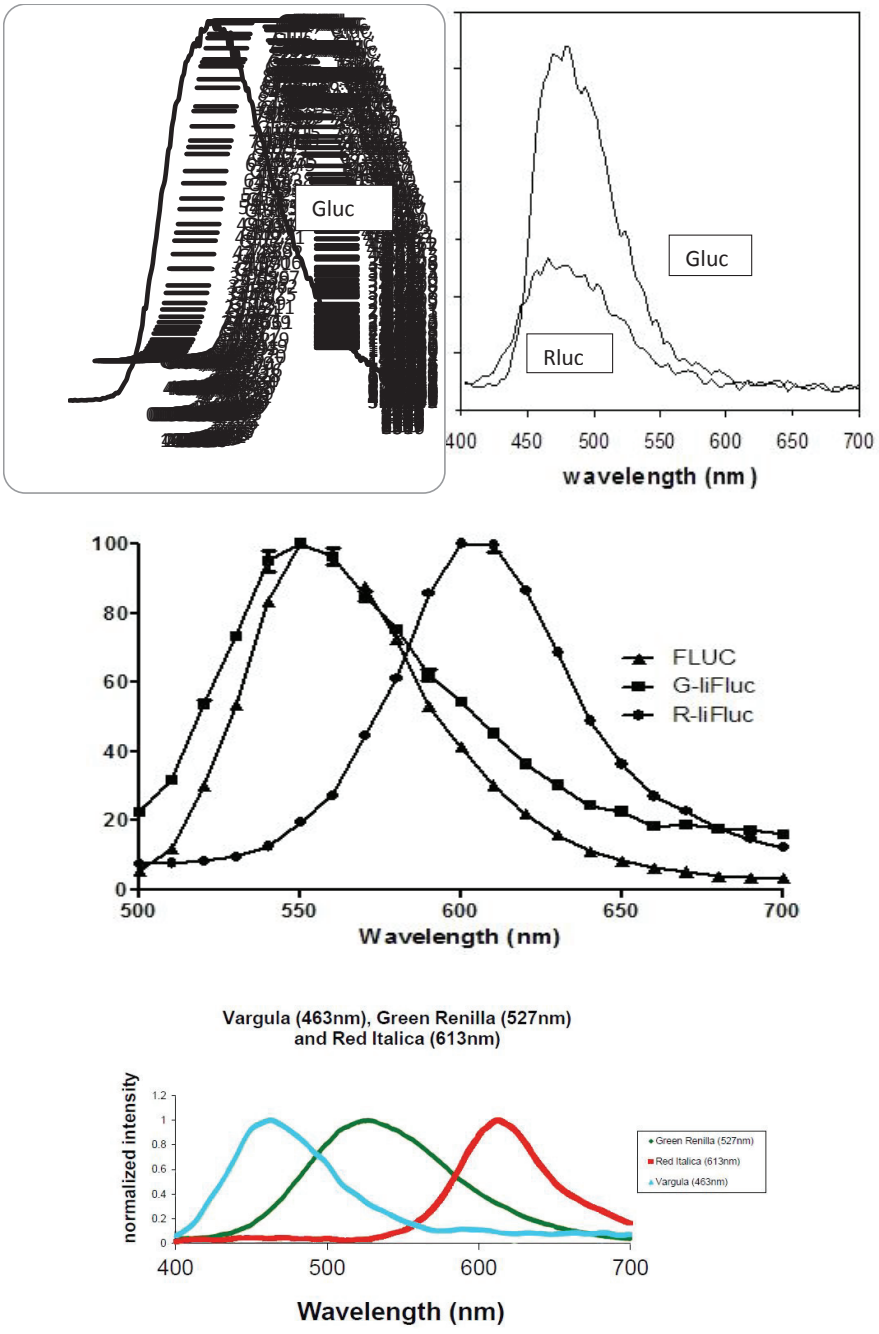

Figure 4: Emission spectra of different luciferases, adapted from chapter VII, [3,5]

