HHS Public Access

Author manuscript

*Nat Protoc*. Author manuscript; available in PMC 2017 November 01.

Published in final edited form as:

*Nat Protoc*. 2017 May ; 12(5): 1055–1076. doi:10.1038/nprot.2017.008.

# Modular low-light microscope for imaging cellular bioluminescence and radioluminescence

**Tae Jin Kim**[1], **Silvan Türkcan**[1], and **Guillem Pratx**[1]

[1]Department of Radiation Oncology, Division of Medical Physics, Stanford University School of Medicine, Palo Alto, California 94304, USA

## Abstract

Low-light microscopy methods are receiving increased attention as new applications have emerged. One such application is to allow longitudinal imaging of light-sensitive cells with no phototoxicity and no photobleaching of fluorescent biomarkers. Another application is for imaging signals that are inherently dim and undetectable using standard microscopy, such as bioluminescence, chemiluminescence, or radioluminescence. In this protocol, we provide instructions on how to build a modular low-light microscope (1-4 d) by coupling two microscope objective lenses, back-to-back from each other, using standard optomechanical components. We also provide directions on how to image dim signals such as radioluminescence (1-1.5 h), bioluminescence (~30 min) and low-excitation fluorescence (~15 min). In particular, radioluminescence microscopy is explained in detail as it is a newly developed technique, which enables the study of small molecule transport (eg. radiolabeled drugs, metabolic precursors, and nuclear medicine contrast agents) by single cells without perturbing endogenous biochemical processes. In this imaging technique, a scintillator crystal (eg. $CdWO_4$) is placed in close proximity to the radiolabeled cells, where it converts the radioactive decays into optical flashes detectable using a sensitive camera. Using the image reconstruction toolkit provided in this protocol, the flashes can be reconstructed to yield high-resolution image of the radiotracer distribution. With appropriate timing, the three aforementioned imaging modalities may be performed altogether on a population of live cells, allowing the user to perform parallel functional studies of cell heterogeneity at the single-cell level.

## Introduction

Both bioluminescence and radioluminescence signals are dim phenomena that require a highly sensitive microscope for imaging at the cellular level. Bioluminescence microscopy is an attractive method for studying biological processes with high sensitivity. This is possible as bioluminescence captures the endogenous light emission from cells[1,2], without the background signal (e.g. autofluorescence) and phototoxicity associated with external light

Correspondence should be addressed to G.P. (pratx@stanford.edu).

**Author Contributions:** T.J.K. designed and built the low-light microscope system; T.J.K. and G.P. developed the graphical user interface version of ORBIT; T.J.K. and S.T. prepared radiolabeled samples; T.J.K. performed experiments and analyzed data; T.J.K. and G.P. prepared manuscript; S.T. provided manuscript feedback; G.P. provided guidance for the entire project.

**Competing Interests Statement:** The authors declare no competing financial interests.

sources. These features make it possible to track biological signals longitudinally over extended periods of time, with temporal resolution ranging from a fraction of a second to tens of seconds, and spatial resolution on the order of a few microns. Examples of bioluminescence microscopy include protein-protein interactions have been quantified using either bioluminescence energy resonance transfer[3] or luciferase complementation imaging[4]. Calcium levels have also been measured longitudinally using a luciferase variant known as aequorin[5]. Finally, luciferase has been shown to be a suitable reporter of gene expression, which can be used to follow circadian rhythms or other slow cellular oscillations[6].

Radioluminescence is another dim phenomenon that can be used to measure cellular transport of radiolabeled compounds. Conventional radionuclide labeling techniques are well established for probing molecular processes involving small molecules (drugs, metabolites, etc.), since virtually any small molecules can be tagged with a radioisotope[7]. Such techniques are commonly used in conjunction with *in vivo* radionuclide imaging such as positron emission tomography and autoradiography, or *ex vivo* radionuclide counting. However, these conventional radiometric assays are limited to bulk samples composed of many cells.

Radioluminescence microscopy is a recently developed technique that extends radionuclide assays to the single-cell level, thus providing a quantitative assessment of molecular transport of radiolabeled probes by single cells. In order to perform radioluminescence microscopy, a scintillator crystal[8] is placed near the cells and emits dim optical flashes each time a beta particle (electrons or positrons) emerges from the radiolabeled cell. By capturing images of this low-light phenomenon, we can image the distribution of the radiolabeled probe in single cells. To form an image, two approaches may be employed. The fastest and simplest method is to take a single long exposure (30-300 s), which integrates the optical signals of many radioactive decays and thereby provides an approximate image of the radionuclide distribution. However, this image is only qualitative and its suffers from a few distortions. A more quantitative image can be obtained by capturing many camera frames with short exposure (typically 10-100 milliseconds each) and by processing each frame separately to extract the location of individual radioactive decay events. These events can then be digitally counted to create a composite image showing their spatial resolution. The resulting image is intrinsically digital (since it corresponds to the number of detected events) and therefore it can be used to obtain quantitative information on the uptake of a radionuclide in cells. Previously, we have used this technique to characterize cell metabolism based on endpoint and time-dependent transport of glucose[9], cell proliferation using a radioactive thymidine analog[10], and drug binding in a humanized mouse model of non-Hodgkin lymphoma[11].

