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.

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-live 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 renillidutes (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 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
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 let 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 more accessible and highly intriguing tool opening new avenues to the biomedical research community.

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

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