Aside from light emission characteristics these luciferases have different kinetics of light output, sensitivity and enzymatic stability. Rluc and Gluc both display a flash-type kinetic reaction, in which the emitted light decays to background level within minutes. Fluc on the other hand displays a glow type kinetic that decays over a longer period of time.[68] Further, Fluc has a half-life of 4 hours in contrast to the half-life of Gluc, which is around 5 days.[68] These different characteristics make each reporter suitable for a different type of application. Whereas the glow kinetics of Fluc is favorable for high throughput screening *in vitro,* the absence of natural secretion makes the real-time monitoring from the same cells impossible. Gluc on the contrary is perfect as a secreted reporter, but its flash characteristics make it unsuitable for high throughput screening**.**

For *in vivo* applications, we can point out a comparable dilemma: Fluc is more sensitive due to its emission towards the red side of the spectrum, however, unlike Gluc, its activity cannot be monitored in the blood and therefore is not suited for monitoring of few circulating cells. Since Fluc and Rluc/Gluc utilize different substrates, they can be used together for dual BLI to monitor two different processes simultaneously.

2.3 Secreted blood reporters

Traditional enzyme-based reporter systems using cytosolic markers have been successfully used in different fields; however, they generally require tissue lysis and are therefore not suited for frequent imaging.[86] On the other hand, reporter proteins which are naturally secreted and can be detected in the cell-free conditioned medium in culture or in animal body fluids (blood/urine) were shown to be useful tools for non-invasive and real-time monitoring of different biological processes including tumor development, growth and response to different therapies, time-course embryo development, viral dissemination, gene transfer as well as the fate of genetically engineered cells in animal models. [68,87,88] The most commonly used blood reporters are the secreted alkaline phosphatase (SEAP), soluble marker peptides derived from

human carcinoembryonic antigen and human chorionic gonadotropin, as well as *Gaussia* luciferase. [86]

SEAP was the first described blood reporter; Berger et al. [89] used SEAP for quantification experiments in cell culture and created a fully active secreted protein by introducing a termination codon. Thereafter, this reporter was applied for *ex vivo* analysis of gene transfer in a mouse model. [90] SEAP can be constitutively expressed and efficiently released from transfected cells and is the most common reporter to monitor biological processes *ex vivo.* [86] Bettan et al [90] and Cullen and Malim [91] observed that changes in SEAP levels in medium of Chinese hamster ovary cells are directly proportional to changes in intracellular SEAP mRNA and cell number and hereby they initiated the widely use of SEAP as a secreted serum reporter. [86,92] Its use has been extended to the clinic, where its serum levels have been used to monitor systemic and cervical antibodies after vaccination with HPV16/18 AS04-adjuvant vaccine.[92]
Due to its high molecular weight (64kDA) expression level in the blood is low, requiring relatively large samples to be measured. Further, most blood samples contain naturally occurring serum alkanine phosphatases, which can interfere with the SEAP assay.[93]

Soluble marker peptides are of great use in cancer virotherapy studies since they can be introduced into a virus to monitor its spread and elimination *in vivo*. Furthermore, they can even measure the profile of viral gene expression as well as the kinetics over time.[86,87] An optimal soluble marker peptide should lack biological activity, the half-life should be constant in the circulation, it should have a limited immunogenicity and a validated assay should be available.[87]
Edmonston vaccine strain of measles virus (MV-Edm) has been effectively engineered to express different soluble marker peptides, including human carcinoembryonic antigen (hCEA) and the *β* subunit of human chorionic gonadotropin (*β*hCG).[94,95] Recently strains of this virus have been used to infect and destroy cancerous cells without affecting the surrounding tissues. This specificity can be explained by an over expression of CD46, a measles virus receptor, on the tumor cells. Simultaneously, these strains were modified to express certain soluble marker

peptides, such as CEA, and thereby the viral gene expression and replication could be monitored *in vivo*.[87]

