

Bioluminescence Microscopy

New Avenues in Live Cell Imaging



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In contrast to fluorescence methods, bioluminescence microscopy does not need excitation by light. As photon emission results from a chemical reaction, results are highly specific and quantifiable. Until recently bioluminescence microscopy was difficult to approach as a result of rather dim signal intensities. Due to better probes and especially thanks to better and more specific instrumentation this technique has now become much more accessible and can in many situations outperform fluorescent approaches.

Bioluminescence Microscopy: Advantages and Challenges

In bioluminescence microscopy we detect light that is produced due to a chemical reaction of an enzyme (luciferase) with its substrate (luciferin). Similar to the much better known fluorescence approaches, bioluminescence is a technique that can be used for non-invasive analysis of molecular functions in living cells and tissues (fig. 1). In contrast to fluorescence where the light to be produced is generated by the absorption of photons, bioluminescence does not need excitation light. A second important difference is energy-dependency (fig. 2). Both these characteristics give bioluminescence microscopy several conceptual advantages:

In a traditional fluorescence microscope the light path is generally more complex, as specially designed filter sets (plus other optical elements, see below) are used to separate emission from excitation light. This filtering is challenging as the excitation light is by principal much stronger than the corresponding emission light. This problem is non-existent in bioluminescence. A second challenge in flu-

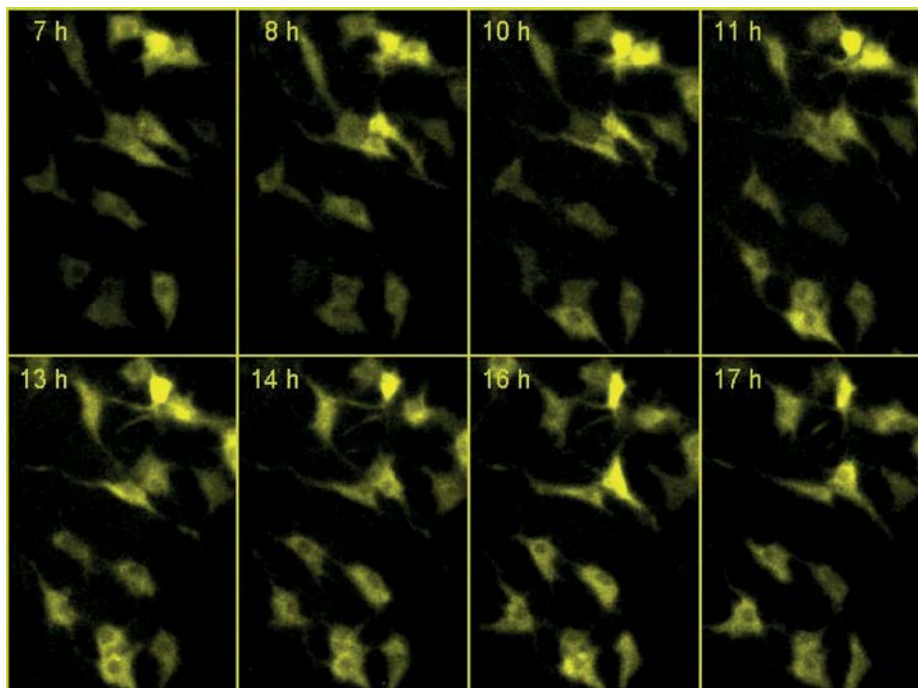


Fig. 1: Bioluminescence time lapse microscopy of NIH3T3 fibroblasts stably expressing Bmal1-luciferase [6] (only selected images of the full time-lapse are shown). Images were taken with the LV 200 bioluminescence microscope with 30 minutes exposure times.

orescence comes from autofluorescence: Components other than fluorochromes in and outside of cells emit fluorescent light in a non-specific manner. Again autofluorescence is totally absent in bioluminescence leading to extremely low levels of background light. Although fluorescence signals might be often stronger in absolute values (see below), the signal to noise ratio can thus still be as good or better in bioluminescence. Consequently bioluminescence microscopy is ideal to quantify expression levels by observing small signal changes close to background levels.

Another advantage for bioluminescence comes from the aforementioned energy- (ATP-) dependency: Only physiologically active and intact cells will produce light. In fluorescence dead or dying cells do actually express a very high autofluorescent signal.

The third important issue is phototoxicity and photobleaching. In fluorescence microscopy photobleaching decreases signals over time while phototoxicity leads to damaged or dead cells due to light-induced generation of free radicals. Both these phenomena are again non-existent in bioluminescence.