It should be noted that other approaches also exist for high-resolution imaging of radionuclides. Film microautoradiography, the standard method for high-resolution imaging of radionuclides in cells, uses a specially made photographic emulsion to capture the passage of ionizing particles out of the cells[12]. Compared to radioluminescence microscopy, this technique is advantageous for imaging large area tissue sections and for low-energy radionuclides such as $^{3}H$ and $^{14}C$ due to the high spatial resolution. However, the preparation of thin emulsions is technically difficult to implement and the captured image is

not easily amenable to automated computer analysis for accurate quantitation of radiotracer concentration. The signal can also be non-linear due to saturation of the silver halide grains. Finally, standard measurements require long exposure times due to low sensitivity, and cells must be fixed prior to imaging, which precludes kinetic imaging of cellular transport by single cells. Fully digital approaches, such as the iQID camera[13] and the BetaIMAGER[14], are also available for imaging radioactivity in thinly cut tissue samples, but without live-cell and fluorescence microscopy capabilities.

The main challenge with measuring the aforementioned low-light phenomena is that they are generally too dim for conventional fluorescence widefield microscopes. This issue has motivated the development of two commercial bioluminescence microscopes, the LV200 from Olympus[15,16] and the Cellgraph from ATTO[17]. These systems combine efficient light collection with highly sensitive cameras such as electron multiplying charge coupled devices (EMCCD) or intensified charge-coupled devices (ICCD). However, being primarily optimized for bioluminescence microscopy, these systems offer little flexibility for implementing other low-light imaging approaches such as radioluminescence imaging. In addition, they suffer from high background (spectral bleed-through) during fluorescence imaging because the sample is illuminated using a transillumination source.

Recently, we developed a “low-light microscope” (LLM) to offer a cost-effective and modular alternative to commercial bioluminescence microscopes[18]. This system is entirely constructed from commercial lenses and optomechanical parts. In particular, the tube lens (image forming lens), which is proprietary on commercial bioluminescence microscopes, is a high-end objective lens (CFI Plan Apochromat λ 4×, Nikon) placed backwards in the optical path. The design of the microscope enables a level of performance comparable to commercial bioluminescence microscopes (e.g. LV200), except for a minor reduction in field-of-view (~30% area) due to the smaller physical aperture of the 4× objective used as tube lens[18]. Since the microscope is highly modular, the setup can be tailored to the researcher's needs, and additional capabilities such as microfluidics, intravital imaging or motorized X-Y stage can be added[19].

An epifluorescence illumination source can be incorporated to enhance the contrast of fluorescence imaging. Commercial bioluminescence microscopes use a transillumination configuration for fluorescence imaging, which creates high background signal due to bleed-through of the excitation light through the emission filter. It should be noted, however, that the epifluorescence module will cause additional vignetting (20% reduction in imaging area) compared to the LLM without the epifluorescence module. This is due to the additional spacing required between the objective and tube lens to fit the dichroic mirror. If a larger imaging area is desired, the tube lens should be placed close to the objective lens without leaving space for the epifluorescence illumination module. Table 1 summarizes the advantages and shortcomings of the proposed setup compared to the commercial microscopes.

The protocol also provides details on performing bioluminescence and radioluminescence microscopy. Special attention is devoted to radioluminescence because protocols for this type of imaging have not been presented elsewhere in detail. Of note, the instrument

described in this protocol can also be used for epifluorescence imaging with very low excitation, albeit with lower spatial resolution and magnification. This feature could be useful to complement the information obtained using bioluminescence and radioluminescence imaging or to follow the fluorescence signal of single cells over extended periods of time with low phototoxicity.

## Experimental Design

**Low-light Microscope Assembly—**Image brightness, or light gathering power, is defined as the intensity of the light that reaches the camera sensor. This metric increases as more light is collected by the objective (higher numerical aperture) and as the light is spread out less when reaching the detector (lower magnification). As a result, brightness can be calculated as,

$$B=\left(\frac{NA}{M_{\text{effective}}}\right)^2 \cdot T \cdot 10^4, \quad (1a)$$

$$M_{\text{effective}}=\frac{f_{\text{tube}}}{f_{\text{objective}}}, \quad (1b)$$

where $NA$ is the microscope objective numerical aperture, $T$ is the transmission efficiency, $M_{effective}$ is the effective magnification of the microscope, $f_{tube}$ is the focal length of the tube lens and $f_{objective}$ is the focal length of the microscope objective.

The principle behind the LLM is to allow more light to converge on each pixel through high numerical aperture and low magnification[15]. According to equation (1a), a combination of high $NA$ and low magnification $M_{effective}$ will greatly enhance image brightness. While the most desirable solution is to find a microscope objective that features high NA at low magnification, such objective lens does not exist. Therefore, low magnification is achieved through demagnification using a custom tube lens with short focal length ($f_{tube}$) (Fig. 1).

