REPORTER EXPRESSION:
Helpful Tips on Model Development

In Vivo Optical Imaging is a highly sensitive, noninvasive technique that employs either bioluminescent and fluorescent reporters or exogenous probes for the visualization of biological processes and molecular events. Both bioluminescent and fluorescent reporters emit light that is detected and captured to produce optical data. However, the difference between these two reporter systems lies in the mechanism by which light is emitted.

In animal models of bioluminescence, light is produced and emitted as a result of a chemical reaction comprising a luciferase enzyme acting on its chemical substrate, with luciferin and coelenterazine being the most common. In bioluminescent bacterial models, light typically occurs via an oxidation reaction that creates energy in the form of a blue-green visible light. With fluorescence, excitation light is shone on a fluorescent protein such as a Green Fluorescent Protein (GFP) or an antibody labeling dye like AlexaFluor 680 dye. The fluorophore absorbs a photon from the excitation light and emits a photon with a longer wavelength. Fluorescent reporters are available in many types including expressed proteins, dyes, microspheres, and nanoparticles.

Common strategies used to achieve *In Vivo* Optical Imaging of biological systems include genetically engineered cell lines or exogenous imaging probes. Cell lines are engineered for the stable expression of a luciferase enzyme and/or fluorescent proteins. This enables the quantitative analysis of disease progression in different disease models including subcutaneous, orthotopic, and ectopic models. Light-emitting reporter constructs may also be used for the indirect visualization of transcriptional and post-transcriptional gene expression, gene regulation, and interactions between proteins in living animal models. In contrast, exogenous imaging probes are simply injected into animals to investigate disease burden and biological processes, such as angiogenesis, proliferation, and apoptosis.

The following are the two common methods used to achieve fluorescent or luminescent reporter expression in mice:

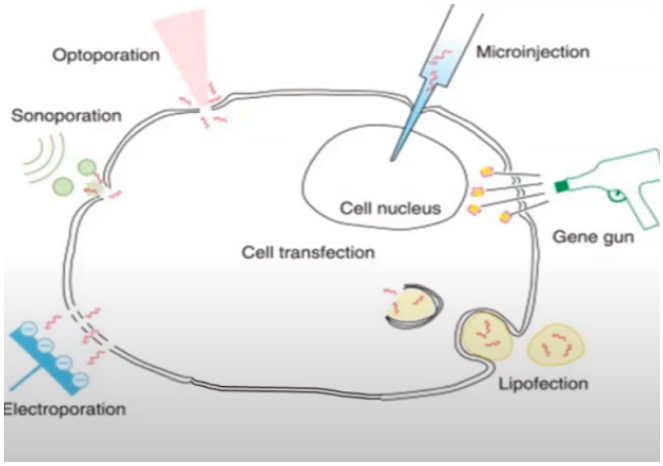
1. CELL TRANSFECTION

Transfection is the introduction of new genetic material into target cells by use of a physical or chemical process. There are various protocols and mechanisms that may be used for cell transfection including:

- A variety of chemical carriers such as calcium phosphate, cationic polymers, liposomes, and micelles.

- Targeted modification by CRISP/Cas9.

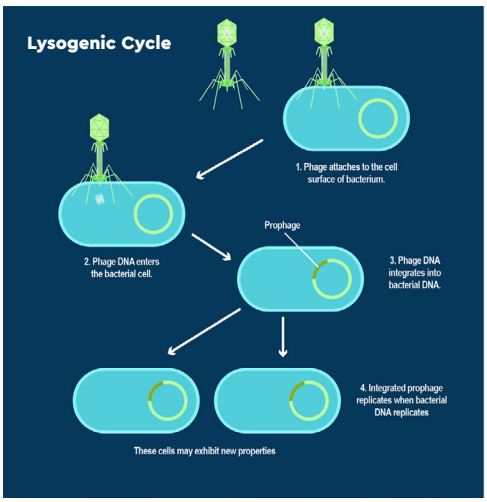
- Physical treatments used in cell transfection protocols include electroporation, sonoporation, optoporation, microinjection, gene gun, and lipofection.

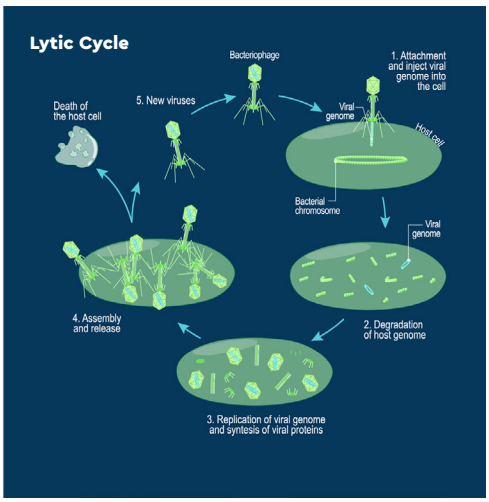


https://www.sciencedirect.com/science/article/pii/B9781907568671500162

2. CELL TRANSDUCTION

Transduction is a process by which foreign genetic material is introduced into the target cells via a viral agent. The viral particle used may be in either a lysogenic or a lytic phase, each having its unique application in optical imaging.



