

# Optical imaging in pills: techniques, instruments and applications

F. Boschi,<sup>1</sup> L. Calderan<sup>2</sup>

<sup>1</sup>Department of Computer Science, University of Verona, Strada le Grazie 15, 37134 Verona, Italy

<sup>2</sup>Department of Neurological, Biomedical and Movement Sciences, University of Verona, Strada le Grazie 8, 37134 Verona, Italy

Corresponding author: Federico Boschi

Department of Computer Science, University of Verona, Strada le Grazie 15, 37134 Verona, Italy

Tel. +39.045.8027272

E-mail: federico.boschi@univr.it

## Summary

Optical imaging (OI) is an emerging field based on the detection of light photons in the ultraviolet, visible and infrared regions of the electromagnetic spectrum. According to the definition, the term “optical imaging” typically excludes classical microscopy techniques in favour of larger scale imaging methods which are devoted to the acquisition of images of small (few centimetres) sized samples. Biomedical OI is focused on the detection of biological samples with special attention to the *in vivo* acquisitions. Recent development of very sensitive detectors (photomultipliers and charge coupled devices – CCDs) allowed the detection of very weak light signal in biological samples not only in the visible range but also in the near infrared (NIR) region of the spectrum, which is noteworthy for the biological applications. The parallel advance of the light sources (lasers and LEDs) allowed to produce high intensity light beam with very powerful optical characteristics at relatively low costs. These two technological improvements, combined with the advances in the chemical field by the synthesis of new contrast agents with luminescent properties, led to the increasing importance of the OI techniques in the molecular imaging landscape. Here we review the conceptual bases of OI, the three main techniques (i.e. Bioluminescence, Fluorescence, and Cerenkov luminescence imaging), and some applications of OI in our laboratory.

**Key words:** optical imaging, bioluminescence, fluorescence, Cerenkov luminescence imaging, *in vivo* imaging

## Light propagation in biological tissues

The term *optical imaging* (OI) “typically excludes classical microscopy techniques in favour of larger scale imaging methods” (Nature.com, 2015) which are devoted to the acquisition of images of small (few centimetres) sized samples. In order to understand the advantages and limits of OI, it is fundamental to highlight what it happens when the light passes through the matter. The most important processes are the scattering and the absorption of photons.

A light beam, travelling through a slab of biologic material, encounters many different structures with different optical refractive index (membranes, cytoplasm, organelles, extracellular matrix) and it is forced to change its original direction.

From another point of view, each photon of the beam can be deviated by the interaction with the electrons of the matter. The phenomenon is called “scattering” and it is responsible of the diffusion of light as it occurs, for example, when a finger is placed in front of the red LED of a television set. A photon can undergo 10-1000 or more scattering events; the number depends on both the wavelengths of the photon and the optical properties of the matter. Each scattering event can be fatal for the photon which can be absorbed by a molecule. If it happens, the molecule is excited to a higher vibrational energy level and rapidly losses part of the energy as heat and relaxes to the ground state with the emission of a photon with lower energy. The process is almost immediate ( $10^{-8}$  s) and it is called “fluorescence”.

The photons' absorption is crucial for OI. Instead, the number of photons in a light beam decreases as the travelled distance increases, with an exponential law (Lambert-Beer law). The "mean free path before absorption" of the photons, i.e. the distance which reduces the photons' number to about 1/3 (which depends on the wavelength and the optical properties of the matter) is about 0.4 cm for blue photons (450 nm) and 1.8 cm for NIR photons (800 nm), in a tissue with the optical properties of muscle. It is clear that only shallow light sources are detectable and the technique is prevalently appropriate for small laboratory animals. Moreover, acquisition in the NIR range is more favorable with respect to the other spectral regions. In fact the absorption of hemoglobin, water and lipids, which are the most important absorbers in biological tissues, is reduced between 650 and 800 nm which is the so called "optical transparency window". For this reason the availability of CCD cameras able to detect NIR photons has increased the diffusion of the OI technique. Finally, further technological advances in the sensitivity of the detectors could lead to the imaging of deeper sources.