The LLM uses a Nikon λ 4× objective as tube lens. The 50 mm focal length of this objective is substantially shorter than that of conventional tube lens (200 mm, 180 mm, and 165 mm for Nikon/Leica, Olympus, and Zeiss, respectively), proportionally reducing the effective magnifying power. Furthermore, with its high numerical aperture of 0.2, the Nikon λ 4× is one of the few objectives that can accommodate oil immersion objectives, generally without loss of performance. To retain high light collection capability, the tube lens should have an entrance pupil diameter comparable or greater than the exit pupil diameter of the microscope objective. Based on this principle, the numerical aperture of the tube lens must satisfy

$$(f \cdot NA)_{\text{tube}} \geq \left( f \cdot \frac{(NA/n)}{\sqrt{1-(NA/n)^2}} \right)_{\text{objective}}, \quad (2)$$

where $n$ is the refractive index of the immersion fluid or air of the objective lens. If the right-hand side of this equation is greater than that the left-hand side, the effective *NA* and light collection of the system will be less than specified by the objective lens. Also, the LLM design requires the working distance of the tube lens to be long enough for the lens to be coupled backwards to a camera. It should be noted that the effective magnification and theoretical brightness of the LLM in equation (1b) varies depending on the manufacturer of the objective (Supplementary Table 1).

Once the microscope is assembled, a camera must be coupled to the tube lens. Given the dim signals, minimizing camera noise is essential. The three major sources of noise are read noise, dark noise, and shot noise. Read noise occurs each time the camera is readout. Since read noise is constant and independent of the signal, its effect can be minimized by amplifying the signal before it is read out. This is achieved either using an electron multiplication register (EMCCD) or an image intensifier (ICCD). The next type of noise, dark noise, occurs at a fixed rate, and therefore its effect is stronger for longer exposures. Deep sensor cooling is effective at minimizing dark noise. Finally, photon shot noise emerges from inherent statistical fluctuations in the number of incident photons. To minimize this effect, one must maximize the number of photons detected per pixel. This can be achieved by increasing the exposure time, the detector quantum efficiency, the camera pixel size (binning), or the light collection efficiency (numerical aperture).

Accounting for the aforementioned requirements, EMCCD cameras are best suited for radioluminescence microscopy since they can capture dim signals with short exposures (as low as 10 ms per frame). Such short exposures are required because it is not possible for the computer software to process multiple radioactive decays that overlap spatially in the camera frames. With a short exposure, there are fewer decays per frame on average. Another advantage of using EMCCD cameras is that their larger pixel size (typically 13 or 16 μm) allows more light to converge on each pixel. This effect increases the signal-to-noise ratio without degrading spatial resolution, which is fundamentally limited by the physics of beta particle propagation. For bioluminescence imaging, a deep-cooled CCD camera with relatively large pixel size is a cost-effective alternative, especially when complemented by on-chip pixel binning and long exposures[15].

In addition to the microscope system, the entire microscope environment should be physically as dark as possible by enclosing it inside a light-tight box. Few commercial products are available for this purpose; therefore, the best option will be to manufacture a custom enclosure. Since imaging cannot be performed with the enclosure door open, a motorized stage is required to focus the microscope.

The level of expertise required to assemble the LLM is moderate-high. Although the assembly procedure is relatively simple, the system needs extensive calibration to achieve

good performance. In particular, the epifluorescence illumination must be calibrated using the Kőhler method to deliver even illumination throughout the specimen and prevent an image of the lamp from appearing on the imaging plane (Supplementary Fig. 1).

**Bioluminescence Microscopy—**Bioluminescence signals can be highly specific to a given biological process due to the near-absence of background noise. However, since the signal itself is extremely dim (as low as 3-4 photons/$mm^2$·s), bioluminescence microscopy requires a sensitive microscope with specialized optical train and low-noise camera. The LLM is sensitive enough to perform bioluminescence imaging of single cells, with performance equal to or better than commercial systems.

Bioluminescence microscopy can image the expression of a bioluminescent reporter gene, such as firefly luciferase[20] or Renilla luciferase[21]. The reporter gene can be linked to a promoter sequence to probe for temporal transcriptional activity on the single cell level[22]. While light emission is the brightest right after the luciferase substrate (luciferin or coelenterazine) is introduced into the media, it is recommended to delay imaging for ~10 min, until the luciferin is uniformly taken up by the cells and luminescence emission rate reaches a steady state[23]. Imaging parameters (exposure time, multiplication gain, pixel binning) should be optimized to mitigate noise while maximizing spatial and temporal resolution.

**Radioluminescence Microscopy—**Radioluminescence microscopy can be used to quantify small molecule uptake in single cells using radionuclides. Suitable radionuclides are those that decay by emitting alpha or beta particles. Short-lived isotopes are also preferred because they do not produce long-term radioactive waste and have high specific activity.

In order to detect the invisible radioactive emission, a scintillating crystal is positioned in close proximity to the cells. Ionizing particles emanating from the sample deposit energy as they travel through the scintillator and produce scintillation light all along the way[24]. There are a few possible methods to position scintillators depending on different applications. Typically, the cells are grown in an imaging dish, and a scintillator plate (similar to a small cover slip) is gently dropped on top of the cells. Alternatively, the cells can be grown directly on the scintillator itself to ensure very close contact between the cells and the scintillator to achieve optimum imaging performance. Finally, non-adherent cells can be immobilized between the imaging dish and the scintillator using a temperature-sensitive gel such as Matrigel. As for tissue imaging, radioactive tissue sections can be sandwiched between the scintillator and a glass slide.