What are the challenges of bioluminescence microscopy? The main challenge was and to some extent still is the fact that bioluminescence signals are generally much weaker than fluorescence. Weak signals result in a) poor time resolution and/or b) poor spatial resolution.

Fortunately, optimized microscopical setups and better probes have improved this situation (see next chapter). Other challenges with less impact are variability of the half-life of genetically encoded luciferases and variability in their enzymatic activity.

Evolution of Probes and Instrumentation

As stated, a big challenge in bioluminescence microscopy is signal intensity. To overcome this, advances have been undertaken on two fronts namely a) the development of better probes and b) the development of better instrumentation.

For probes efficient light emission is obviously the most important factor. Other factors to consider are signal stability and expression efficiency. Spectral properties can additionally be of interest for multi-channel approaches.

Luciferases have been isolated from several organisms (fig. 3). Besides the classical firefly (*Photinus pyralis*) luciferase, luciferase genes have been isolated from copepods (*Gaussia princeps*), from renillidae (sea pansy = *Renilla reniformis*) [1], from oplophoridae (deep sea shrimp = *Oplophorus gracilirostris*) [2] and from Elateridae (click beetle). Compared to firefly luciferase, the luciferases from *Gaussia* and *Oplophorus* are significantly brighter (ca. 100 fold for *Gaussia*, ca. 150 fold for *Oplophorus*) [2,3], giving them a clear advantage. To their disadvantage is that *Gaussia* and *Oplophorus* have both their emission peaks at 470 nm (in contrast to the 560 nm emission light of firefly luciferase). This 470 nm emission wavelength could be a disadvantage for tissue imaging as light scattering is increased towards shorter wavelengths. Eluc is a modified firefly luciferase reported to be up to ten times brighter than wild type luciferase [4].

Instrumentation improvements have been realized by a) redesigning the microscope setup and b) by improved detection sensitivity. The first instruments for bioluminescence microscopy used traditional microscope setups. Parts of traditional setups are extra magnification lenses, filters, mirrors and other optical elements that are built into the path between the specimen and the camera. Each of these elements increases the minimal distance between specimen, plus eats up light intensity. None of these elements is needed for bioluminescence.

The only commercial setup, where dedicated beam-path optimizations have been undertaken so far is the LV (Luminoview) 200 from Olympus. In this instrument the light path containing only one tube lens, is shortened to less than half compared to traditional microscopes. This beam-path improvement leads to an over 10x increase in light collection efficiency when compared to traditional microscope setups. Additional (comfort-) improvements come from a light-tight enclosure that protects camera and specimen from outside light. In traditional microscope setups great care had to be taken to design an extremely dark room in order to do bioluminescence microscopy.

Similarly important as the microscope setup, is the camera. Highest quantum efficiencies are currently realized with electron multiplying CCD (EMCCD) cameras. The advantage of this chip construction lays in the fact that the electron multiplication is happening before the read-out. This means that charge (photon induced but also unwanted thermally generated charge) from each pixel is multiplied directly on the sensor. The thermally generated electrons are responsible for dark current noise, the most important noise in these cameras. Fortunately this noise is temperature dependent [5]. By cooling the camera chip it can be kept small. In our setup (Hamamatsu EMCCD C9100-13) we use water- plus Peltier-cooling and work with a camera chip cooled down to -92°C.

Application Examples

We have previously shown that cultured cells contain autonomous and self-sustained clocks using long-time fluorescence live cell imaging [6]. In later experiments we wanted to test robustness of circadian rhythms against changes in temperature or global transcription rates. For these experiments fluorescence microscopy could not be used: As temperature changes and drug treatments were needed, cells did not tolerate any additional phototoxic stress for more than a few hours. In contrast by using bioluminescence time-lapse microscopy of NIH3T3 fibroblasts expressing luciferase under the control of a circadian

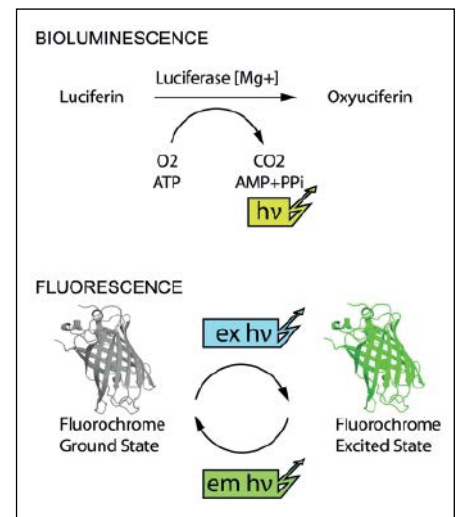


Fig. 2: Light production scheme in bioluminescence and fluorescence microscopy.

promoter (Bmal1-luc cells [7]), circadian gene expression could be monitored and quantified over several days.