Recently, *Gaussia* luciferase has been characterized as a blood reporter. The major advantage of using Gluc as a secreted blood reporter is that its *ex viv*o blood analysis can complement *in vivo* bioluminescence imaging. Thereby Gluc assay has the ability to localize the signal *in vivo* while monitoring the tumor response *ex vivo*.[75] This multifaceted approach gives Gluc great advantage as a blood reporter. Furthermore, Gluc also has a much shorter assay time with increased sensitivity and linear range over other secreted blood reporters. [75,68,80]
Gluc has been used extensively as a blood reporter. The Gluc blood assay was for instance used to monitor the response to treatment and metastasis of human breast cancer in animal models. Besides the good correlation between the primary tumor volume and the Gluc level in the blood and urine, more importantly the Gluc assay revealed early detection of tumor growth and metastasis in these animal models which was not accomplished by typical *in vivo* imaging techniques.[82]
In addition, Gluc can be used for the evaluation of transcriptional regulation associated with signaling pathways. These pathways can be dysregulated in many human disorders including cancer.

2.4 BLI in cancer: strategic approaches

BLI has shown to be a valuable tool in the understanding of tumor biology and the intricate processes associated with tumorigenesis. Its ability to report from the subcellular level, to track both proteins and cells, to visualize gene activation and to monitor responses to cancer therapies has allowed the development of many new research strategies. Its key roles in cancer are summarized below.

Imaging of apoptosis. Apoptosis, or programmed cell death is executed through activation of cysteine aspartyl proteases (caspases) and is very often disrupted in cancer. This process has recently been monitored by fusing an inactive luciferase reporter gene to a caspase cleavage site. An example of such a reporter is the caspase 3 – Gluc reporter. Gluc is fused to the estrogen receptor (ER) regulating domain and is thereby rendered inactive. Since the two genes are separated by a

caspase cleavage site (DEVD), Gluc will be released in the presence of caspase 3, resulting in light emission when apoptosis occurs.[96-98]

Imaging of tumor therapy. BLI allows for the quantitative measurement of tumor growth and response to therapy. The amount of luciferase expression correlates directly to the amount of tumor cells.[99] First, luciferase reporter genes are *ex vivo* introduced in GBM cells for stable expression. Upon transplantation, tumor signal can be measured by cooled CCD camera or in the blood using the luciferin substrate; making it possible to track growth, metastasis and response to therapy in animal models of GBM.[100,101]

Drug discovery. The screening of drug libraries is a very time consuming process. BLI allows for sensitive and relatively quick readout of photon emission. This way screening of thousands of compounds can be done in an effective way, using a cell-based assay. Luciferases are used as cell viability markers and by interpreting their light output, molecules with specific toxicity towards cancer cells can be identified.[102] Gluc is especially useful in this setting, since it is secreted. Sampling aliquots of conditioned medium over time allows noninvasive evaluation of cell fate in real time.[103] Luciferases can also be used for the screening of gene-targeted drugs. Reporter genes containing a DNA binding sequence of the gene of interest (GOI) driving the expression of a luciferase can be introduced. P53, the most studied tumor suppressor gene, is an important target for drugs. By using a p53 reporter vector, small molecules that affect the transcriptional activation can be identified.

Imaging of (Cancer) Stem Cells. Cancer Stem Cells are thought to play a major role in the initiation and progression of GBM. Understanding and tracking of these cells could provide new insights in tumor biology. Further, a variety of 'regular' stem cells (mesenchymal, neural, embryonic, hematogenic) can be used as drug delivery vehicles, since they are known to "home" to the tumor site upon iv administration.[104,105] This way, delivery issues related to the use of viral vectors (low transduction percentage, targeting) can be circumvented. An RFP-luciferase-thymidine kinase reporter protein has been used to label mesenchymal stem cells and track them in mice using BLI and PET.[106] Other stem cell tracking assays based on Gluc have been validated in animal models for several stem cell lines.