The scintillation light can then be captured by focusing the microscope objective on the edge of the scintillator plate and as close to the cells as possible (Fig. 2). Due to the limited depth of field, the microscope effectively sees a cross-section of the 3D scintillation track that is projected onto a 2D plane. Thus, accurate focus is crucial for radioluminescence microscopy. Also, because the scintillation light originates a few microns away from the cells, brightfield and fluorescence imaging can only be used for coarse focusing. Furthermore, ionization tracks can extend to hundreds of microns inside the scintillator;

therefore, it is possible to visualize sharp ionization tracks and yet be out of focus from the cells, as shown in the Expected Results section.

An image of the integrated scintillation light can be obtained by acquiring a long exposure (30 - 300 s) with low-to-medium electron-multiplication gain. Such an image represents an analog measurement in which the individual scintillation flashes overlap with one another. While this acquisition mode provides only semi-quantitative information, it allows the user to quickly assess the distribution of the radioactivity.

For a sharper and fully quantitative image, multiple images of the scintillation flashes must be acquired and individually processed. Each scintillation flash corresponds to the stochastic decay of a single radiolabeled molecule. We use an algorithm to automatically identify and accurately localize radioactive decays based on the position and shape of the corresponding scintillation flash. In order for this reconstruction process to work, a few parameters should be considered during the image acquisition procedure:

1. *Exposure time*. Since radioactive decay is a random process, the scintillation flashes occur in a random fashion. To prevent multiple flashes from overlapping, it is important to select a sufficiently short exposure time. Depending on the number of cells imaged and the radioactivity of each individual cell, the scintillation flashes occur at a rate of 1-300 flashes per second. A rule of thumb is that there should be no more than 10 ionization flashes per frame, which requires exposure time to be on the order of 30 to 1000 ms.
2. *Number of frames*. As for any counting processes, the noise in radioluminescence microscopy is dominated by shot noise, which follows Poisson statistics. Too few camera frames will lead to noisy images and uncertain quantitation. To achieve a noise level of 5%, roughly over 400 counts/cell should be acquired. In practical terms, this translates to 10,000 to 15,000 frames per acquisition, and 15 to 30 min per acquisition, respectively.

Once the raw frames have been acquired, the distribution of the radionuclide probe is reconstructed using a methodology called “optical reconstruction of the beta ionization track”, or ORBIT[24]. Briefly, the method comprises three steps. First, the raw frames are filtered to decrease noise and segmented to isolate individual scintillation tracks. Next, individual scintillation tracks are extracted and processed to estimate the position of the molecule that caused the scintillation flash. Through this process, up to 20% of the tracks may be discarded if they are deemed unsuitable for accurate positioning (overlapping of multiple tracks, poor focus, or complicated track trajectory). Finally, the positions of individual molecules are aggregated to form a quantitative image of the radiotracer distribution. With proper calibration, the distribution can be expressed in absolute units of $Bq/mm^2$ (1 Bq = 1 radioactive disintegration per second).

In a typical radioluminescence experiment, the reconstructed images are analyzed to yield quantitative measurements of single cells. Radioluminescence imaging is a highly quantitative approach, sensitive to trace amounts of radioactivity (equivalent to <1 attomole). A typical analysis pipeline starts by placing regions of interests (ROIs) on individual cells. To minimize variability between ROIs, a single ROI template should be used for all cells—

typically a circle of a fixed radius. This is important because (1) the boundary of the radioactivity is not clearly defined on the radioluminescence image, and (2) ROIs of different sizes receive variable contribution from the image background. ROIs should be placed only on cells that are spaced far enough from one another to avoid crosstalk[18]. Once the ROIs have been placed, the number of recorded decays can be tallied and converted into units of number of molecules initially present inside a single cell,

$$N_0 = \frac{D}{S \cdot Y} \cdot \frac{1}{1 - e^{-\lambda T}} \left[ {}^{\text{molecules}} / {}_{\text{pixel}} \right], \quad (3)$$

where $D$ is the number of counts detected with the LLM, $S$ is the sensitivity calibration factor, $Y$ is the radioactive yield of particles, $\lambda$ is the decay constant of the radionuclide, and $T$ is the total exposure time. Derivation of the equation and additional details are included in Supplementary Note 1.

In summary, LLM enables radioluminescence imaging of single cells, providing information on heterogeneous biomolecular process involving small molecules, such as diagnostic and therapeutic agents. The LLM is also a multi-modal imaging tool that can capture fluorescence and bioluminescence signals on the same sample. It should be noted that, while the capabilities of radioluminescence microscopy are unmatched, the method suffers from low throughput (~100 cells/acquisition), long acquisition times (15-30 min), and lower resolution than purely optical microscopy methods.