Small-sized animals are generally needed for OI investigations for the obviously reduced optical thickness of their tissues. Special role is represented by the athymic Nu/Nu mice which have a thin skin layer, furless and without the hair bulb: skin, fur and hair bulbs are high light absorbers. Unfortunately Nu/Nu mice cannot support many experimental model of pathologies, so other strains must be used with the consequence of a loss in the light signal. Sometimes, it can be useful to shave the animals before the OI acquisitions, but care has to be taken to avoid even small wounds which are well visible in the optical images.

The OI technique was borne as a planar technique capable to only acquire 2D images of the samples. Nowadays, OI is a full tomographic technique by which 3D localization can be acquired of the light sources inside the bodies. Two different approaches are used: the first one is the *multi-view* approach based on the acquisitions of images from different viewpoints (this is obtained by rotating the sample or the detector); the second one is the *multi-spectral* approach which is based on the acquisition of images with many different wavelength emission filters.

## OI techniques

All the light emitting processes are virtually detectable with the OI techniques. The most important are bioluminescence, fluorescence and Cerenkov emission.

### Bioluminescence Imaging (BLI)

Bioluminescence is the production and emission of light by a living organism. It is a form of chemiluminescence. The most important bioluminescence process for molecular imaging involves the light-emitting substrate luciferin and the enzyme luciferase (most frequently the *Firefly luciferase*). The enzyme-substrate reaction requires oxygen and adenosine triphosphate (ATP) and emits light with a broad emission spectrum peaked around 560 nm (Luker and Luker, 2008).

Transfected cells for luciferase expression can be used and generally transferred in the living system by inoculation (i.e. cancer cells for tumor development) or injected in different anatomical districts (i.e. stem cells in the tail vein). Luciferine is then injected intraperitoneally and the light production reaches the maximum 12-15 minute after injection and is stable up to 40 minutes. This technique allows the detection of only living cells expressing luciferase. Other cells shows a neglecting light signal background, so the images show a high signal to background ratio. Finally, the greater the number of transfected cells the stronger is the light signal, thus the number of transfected cells can be easily monitored allowing to study the tumor progression or the antitumor drug efficacy. The exposure time in BLI is in the order of 5 minutes. Tomographic acquisitions can last up to 30 minutes. New transfected luciferase expressing cells emit high luminescence signal allowing a very rapid imaging *in vivo*, with only 1 second exposure time for planar images and 6 seconds for tomographs.

### Fluorescence Luminescence Imaging (FLI)

FLI is devoted to the acquisition of photons coming from fluorescent systems. They can be endogenous molecules (collagen, hemoglobin), fluorescent proteins (GFP) or fluorescent dyes (Luker and Luker, 2008; Balas, 2009). FLI needs an external light source and the emitted light is to be detected during illumination. Filters are used to discriminate the emitted photons (with lower

energy) from the exciting photons (with higher energy). Recently, the huge improvement in the nanomaterial field led to the synthesis of many nano-sized materials with optical properties useful for OI. In particular, nanoparticles (objects with the three dimension smaller than 100 nm) were and still are developed for imaging applications. Among the fluorescent nanoparticle, quantum dots (QDs, semiconductor nanocrystals) are very useful thanks to the very wide range of excitation wavelengths and the specific emission. Their emission is tunable and depends on the size of the particle core. QDs are generally composed by a core and one or more outer shells, protecting the core and improving solubility in water. The chemical composition of the particles is an important issue, since toxic materials are often used for the synthesis of some nanoparticles.

Generally, the fluorescence emission is stronger than the bioluminescence emission but the background too is higher, mainly due to tissues' autofluorescence. Depending on the excitation and emission wavelength selected for the experiments, a specific animal diet with alpha-alpha free food can be used to reduce intrinsic autofluorescence.