In a related project, bioluminescence microscopy with neuronal precursor cells helped to demonstrate that circadian gene expression is already apparent during early stages of development [8].

In a very different subject bioluminescence microscopy showed to be equally useful to precisely quantify short-time events. In both prokaryotes and eukaryotes, transcription has been described as being temporally discontinuous, most genes being active mainly during short activity windows interspersed by silent

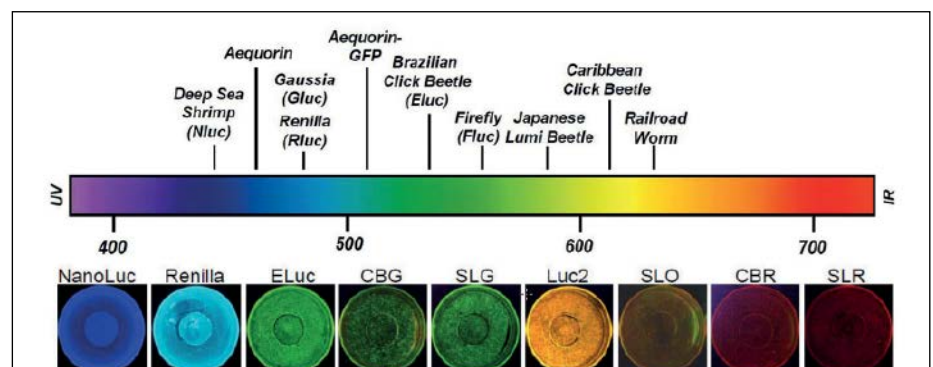


Fig. 3: Colors of bioluminescence: NanoLuc (deep sea *Oplophorus gracilirostris* luciferase, Promega [2]), Renilla (Rluc from *renilla reniformis*), Eluc (Enhanced Beetle Luciferase [4]), CBG (click beetle green luciferase [11]), SLG (green-emitting luciferases, Toyobo), Luc2 (synthetic firefly luciferase Promega), SLO (orange-emitting luciferase [12]), CBR (red-emitting click beetle luciferase, Promega), SLR (red-emitting beetle luciferase from *Phrixothrix hirtus*, Toyobo). Image kindly provided by Olympus Corporation, Tokyo.

periods. To characterize this in more detail transcription rates needed to be monitored at higher temporal resolution. This was done by establishing various cell lines expressing a short-lived luciferase protein from an unstable mRNA. Using high camera binning (4x4 pixels) and photon counting mode we could record and quantify transcription levels for up to 72 hours with a time resolution of five minutes. This allowed to characterize transcriptional kinetics of endogenous mammalian genes and led to the conclusion that mammalian genes are transcribed with widely different bursting rates having each its characteristic kinetics signature [9].

In a recent paper we describe circadian gene expression in pancreatic human islets. For this project we needed additional channels besides bioluminescence. To do this we added a CoolLed light source to our bioluminescence microscope. As there is no room for dichroic mirrors in the system, we used highly selective emission filters inserted in a filter wheel between specimen and camera. With this setup we could do time-lapse experiments combining bioluminescence with fluorescence and transmission channels [10] (fig. 5).

Summary and Outlook

Bioluminescence microscopy offers new avenues in live cell imaging and can replace fluorescence microscopy approaches if phototoxicity becomes critical. This is frequently the case in long-term recording experiments and/or if the experimental setup implements unavoidable stress. In contrast to fluorescence approaches, bioluminescence microscopy does not need excitation by light (with potentially phototoxic effects). In addition, bioluminescence results are highly specific and quantifiable. In distinction to fluorescence microscopy where autofluorescence of specimens as well as reflections or contamination from the excitation light can contribute to signal intensities, bioluminescence signals correspond in a one-to-one fashion to molecular events.

Until recently bioluminescence microscopy was a difficult task. Improvements in probes and better-designed instrumentation have made the technique a more accessible and highly intriguing tool opening new avenues to the biomedical research community.