## Materials

### Equipment

#### Low-light Microscope

- Tube lens (Nikon Co., CFI Plan Apochromat λ 4×)
- Objective lens with high *NA* (Nikon Co., CFI Plan Apochromat λ 20× recommended)
- Scientific EMCCD camera (Hamamatsu Photonics K.K., ImagEM C9100-13)
- Optional refrigerated water circulator (Julabo Inc., F25)
- Light-tight box (Qsonica LLC., cat. no. 432B2; Newport Co., Light Tight Optical Table Enclosures; Thorlabs Inc., Optical Enclosures; or custom-built enclosure)
- Calibration grid (Thorlabs Inc., cat. no. R1L3S3P)
- Bullseye and tubular spirit level
- Blackout fabric (Thorlabs Inc., cat. no. BK5)
- Screw set (Thorlabs Inc., cat. no. HW-KIT2)
- SM1 and SM2 spanner wrench (Thorlabs Inc., cat. no. SPW606 and SPW604)
- Hex key set (Thorlabs Inc., cat. no. TC2)

- Computer (128 GB solid-state drive for fast data acquisition; 1 TB hard-disk drive for long-term data storage; 8 Gb memory; quad-core processor; PCIe frame grabber, eg. AS-PHX-D24CL-PE1)
- Optics and optomechanical components for simplified version of LLM. See Table 2 for detailed component names and catalog numbers.
- Optics and optomechanical components for epifluorescence module. See Table 3 for detailed component names and catalog numbers.

### Software

- Microsoft Windows (Version 7 or greater) operating system
- MATLAB (version 2010 or greater)
- Hamamatsu HCImage (Version 2.0.1.16 or greater)
- Z-stage software controller (Thorlabs Inc., APT Version 3.11.0)
- ORBIT MATLAB package. Download software from Supplementary Data or from http://med.stanford.edu/pratxlab/research/downloads.html
- NIH ImageJ (or equivalent image viewer). Download from http://rsb.info.nih.gov/ij/download.html
- Optional web-based box plot toolbox for data analysis. Access from http://shiny.chemgrid.org/boxplotr/

### Radioluminescence Microscopy

- Dose calibrator (Biodex Medical Systems Inc., cat. no. 086-335)
- Lead L-block shield (Biodex Medical Systems Inc., cat. no. 042-419)
- Lead bricks, quantity 16 (Biodex Medical Systems Inc., cat. no. 002-248)
- Lead pig (Biodex Medical Systems Inc., cat. no. 001-706)
- Geiger-Mueller counter (Ludlum Measurements Inc., cat. no. 48-1605 and 47-1539)
- $CdWO_4$ scintillators, 1 cm × 1 cm × 0.5 mm, both sides polished (MTI Co., cat. no. CdW-101005S2) or other suitable scintillator[25]
- Glass-bottom imaging dishes (Cellvis, cat. no. D35-20-1-N)
- CO2 cell incubator, approved by Environmental Health and Safety department for radioactive samples (Thermo Scientific Inc., cat. no. 3403)
- 100 ml Pyrex beaker
- 50 ml graduated Pyrex cylinder, quantity 2 (Fisher Scientific Co., cat. no. S76101C)
- Optional Microm HM550 cryostat microtome (Thermo Scientific Inc.)
- Optional low-profile microtome blade (Fisher Scientific Co., cat. no. 3050822)

- Optional disposable base mold (Fisher Scientific Co., cat. no. 41-742)
- Optional pain brush, small

## Reagents

### Radioluminescence Microscopy

- Standard cell culture environment
- DMEM culture media (Thermo Fisher Scientific Inc., cat. no. 11995-065) supplemented with 10% FBS and 1% penicillin-streptomycin
- Glucose-free DMEM media (Thermo Fisher Scientific Inc., cat. no. 11966-025) supplemented with 10% FBS
- Phenol-red-free DMEM (Thermo Fisher Scientific Inc., cat. no. 21063-029) supplemented with 10% FBS
- Phosphate buffered saline (Thermo Fisher Scientific Inc., cat. no. 10010-023)
- Radiotracer solution, such as 2-deoxy-2-[$^{18}$F]fluoro-D-glucose ($^{18}$F-FDG; PETNET Solutions or local radiochemistry facility). An initial stock of 100 MBq in a small volume (<1 ml) is recommended. Freshly made radiotracer is preferred due to the higher concentration and specific activity.
- Optimal cutting temperature (OCT) compound (VWR, cat. no. 25608-930)
- Matrigel (Fisher Scientific Co., cat. no. CB-40234C)

### Scintillator Cleaning

- Concentrated sulfuric acid (Fisher Scientific Co., cat. no. A300S-500)
- Hydrogen peroxide, 30% (Fisher Scientific Co., cat. no. H325-500)

### Bioluminescence and/or Fluorescence microscopy

- Standard cell culture environment
- DMEM culture media (Thermo Fisher Scientific Inc., cat. no. 11995-065) supplemented with 10% FBS and 1% penicillin-streptomycin
- Optional Phenol-red-free DMEM (Thermo Fisher Scientific Inc., cat. no. 21063-029) supplemented with 10% FBS
- Luciferase-expressing cells
- D-luciferin reagent (Sigma-Aldrich Co. LLC., cat. no. L9504-10MG)
- NucBlue Live ReadyProbes reagent (Thermo Fisher Scientific Inc., cat. no. R37605)

## Reagent Setup

**Piranha solution—**Pour 9 ml of sulfuric acid (98%) into a graduated Pyrex beaker, and slowly pour in 3 ml hydrogen peroxide, 30% until the ratio is 3:1 (sulfuric acid: hydrogen

peroxide). ▲ CRITICAL Prepare solution right before cleaning the scintillator. ! CAUTION Harmful; prepare in chemical hood with full protection including protective eyewear, rubber apron and neoprene gloves; ensure there are no other organic compounds in the cleaning process other than the used scintillators; never leave hot Piranha solution unattended; never increase the hydrogen peroxide concentration to more than 50%.