The exposure time in FLI is of the order of one second only. Tomographic acquisitions can last up to 10-15 minutes.

### Cerenkov Luminescence Imaging (CLI)

CLI is a very recent technique based on the detection of charged particles travelling in the biological tissues with a velocity greater than the speed of light through the material (Boschi and Spinelli, 2014). The charged particles are generally electrons or positrons (which are positive charged electrons) emitted by radioactive nuclei. Radiotracers usually employed for humans in Nuclear Medicine are thus bimodal tracers visible by both OI and PET (Positron Emission Tomography). One of them is  $^{18}\text{F}$ -FDG which is a modified glucose molecule used to mark tumors and high active organs (brain, heart). A list of radiotracers optically detectable and their employment can be found in Boschi and Spinelli, 2014.

The exposure time in CLI is in the order of 5 minutes. Tomographic acquisitions need 5-6 images requiring a total of 25-30 minutes.

## Instruments

Generally, the instruments for OI are called "optical imagers". The heart of the instruments is a very high sensitive CCD camera with  $512 \times 512$  up to  $2048 \times 2048$  pixels. The CCD is cooled at minus 40 up to minus 90 °C to reduce the electronic noise. For FLI one or more light sources (lasers, LEDs or lamps) are needed to illuminate the samples. Instead, no sources are required in case of BLI. In order to prevent the contamination of ambient light, the samples are placed in a dark chamber on a stage which is generally heated to keep the animals warm during anesthesia. A camera for pre-anesthesia is generally equipped with the instrument.

The field of view (FoV) can be set from few centimeters to 20-30 cm allowing the imaging of more than one animal at the same time (up to five) reducing the total experimental time. For fluorescence modality, a set of excitation and emission filters is used to select the proper light to excite the fluorophores and to discriminate the right wavelengths allowed to reach the detector.

The acquisition modality is very easy, comparable to taking a picture with a standard photographic camera. To control the amount of detected light the researcher can set the exposure time, the diaphragm, the binning, (*i.e.* the sensitivity of the CCD) and the focus height. The resolution can vary depending on the FoV and the pixel size; in theory, it is possible to reach a resolution of about 20 micron for *in vitro* acquisitions, whereas, for *in vivo* applications, the resolution is reduced because the images are blurred due to the scattering of the photons in the tissues.

The luminescence images are presented in pseudo-colors related to the intensity of the collected light and superimposed on a photograph of the samples in order to co-localize the signal sources. Some instruments, equipped with a Computed Tomography module can fuse optical images with CT anatomical information, generally the skeleton of the mouse.

The quantification of the light emission is done by tracing a region of interest on the anatomical images and by measuring the total flux emitted or the efficiency (the number of emitted photons divided by the number of photons incoming on the samples) in case of FLI.

The instruments have lower costs with respect the other imaging systems based on different

techniques. Contrast agents, anesthesia and related filters are the only consumable needed for the applications.