Imaging of hypoxia and angiogenesis. Hypoxia and angiogenesis are very closely related; they are two of the hallmarks of GBM. Upon hypoxic circumstances, different growth factors, transcription factors and cytokines are induced (as described in the GBM section). The main transcription factor found in a hypoxic state is the hypoxia-inducing factor (HIF1). HIF1 binds to HRE (HIF responsive element) to maintain transcription factor activation. Reporter luciferases coupled to the HRE element are designed to allow the visualization of hypoxic areas in the tumor and to help developing HIF targeted therapies.[107,108] A similar system exists to measure angiogenesis. These luciferase reporters are coupled to VEGFR2, allowing visualization of areas high in angiogenic activity upon activation of the receptor.[109]

BLI and gene therapy. Gene therapy is a promising approach in the battle against cancer and many other diseases. BLI reporter luciferases allow the evaluation of viral tropism, transduction efficiency and replication[110,111], providing the tools to further optimize this method. Since the efficacy of gene therapy mediated approaches is dependent on proper transduction, it is important to validate whether or not enough expression has been established. Otherwise, a lack of effect will be blamed on the chosen strategy, and a potential successful approach is rendered ineffective for the wrong reasons.

2.5 BLI and cancer: hot topics and limitations

The ability of BLI to give insight in both interactions between cells and their environment and intracellular processes makes BLI a very important tool in cancer research. Furthermore, it can facilitate the identification of cancer treatment by validating novel drugs in animal models, bridging the gap between the laboratory and the clinic. However, some limitations need to be overcome to further increase the value of BLI in (cancer) research. As for this moment, the use of BLI is limited to animal models, due to its relatively weak light emission (due to signal quenching and scattering through tissue) and potential substrate-associated toxicity).

Light emission and signal quenching. Both Gluc and Rluc emit light in the blue range of the spectrum. This causes that part of their light is absorbed by pigmented molecules as hemoglobin and melanin and is scattered through tissue, limiting its *in*

vivo sensitivity. Still, sufficient signal can escape these barriers and is detected by a cooled CCD camera or in the blood. Current research is focusing on the engineering of luciferase variants emitting light in the red region of the spectrum or with an even brighter intensity.

Signal Stability. Gluc emits light in a flash type bioluminescence reaction, resulting in a peak signal as soon as substrate is added, followed by rapid light decay. This is favorable for readings with a CCD camera, since accumulation of signal over time is limited, allowing for several reads in a relatively short amount of time. Since Gluc is secreted, it is also very suitable as a cell viability marker in drug screens. Aliquots of the conditioned medium can be assayed over time, allowing functional analysis of drug kinetics. However, due to the rapid light decay, the use of a luminometer with a built-in injector is essential for immediate reading of signal once substrate is added, one well at a time. Too much time delay will be accompanied by a drop in Gluc signal, not caused by efficacy of the drug, but by kinetic instability, potentially confounding results. Therefore, a Gluc variant with a more stable light output would be highly desirable for use in drug screens. A more stable type of Gluc has recently been discovered; however, this luciferase only exhibits its increased stability in the presence of the detergent Triton-X100. Interactions between the detergent and the to be tested drugs cannot be excluded, limiting the value of the assay.