**D-Luciferin substrate solution—**Create aliquot of concentrated D-Luciferin solution (~60 mM) by mixing 10 mg of D-Luciferin with 600 μl of sterile water. Filter sterilize through a 0.2 μm filter, if possible.

■ PAUSE POINT The D-Luciferin solution can be stored at -80°C for over 6 months.

## Equipment Setup

**Installing ORBIT in MATLAB library—**Unzip the three files in the ORBIT package directly into the user MATLAB folder. The user may type in the command 'path' in order to locate the folder (eg. C:\USER\DOCUMENTS\MATLAB\). The user may refer to the manual supplied by the authors for detailed instructions.

## Procedure

▲ CRITICAL Steps 1 – 35 explain the assembly and calibration of the modular low-light microscope. Steps 36 – 87, Box 1, and Box 2 describe the imaging procedure for radioluminescence, bioluminescence and fluorescence microscopy. Refer to the flowchart in Fig. 3 for a general overview of the entire protocol.

### Assembling low-light microscope base and camera mount ●TIMING 2-4 h

1. *Microscope base (see* Supplementary Fig. 2 *for detailed photographs).* Attach right-angle mounting plate on the 8″ × 8″ aluminum breadboard using a pair of 5/8″ length cap screws on each slot.
2. Attach 12″ × 24″ aluminum breadboard to the right-angle mounting plate using a pair of 5/8″ length cap screws on each slot.
3. Check that the bottom surface of the standing aluminum breadboard is flush with the work surface.

   ! CAUTION Ensure that the 8″ × 8″ aluminum breadboard is resting on a stable and flat surface. The system may become heavy enough to topple over if placed on an uneven surface, causing injury or damage to the system.
4. *Camera mount (see* Supplementary Fig. 3 *for detailed photographs).* Attach the 1″ length translation stage vertically to the 12″ × 24″ breadboard (microscope base), with the micrometer facing upwards. Secure the translation stage using two 3/8″ long cap screws.

   ▲ CRITICAL STEP Components required to assemble the mount will vary depending on the dimensions of the camera.

5. Attach 1″ length post holder to the translation stage using a 0.5″ length set screw (1/4″-20).

6. On the camera body, attach 0.75″ length optical post to the camera using a 0.5″ length set screw (1/4″-20).

   ▲ CRITICAL STEP Assembly may require longer or shorter posts.

   ! CAUTION Ensure that the camera detector is covered with the supplied cover lid until the assembly is completed. The tube lens or other compartments may fall on the detector, causing irreversible damage

7. Insert the camera into the post holder and tighten thumb screw.

   ▲ CRITICAL STEP Allow extra space at the bottom of the camera for camera cables. Also, ensure that the distance between the 12″ × 24″ breadboard surface and the center of the camera port is 1.5″ to 2.5″.

   ! CAUTION In order to prevent the camera from swiveling, possibly causing damage to the camera, ensure that all the optical components are securely tightened using hex keys.

8. Place a bullseye level on top of the camera detector port and confirm that the camera is vertically aligned (an example of how to use a bullseye level is shown in Supplementary Fig. 4).

### Assembling tube lens component

9 Install tube lens module (Option A) or tube lens with dichroic filter cube (Option B). If the primary purpose is to measure bioluminescence or radioluminescence, it is advisable to proceed with Option A, which is simpler to implement. If epifluorescence illumination is required, follow Option B.

**A Assembling simplified tube lens module** •TIMING **1 h:** ▲ CRITICAL See Supplementary Fig. 5 for a series of photographs that show this process in detail.

i. Use a SM1 to M25 adapter to attach the Nikon CFI Plan Apochromat λ 4× lens to SM1 lens mount.

ii. Connect a 2″ length optical post to the lens mount.

iii. Slide the lens mount into a right-angle end clamp and tighten the thumbscrew.

iv. Connect the right-angle end clamp to a 3″ length optical post and insert the post into a post holder.

v. Attach the post holder to a mounting base using a 3/8″ length cap screw and secure the mounting base on the 12″ × 24″ breadboard using a 5/8″ length cap screw.

vi. Check that the Nikon 4× lens is approximately centered on the camera port.

▲ CRITICAL STEP Allow approximately 10 mm spacing between the lens edge and the camera port to provide enough space when calibrating the magnification power.