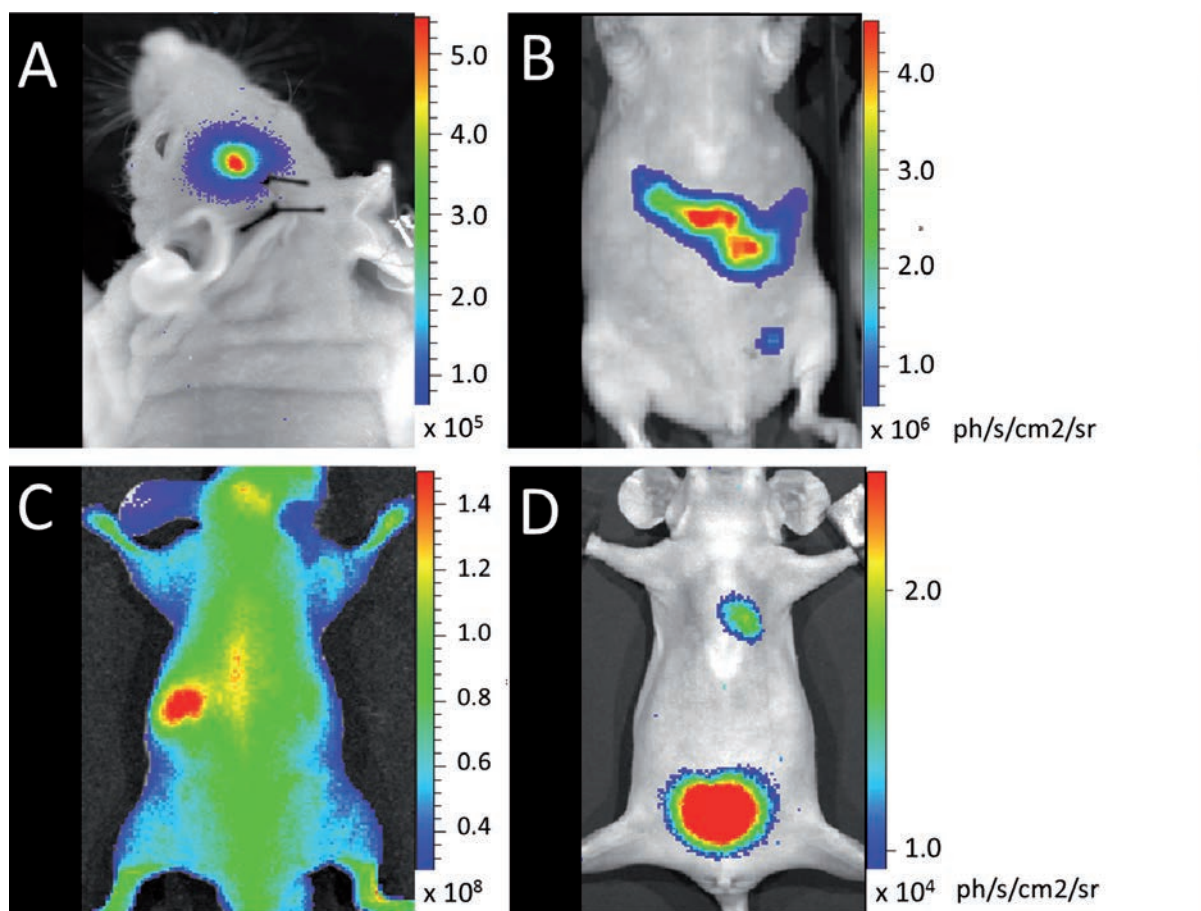
## Applications

A great variety of cellular and molecular processes *in vivo* can be visualized by OI, including protein interactions, protein degradation and protease activity. Our applications are focused in preclinical research including cancer imaging, drug biodistribution, drug targeting, stem cell homing, new nanoparticle design and CLI improvement. The laboratory is equipped since 2006 with a IVIS 200

(upgraded to IVIS Spectrum in 2012) purchased by Xenogen Corporation (now Perkin Elmer).

## BLI applications

In our laboratory BLI is mainly used in oncology to monitor tumor progression (Figures 1 and 2), with particular attention to pancreatic cancer (Ritelli *et al.*, 2015; Pozza *et al.*, 2015), colon rectal cancer (Minicozzi *et al.* 2011), and brain cancer, and to test potential anticancer drugs in collaboration with pharmaceutical companies. We also explored the sensitivity and the diagnostic potential of combined OI and magnetic resonance imaging (MRI), and we investigated the relation bet-