Measuring interaction. One limitation to current bioluminescence imaging is that typically only one luciferase reporter is used to measure one parameter of tumor development. As a highly malignant form of brain tumor, GBM progression is a complex process involving communication between tumor cells, surrounding "normal" cells, and the vasculature.[112] Simultaneous measurement of multiple factors of tumor growth as well as the tumor's response to therapeutic agents would be extremely helpful in getting insight in the complex interactions of cancer growth. Since Fluc and Gluc/Rluc use different substrates, recently a combination of these luciferases has been used for dual reporting allowing the simultaneous monitoring of two events.[113]

TRAIL. TRAIL (tumor necrosis factor related apoptosis inducing ligand) is a member of the TNF family and is one of the most commonly explored cancer therapeutics,

because it binds to death receptors found specifically on tumor cells. Therefore, TRAIL is able to cause a widespread apoptotic effect with minimal cytotoxic effects on normal tissues.[114,115] In preclinical studies, impressive growth inhibition and cytotoxicity against malignant tumor cells of various origin was observed, including lung, breast, colon, bladder, brain and T cell malignancies.[116] However, there also appears to be a group of tumors, including GBM, that is resistant to TRAIL-mediated apoptosis. Upregulation of the Bcl2 associated Athanoge (BAG3) genes and multiple other genes have been described, causing resistance at various points along the apoptotic pathway. The second issue in the use of TRAIL for glioma therapy is its inability to cross the blood brain barrier. In a recent Gluc-based drug screen of the Tannous lab, molecules were identified that sensitized GBM cells for TRAIL. This resulted in the selection of Lanatoside C, an FDA approved cardiac glycoside that is capable of crossing the blood-brain barrier.[103] Lanatoside C on its own was found to induce a non-apoptotic necroptosis-like cell death. The combination of the dual cell death mechanisms displayed by TRAIL (apoptotic) and Lanatoside C (necroptotic) appeared to be very successful and provides a basis for the development of new glioma therapy.[103]

2.6 *In vitro* Glioma Research and Mycoplasma

Mycoplasma species are the smallest organisms known and thereby not seen by microscope. Mycoplasma are resistant to commonly used antibiotics and therefore are frequent contaminants in regular cell culture with an incidence up to 70%.[4,117,118] These contaminants have been shown to affect different pathways in cell culture yielding to false interpretation of data. Whether *in vitro* or *in vivo*, all molecular techniques described above can be severely affected by mycoplasma contamination with almost all cellular processes.[117,119,120] Logically, these facts underlie the importance of detecting and clearing Mycoplasma contamination in all experimental research laboratories.

While many different species of mycoplasma have been isolated, only few are responsible for 90-95% of mammalian cell contaminations, namely: *M. orale*, *M. hyorhinis*, *M. arginini*, *M. fermentans, M. hominis* or *A. laidlawii*. The most common

form of Mycoplasma is the *M. orale*, found in the oral cavity of healthy humans and accounts for 20-40% of all mycoplasma infections in cell cultures (Table 5).[4] With the knowledge of this widespread contamination and its disastrous effects, one can understand that a detection method which is simple, sensitive, specific and inexpensive is needed.

Currently a wide range of detection methods are being used, of which the classical culture assay with the polymerase chain reaction (PCR) is known to be the most reliable nonetheless complex and time-consuming approach. PCR is based on the amplification of mycoplasma DNA, it has the advantage to distinguish different species of Mycoplasma and it provides a sensitive and specific method to quickly identify contamination and monitor growing cell cultures. Nevertheless PCR requires careful preparation of the sample, attention to polymerase inhibitors in the cell cultures and often has false positive contaminants.[121] Traditional bacterial culture has the advantage of actually observing the colonies in combination with a high sensitivity and inexpensiveness. However this assay disqualifies itself by being even more time consuming; expertise is required and simply not all common Mycoplasma species can be cultured.[4] In addition to culture and PCR, other assays have been described, such as fluorescent DNA staining, enzyme-linked immunosorbent assays and RNA amplification, with each having significant drawbacks. Fluorescence DNA staining lacks the ability to reveal all the common species, enzyme-linked immunosorbent assays have a low sensitivity and RNA amplification requires a rather complex protocol.[4,117,121] Currently a simple bioluminescence-based mycoplasma detection kit is commercially available, but it has low sensitivity and its use for larger stocks of cell culture is limited due to high cost.