? TROUBLESHOOTING

**vii.** Place the bullseye level on top of the SM1 lens mount and confirm that tube lens is horizontally leveled.

**viii.** Cover the spacing between the tube lens and the camera detector with light shield: aluminum foil can be wrapped around the camera body if a customized light shield is unavailable.

**B Assembling tube lens module with dichroic mirror mount for epifluorescence imaging** •TIMING **1-2 h:** ▲ CRITICAL See Supplementary Fig. 6 for a series of photographs that show this process in detail.

**i.** Attach the Nikon CFI Plana Apochromat λ 4× lens to the dichroic filter mount using a SM1 adapter.

▲ CRITICAL STEP The cube should be oriented so that the Thorlabs logo on the dichroic filter cube top appears upside down and the Nikon 4× lens is mounted on the bottom side of the cube.

**ii.** Connect a 3″ length optical post to the dichroic cube mount using a 0.5″ length set screw (1/4″-20).

**iii.** Insert two right-angled post clamps through the optical post and tighten the thumbscrews.

**iv.** Connect 2″ length optical post to each of the two clamps, and secure both posts into post holders.

**v.** Attach mounting bases to the post holders using 3/8″ length cap screws, then mount the entire assembly onto the 12″ × 24″ breadboard using two 5/8″ length cap screws.

**vi.** Check that the Nikon 4× lens is approximately centered on the camera port.

▲ CRITICAL STEP Allow approximately 10 mm spacing between the lens edge and the camera port to provide enough space when calibrating the magnification power.

? TROUBLESHOOTING

**vii.** Place the bullseye level on top of the dichroic cube mount and confirm that it is leveled horizontally and properly centered on the camera detector.

**viii.** Block the cube mount port facing the 12″ × 24″ breadboard with a SM1 threaded cap.

### Assembling objective and specimen stage •TIMING 4-6 h

**10** *Motorized microscope objective stage (see* Supplementary Fig. 7 *for a series of photographs that illustrate this process).* Remove the micrometer from the 0.5″ translation stage and attach the servo motor actuator.

**11** Attach the translation stage to the microscope base (12″ × 24″ breadboard) using a pair of 5/8″ length cap screws. Use a tubular spirit level to confirm that the stage is horizontally leveled (Supplementary Fig. 4).

**12** Add a spacer to a 0.75″ length optical post by screwing in an 8-32 nut, and connect the optical post to a cage plate.

**13** Place the microscope objective (Nikon Co., CFI Plan Apochromat λ 20×) through the objective lens holder (cage plate) and secure with nylon-tipped setscrew.

**14** Insert the objective lens holder assembly into a 2″ length post holder and tighten the thumbscrew.

**15** Attach the mounting base to the 2″ length post holder using a 3/8″ length cap screw.

**16** Secure the mounting base to the translation stage using a 5/8″ length cap screw.

▲ CRITICAL STEP Ensure that the microscope objective lens is centered on the tube lens mount (from Step 9) by gently lifting the translation stage (Supplementary Fig. 8). If the objective lens is properly positioned, the translation stage will slide smoothly without resistance. If the objective lens does not move, re-align the optical posts until the lens can be lifted without rubbing against the threads.

**17** *Assemble microscope specimen stage (see* Supplementary Fig. 9 *for detailed photographs).* Fabricate 3″ × 3″ glass stage base by bonding 3 pairs of 1″ × 3″ glass slides with superglue. Allow sufficient time for glue to dry (additional information on fabricating the glass stage can be found in Supplementary Fig. 10).

**18** Secure the glass stage to the XY translation mount.

! CAUTION Do not apply too much force when tightening the set screws to hold the glass stage. If the superglue is unevenly distributed near the base of the glass stage, the glass may break when the XY translation mount is mounted.

**19** Connect a 1.5″ length optical post to the XY translation mount.

**20** Insert the optical post through a right-angle clamp and tighten the thumbscrew.

**21** Insert a 3″ length optical post through the vacant slot in the right-angle clamp and tighten the thumbscrew.

**22** Insert the optical post into a 2″ length post holder and attach mounting base using a 3/8″ length cap screw.

**23** Attach the microscope specimen stage to the 12″ × 24″ breadboard using a 5/8″ length cap screw. The top surface of the stage should be approximately 5 mm above the top of the microscope objective lens.

**24** Place the bullseye level on top of the stage and confirm that the stage is horizontally levelled.

! CAUTION When mounting the microscope stage, make sure that all components are tightly secured in order to prevent the stage from swiveling, causing the petri dish to spill. A second optical post can be attached to the microscope stage for added support. Do not apply excessive force on the stage as the glass stage may break.

**25** Connect the microscope Z-stage motor to the controller.

**26** The user may choose to control the stage from the computer or with the jog dial on the controller.

### Assembling illumination source

**27** Assemble transillumination module for brightfield imaging (option A), epifluorescence illumination module (option B), or both (option A and B). If the basic tube lens module with no filter cube has been installed in the previous steps (Step 9 (A)), only option A can be followed.

**A Assembling transillumination module** •TIMING **1-2 h:** ▲ CRITICAL See Supplementary Fig. 11 for detailed photographs. Required optomechanical components may vary depending on the available white light source. Since this module only features a simple collimated source, refer to the epifluorescence illumination setup (Step 27-B) if precise transillumination using Kőhler optics is desired.