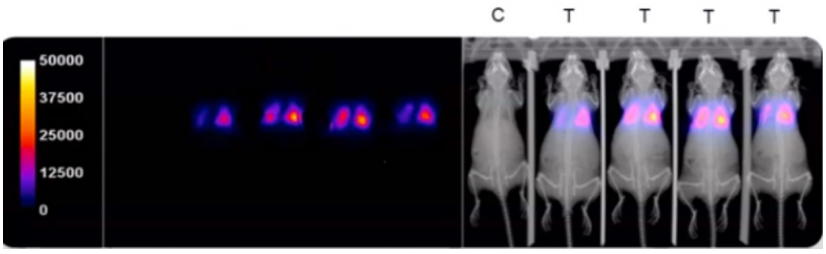


https://www.technologynetworks.com/immunology/articles/lytic-vs-lysogenic-understanding-bacteriophage-life-cycles-308094

- **Lysogenic phase model** - Viral particles in the lysogenic phase are used to introduce viral genomic content into the target cell population, be it a bacterium or a eukaryotic cell. When the target cell population replicates, the viral genome carrying the optical reporter gene is replicated along with it.

- **Application of lysogenic viral particles** - An example of this can be seen in the following study:

    - In one preclinical study, human lung carcinoma a549 cells were transduced by lentivirus to express firefly luciferase and these cells were then injected into mice. These a549 cells become enmeshed in the capillary bed of the lungs. When luciferin, the substrate of luciferase, is injected into the reporter-tagged mice, light is produced which is detected and superimposed onto x-ray to produce optical images.

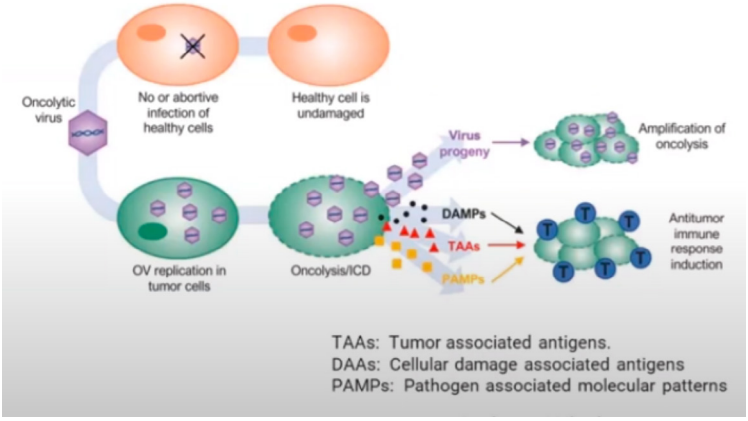


Courtesy of A. Van Praagh, Bruker BioSpin, PCI Lab, 2016

- **Lytic phase model** - In this model, the viral particle is used to lyse the target cell by entering and using the replication machinery inside the cell to cause cell lysis and death.

- For lytic viral particles, the application is notably different:

    - In most cases, the purpose is to target and kill a specific cell population, a recent and popular application within oncology. A viral particle is used to attack tumor cell lines of interest causing the death of the targeted cancer cells. This technique is possible because the cytosolic environment at a transcriptional and translational level is unique within tumor cells. The viral lytic particles can replicate only in the tumor cell environment; healthy noncancerous cells remain unaffected. The secondary effect beyond cell death on the target tumor cells is the release of a range of tumor-associated antigens such as TAAs, DAMPs, and PAMPs for the induction of host antitumor immunity.



M.Davola and K. Mossman, Oncolytic viruses: how lytic must they be for therapeutic efficacy? Oncoimmunology, (2019), VOL. 8, NO. 6

DIRECT INJECTION OF FLI PROBES

Another method to introduce a probe into the animal model system is the direct injection of the probe of interest. There are a variety of techniques for this, where the protocols can be characterized in terms of directedness and probe structure. The following are some of the different types of FLI probes available for direct injection:

- **NON-TARGETING PROBES**
  - **Small organic NIR fluorescent dyes**
    - A common dye of this category is cyanide. The wavelength of light emitted by these dyes is based on the linker polymethylene chain. The longer the chain, the longer the wavelength of light emitted. as TAAs, DAMPs, and PAMPs for the induction of host antitumor immunity.
    - Although these are not structured to be directed towards a particular cell line they can still act in a directed fashion particularly in oncology models due to the enhanced permeability and retention (EPR) effect because of abnormal angiogenesis in tumor cell masses.
  - **Quantum Dots (QDs)**
    - Quantum dots are highly fluorescent semiconductor nanocrystals.
    - The core of the quantum dots is made of a semiconductor material that is coated with a material that allows chemical conjugation with specific ligands, this enables the quantum dot to be directed to a particular antigenic site inside the mouse model.
    - The size range of quantum dots is between 2 and 8 nm in diameter.
    - High quantum yield; the emitted-to-absorbed photon ratio is quite large.
    - The following features facilitate the use of quantum dots in a version of multiplexing:
      - *Narrow emission spectrum.*
      - *Wide excitation wavelength ranges.*
      - *The emission peak of a quantum dot is positively correlated with the diameter of the quantum dot; the broader the diameter the longer the wavelength of light emitted.*
      - *Peak absorption wavelength is not affected by the diameter.*
    - Multiplexing using quantum dots: Multiple populations of ligand bound quantum dots of different sizes are directed to their respective antigenic site within the mouse model. Once all quantum dots are injected, a single excitation event excites all the different quantum dots in their respective locations. The emission wavelength is distinct for each of those locations based on the diameter of the quantum dot in that location. Using a set of emission bandpass filters, each of the emitted wavelengths of lights can be collected and superimposed onto a final image. This allows different targets to be visualized at once.

- **Target-directed Probes**

    - Molecular Conjugates: In these probes, ligands recognizing a specific target are bound to fluorophores.

    - This construct once injected binds to the antigenic site that the ligand is specific for. Some examples of such probes available in the market are:

        1. ***Tumor cell lines***
           In the case of oncology models, an antiPSA immunoglobulin fluorophore conjugate can be obtained that binds specifically to prostate tumor cells.

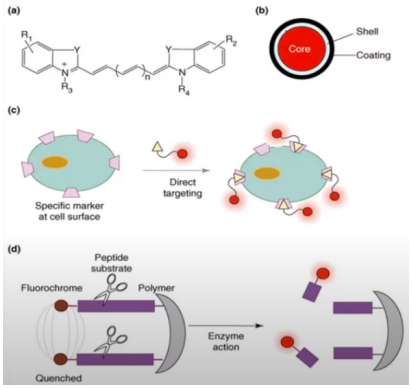
        2. ***Activated lymphocytes***
           To evaluate activated macrophages, an antifolate immunoglobulin fluorophore can be used that binds to the fluorophore receptors expressed in elevated levels in macrophages

        3. ***Exposed intracellular moieties***
           To study apoptotic cells, annexin V conjugate can be used, which binds specifically to exposed phosphatidylserine moieties that are present in higher concentrations only in dead or dying cells.

- **Activatable Probes: Activated by the activity of specific enzymes**
  This class of probes is turned on by the activity of specific enzymes. The activatable probe does not emit fluorescence in the intact state because fluorophores are positioned very close to each other in the structure of the probe conjugate. This allows for the light emitted by one fluorophore following excitation to be absorbed by the other fluorophore, as the Stokes shift is not large. However, this changes in the presence of the enzyme that is specifically able to cleave the linker moiety. The mechanism by which activatable probes work can be summarised in the following manner:

    - In the absence of enzyme: Probe does not fluoresce due to the fluorophore-fluorophore quenching.

    - In the presence of enzyme: Probe linker moiety is cleaved by the enzyme. Consequently, fluorophores separate, quenching stops which allows fluorescence to occur.



Courtesy of Rao, J., et al., Current Opinion in Biotechnology, 2007, 18: 17-25

- **Quantum Dot BRET* Probes: Self-illuminating Quantum Dots**
    - A quantum dot is ligated to multiple copies of a bioluminescent luciferase enzyme.
    - Luciferase enzyme generates blue light upon exposure to its specific substrate.
    - This bioluminescent photon resonance energy is transferred to the quantum dot which excites it, causing the QD to emit its much longer wavelength of light. Therefore an exogenous source of excitation light is no longer required for the quantum dot.
    - The longer wavelength light emitted by the quantum dot has better tissue penetration while the absence of an exogenous light source greatly reduces the background noise normally created by it.
    - Given that quantum dot emitted spectra is a function of quantum dot diameter, investigators can use multiple quantum dot populations of differing diameters to produce multiple quantum dot fluorescent signal peaks.
    - If each quantum dot species is conjugated to a different ligand, each quantum dot will bind to a distinct target site. Thereby, multiple cell populations can be imaged after substrate injection simply by altering the fluorescence emission filter used.

CONCLUSION

In Vivo Optical Imaging is a valuable imaging modality for investigating disease progression and the therapeutic efficacy of drug candidates in preclinical studies. There are many different applications of optical imaging in research related to oncology, neuroscience, gene therapy, drug metabolism, and toxicology. Both bioluminescent- and fluorescent- based optical imaging have helped to accelerate and improve pharmaceutical and academic preclinical research in therapeutic and drug discovery studies, by providing useful molecular data for application in clinical trials. The many different types of light-emitting probes, each having its own unique strengths, can be utilized according to the individualized needs of your preclinical study for the generation of optical data.