Acknowledgements

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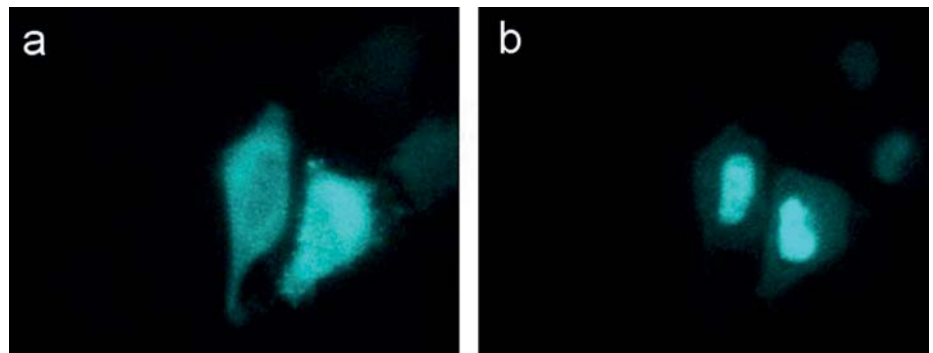


Fig. 4: Dexamethasone-induced cytosol to nucleus translocation of NanoLuc-glucocorticoid receptor (GR) fusion proteins measured in HeLa cells. Images were taken with the LV 200 bioluminescence microscope with 2 seconds exposure times (reprinted with permission from [2]).

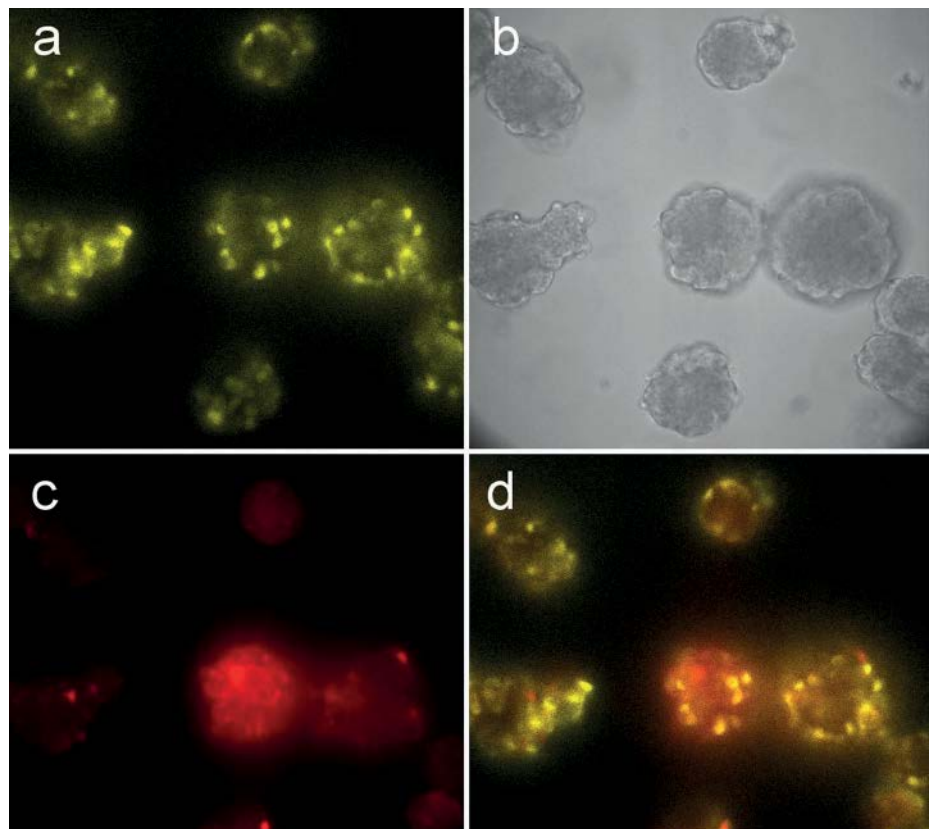


Fig. 5: Combined bioluminescence and fluorescence image of individual human pancreatic islets. a) bioluminescence channel (expression of circadian luciferase reporter in islet cells), b) corresponding transmitted light image, c) fluorescent channel image (tdTomato fluorescent protein under the control of insulin promoter specifically expressed in human beta cells), d) overlay of bioluminescence and fluorescence channels [10].

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Please find the complete list of references online at “www.imaging-git.com”.

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