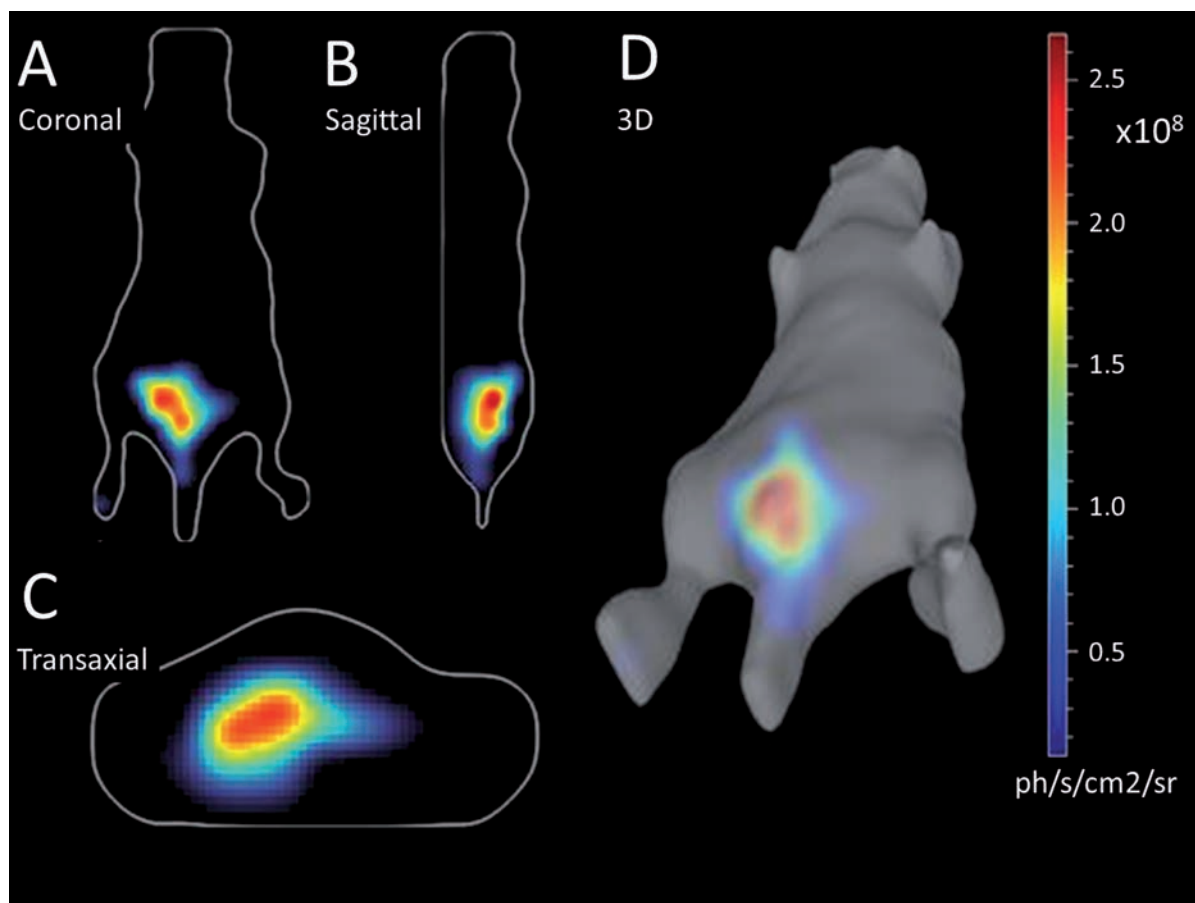


**Figure 1.** Bioluminescence imaging of luciferase expressing glioblastoma in mouse brain (A) and pancreatic cancer in mouse abdomen with metastasis (B). Fluorescence imaging of commercial NIR emitting Quantum Dots nanoparticles, 3 hours after tail vein injection in mouse; the liver uptake is clearly visible (C). Cerenkov luminescence imaging of <sup>18</sup>F-FDG, 1 hour after tail vein injection in mouse; the glucose metabolism of the heart can be observable as the accumulation in the bladder. The colorbars represent the radiance (ph/s/cm<sup>2</sup>/sr) for all the images; a logarithmic scale was used for image (D).

ween OI photon emission and MRI volume of the tumors obtained, for example, by luciferine expressing glioblastoma cells inoculated in the brain of Nu/Nu mice. In another application, we showed that intravenous administration of adipose-derived mesenchymal stem cells represents a promising therapeutic approach for neurological autoimmune diseases before the disease onset. Instead, it significantly reduces the severity of experimental autoimmune encephalomyelitis by immune modulation, and decreases spinal cord inflammation and demyelination. For this purpose we used cells transiently transfected with a plasmid encoding for the firefly luciferase (Constantin *et al.*, 2009).