Table 5: Most common mycoplasma contaminations. [4]

Species	Frequency	Natural host
M. orale	20 – 40%	Human
M. hyorhinis	10 – 40%	Swine
M. arginini	20 – 30%	Bovine
M. fermentans	10 – 20%	Human
M. hominis	10 – 20%	Human
A. laidlawii	5 – 20%	Bovine

3. AIMS AND OUTLINE

The aim of this thesis is to combine the strengths of gene-therapy and BLI for the development of novel reporter systems in order to study glioma tumor biology and its response to therapeutic compounds. We further tried to optimize the currently available BLI luciferases (*Gaussia* luciferase, *Vargula hilgendorfi*) and assays (Gluc blood assay, Mycoplasma detection assay). We explored a new multimodal targeted liposome formulation with increased relaxivity for the treatment and imaging of cancer. Finally we combined the newly developed and enhanced reporters to test a new therapeutic combination for the treatment of Glioma (TRAIL, Lanatoside C).
The research as described above is divided into ten chapters (**chapter III** to **XII**).
Sarah Bovenberg takes responsibility for **chapter III, IV, V**, and **XI**, and Hannah Degeling takes responsibility for **chapter VI** until **X** and **chapter XII**.

Secreted blood reporters are valuable tools for sensitive and fast detection, quantification and noninvasive monitoring of in vivo biological processes.[75,80] The level of these secreted reporters can be measured over time to generate multiple data sets without the need to sacrifice the animal, since only a small amount of blood is required. In **Chapter III** we enhanced the sensitivity of Gluc as a blood-reporter by designing an alternative Gluc blood assay. The enormous advantage of capturing Gluc in a blood-based assay instead of by CCD camera is the ability to follow intracellular processes in real time. Whereas CCD camera obtained pictures show luciferase signal (and thereby reporter gene activation) at a static point in time, the blood assay allows for a dynamic approach, in which a drop of blood can be collected every 5 minutes. The CCD camera does not share this characteristic, since the procedure is time consuming and the BLI reaction takes place inside the animal. Therefore, one has to wait until the previous BLI signal has died out, before a new (reliable) measurement can be made. In the new assay, Gluc is captured from the blood by an antibody-mediated reaction before bioluminescence reaction takes place. This procedure prevents signal quenching by pigmented molecules like hemoglobin, resulting in an over one order in magnitude increase in sensitivity, allowing the detection of few circulating cells and metastases.

In **Chapter V** the development of a triple reporter system based on *Vargula, Gaussia* and *Firefly* luciferases for sequential imaging of three different biological processes is described. We applied this system to monitor the effect of the apoptosis-inducing ligand sTRAIL (soluble Tumor necrosis factor-Related Apoptosis-Inducing Ligand) on GBM tumor cells using an adeno-associated viral AAV vector. As explained earlier, TRAIL is only toxic to cancer cells, since only these cells overexpress TRAIL death receptors. Unfortunately, GBM cells seem to be resistant to TRAIL-mediated apoptosis. To overcome this limitation, in a drug screen a molecule sensitizing GBM cells for TRAIL, called Lanatoside C, was identified. Lanatoside C is a known, FDA approved, cardiac glycoside. We designed a triple luciferase reporter system, to measure the activation patterns and outcome of combined treatment of TRAIL and Lanatoside C in GBM. Since TRAIL cannot cross the blood brain barrier, we circumvented this problem by delivering sTRAIL directly to brain tumor environment by AAV-mediated gene delivery. By engineering the normal brain to synthesize and secrete sTRAIL, it can form a zone of resistance against newly developed glioma, which can be treated with lanatoside C therapy. We used Vluc to monitor AAV gene delivery of sTRAIL, Gluc to monitor the binding of sTRAIL to the glioma death receptor and the consequential activation of downstream events, and Fluc to measure tumor response to combined sTRAIL and Lanatoside C treatment. This work is the first demonstration of triple *in vivo* bioluminescence imaging and will have broad applicability in different fields.