### FLI applications

Fluorescent imaging was firstly applied to the synthesis of a novel conjugate between a near-infrared indocyanine dye and an organic polyamine polymer (polyethylenimine, PEI) with high chemical stability and good optical properties able to bind DNA and detectable *in vivo* (Masotti *et al.*, 2008). Our attention was then focused on the study of biodistribution of commercial QDs (Boschi, 2008; Pozzi-Mucelli *et al.*, 2009) and to help in the design of new fluorescent nanoparticles. In particular, silica nanoparticles loaded with two fluorescent dyes proved to be suitable for *in vivo* optical imaging and, at the same time, for confocal microscopy (Rampazzo *et al.*, 2012).



**Figure 2.** Bioluminescence imaging of luciferase expressing colon rectal cancer in mouse. Coronal (A), sagittal (B), transaxial (C) views and 3D reconstruction (D) of the light sources inside the body. The *multi-spectral* approach was used here using 6 images with different emission filters.

Recently, we studied *in vivo* the biodistribution in mice of solid lipid nanoparticles and nanostructured lipid carriers treated with polysorbate 80: the images demonstrated that after intraperitoneal administration the nanoparticles prevalently accumulated in the liver and spleen and are also able to reach the brain (Esposito *et al.*, 2015).

### CLI applications

In collaboration with dr. Spinelli from the S. Raffaele Institute in Milan, we demonstrated the potentialities of Cerenkov radiation in the biomedical field (Spinelli *et al.*, 2010) and in particular for cancer detection (Boschi *et al.*, 2011). We compared CLI and PET images in case of  $^{18}\text{F}$ -FDG administration. Moreover, we extended the reconstruction algorithm for BLI to CLI in order to obtain a tomographic reconstruction of the Cerenkov sources inside the animal body using the *multi-spectral* view approach. The result was validated by MRI (Spinelli *et al.*, 2011). Using NIR-emitting commercial QDs, we also explored the possibility to increase the detectability of Cerenkov radiation in living animals by shifting the primary Cerenkov blue emission into a red-NIR radiation (Boschi and Spinelli, 2012). Several studies were devoted to optimize Cerenkov detectors and Cerenkov images analysis (Spinelli and Boschi, 2011, 2012), and we obtained the first Cerenkov image in human, in particular from a patient treated with  $^{131}\text{I}$  for hyperthyroidisms

where iodine uptake was imaged in the thyroid gland (Spinelli *et al.*, 2013). For this application we used a portable CCD camera and only 2 minutes of exposure time.

We extended also the optical detection of radiotracers to beta minus emitters and to alpha (Boschi *et al.*, 2011) and gamma emitters, which are regularly employed in Nuclear Medicine. In particular, we observed the  $^{99\text{m}}\text{Tc}$ -pertechnetate (a widely used gamma emitter) in salivary and thyroid glands in mice detecting very faint light signals (Boschi *et al.*, 2013). We referred to the imaging of radionuclides as radionuclide imaging (RLI).

### Future perspective

OI is very cheap, repeatable and fast. It allows very simple samples preparation and it is now largely diffused in the preclinical field, allowing detection of a wide variety of biomolecules and biological processes. The real challenge is the application on humans. Bioluminescence applications are now not applicable but FLI presently is taking the first steps into the clinical field, in particular for the localization of tumor borders during surgery. We also demonstrated that Cerenkov applications may be feasibly applied in humans, so that more numerous and successful applications can be expected in near future.

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