In **Chapter IV** we describe the development of a first of its kind multiplex blood reporter that allows the *ex vivo* evaluation of *in vivo* processes. Since tumorigenesis is an intricate and dynamic process and GBM cells in particular are known for their ability to escape cell death and change characteristics, we developed the first multiplex blood reporter based on secreted alkaline phosphatase (SEAP), Gaussia and Vargula luciferases. This multiplex system differs from the triple imaging system as described in Chapter V in that here all reporters are secreted. As a result, tumor changes can be followed in real time (by simply taking an aliquot of blood), whereas the reporter from chapter V does not allow for real time imaging. Since Vargulla has not been previously used as a blood reporter, first we characterized it as a secreted blood reporter. As a proof of concept the response of three different subsets of glioma cells to the chemotherapeutic agent Temozolomide (TMZ) in the same animal

was monitored. No signal bleeding or substrate cross reaction was observed, proving that these 3 reporters can be used simultaneously in the same animal. This new multiplex reporter system can be extended and applied to many different fields for simultaneous monitoring of multiple biological parameters in real time.

One of the current limitations of BLI for *in vivo* imaging applications is the emission of light in the blue range of the spectrum. As discussed above, pigmented molecules like melanin and hemoglobin absorb the emitted light, while tissue further scatters the signal. Therefore, little signal remains to be picked up by CCD camera, decreasing the sensitivity of BLI for *in vivo* applications. Red light on the other hand, does not get absorbed, and would therefore be an ideal characteristic for a sensitive *in vivo* BLI reporter. Another issue is the flash type bioluminescence of Gluc when used in high throughput drug screens, limiting the numbers of drugs that can be screened.[103] A Gluc variant that catalyzes a glow-type reaction would be extremely helpful in the field of drug discovery allowing for high-throughput approach without the risk of confounding results due to kinetic instability (wildtype Gluc) or to interactions with detergents such as TritonX-100 (GucM43I). Therefore, in **Chapter VI** we used directed molecular evolution to optimize *Gaussia* luciferase for high throughput applications and *in vivo* work. We screened for variants with a higher light intensity, glow-type luminescence and shift in emission spectrum, resulting in the identification of several variants with 10-15nm shifts in emission spectrum and one variant with a glow-type of bioluminescence that remained stable for over 10 minutes.

The exploration for new luciferases hasn't come to an end yet. So far liFluc has only been used for cell-based assays and has only been expressed in bacteria. Therefore, in **Chapter VII** we developed and characterized a codon-optimized variant of liFluc for mammalian gene expression and used it for in vivo imaging of tumors.

Since glioma research relies heavily on tissue culture, and since almost every single intracellular biological processes can be influenced by Mycoplasma contamination – severely confounding results- the use for Gluc as a Mycoplasma detection sensor was explored in **Chapter VIII.** We developed a simple and cost-effective

mycoplasma detection assay using the *Gaussia* luciferase reporter and showed it to be much more sensitive than other bioluminescence-based assays.
In **Chapter IX** we describe the step-by-step protocol for Gluc Mycoplasma assay as created in **Chapter VIII**. Further, we point out critical steps and potential pitfalls regarding the assay for other users.

In **Chapter X,** we developed a new type of liposome as a contrast agent for MRI with a higher efficiency than the conventional liposome. We showed that this new liposome can be used for MRI guided therapeutic delivery to targeted cells due to its biotin inclusion and for ultrasound guided release of its contents due to its thermo-sensitivity.

In **Chapter XI and XII** we provide an overview of the Glioma research field, placing the work developed in this thesis in a broader setting. We review current research strategies, both in experimental setting and in the clinic, discuss the translational gap that exists between those two worlds and reflected on possible future directions.

In **Chapter XIII** we both discuss our own work and suggest what following steps should be taken in order to translate our findings into a clinical setting.

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