**i.** Connect the white light source to one end of 2″ length SM1 coupler.

**ii.** Screw in the SM1 coupler to SM1 lens mount until the mount is approximately positioned at the center of the coupler, and secure the lens mount with an extra SM1 locking ring.

**iii.** Attach the mounted *f*=40 mm doublet lens to the other end of the SM1 coupler.

**iv.** Attach the mirror cube to the other side of the doublet lens mount.

**v.** Use 2″ length optical post, 2″ length post holder, 3/8″ length cap screw, and mounting base to mount the SM1 lens mount assembly onto the 12″×24″ breadboard. Use two 5/8″ length set screws to secure the assembly onto the breadboard.

**vi.** If necessary, make adjustments by rotating the SM1 coupler to achieve collimated beam.

? TROUBLESHOOTING

**B Adding epifluorescence illumination module** •TIMING **1-2 d:** ▲ CRITICAL See Supplementary Fig. 12 for photographs of components and assembly process.

**i.** Attach color LED light source to SM1 coupler, with one of the locking rings removed.

**ii.** Attach translating lens mount to the other end of the SM1 coupler. The Thorlabs logo on the lens mount surface should face the LED light source.

**iii.** Use adapter (SM1 thread to internal SM2 thread) to attach a 2″ length SM2 lens tube to the translating lens mount.

**iv.** Connect 1″ length SM2 lens tube.

**v.** Insert the *f*=40 mm condenser lens into the 1″ length SM2 lens tube, while positioning and securing the lens with two retaining rings. Turn the LED source on and measure the distance where the beam is in focus (smallest and brightest appearance).

▲ CRITICAL STEP While the procedure is based on the lens specifications, re-positioning the lens is required as there may be variations in the actual lens position due to human error. Refer to the lens position indicated in Supplementary Fig. 13.

! CAUTION Do not directly point the light source at yourself or anyone to avoid risks of eye injury. Also, if a UV light source is used, wear appropriate UV safety goggles.

**vi.** Slide in an SM2 slip ring through the SM2 lens tube before moving on to the next step.

**vii.** Continue assembly by attaching a 0.5″ length SM2 lens tube to the 1″ SM2 lens tube containing the condenser lens.

**viii.** Attach an additional 1″ length SM2 lens tube.

**ix.** Attach an SM2 adjustable lens tube.

**x.** Screw an SM2 coupler into the SM2 adjustable lens tube, with both locking rings removed.

**xi.** Use adapter (SM1 thread to internal SM2 thread) to connect an SM1 iris diaphragm to the SM2 coupler.

▲ CRITICAL STEP The center of the iris diaphragm should be positioned where the beam is focused (from Step 27B(v)). Also, refer to Supplementary Fig. 13 for approximate diaphragm location. The position of the diaphragm can be adjusted using the locking ring from the SM2 adjustable lens tube from Step 27B(ix).

**xii.** Attach 0.3″ length SM1 lens tube to the iris diaphragm.

**xiii.** Attach SM1 adjustable lens tube to the SM1 lens tube.

**xiv.** Insert the *f*=40 mm doublet lens into the SM1 adjustable lens tube and secure lens with a retainer ring. Check if the beam is properly collimated by pointing at a surface 1′-2′ from the lens edge.

? TROUBLESHOOTING

**xv.** Continue the assembly procedure by attaching 1″ length SM1 lens tube to the adjustable lens tube containing the doublet lens.

**xvi.** Screw a SM1 coupler into the SM1 adjustable lens tube, with both locking rings removed.

**xvii.** Attach another SM1 iris diaphragm to the remaining end of the SM1 coupler thread (refer to Supplementary Fig. 13 for approximate diaphragm location. The position of the diaphragm can be adjusted using the locking ring from the SM1 adjustable lens tube from Step 27B(xv)).

**xviii.** Attach translating lens mount to the other side iris diaphragm using an SM1 coupler, with both locking rings removed. The Thorlabs logo on the lens mount surface should face the iris diaphragm.

**xix.** Attach another SM1 coupler to the translating lens mount, with only one locking ring removed.

**xx.** *Assemble the horizontal epifluorescence illumination component.* Attach an SM1 coupler to the cube mounted turning mirror.

**xxi.** Attach 1″ length SM1 lens tube to the SM1 coupler.

**xxii.** Insert the *f*=100 mm lens into a 0.3″ length SM1 lens tube and secure it with retainer ring

▲ CRITICAL STEP If microscope objective lens other than Nikon are used, re-positioning the *f* = 100 mm lens may be required (refer to Supplementary Fig. 13 for lens position).

**xxiii.** Attach the 0.3″ length SM1 lens tube.

**xxiv.** Attach the horizontal epifluorescence illumination component to the horizontal port in the dichroic mirror cube.

**xxv.** Attach the vertical illumination component to the horizontal component.

**xxvi.** *Secure the completed epifluorescence illumination module to the 12″ × 24″ breadboard.* Attach 4″ length optical post to the SM2 slip ring located near the LED light source in the vertical illumination component (from Step 27B(vi)).

**xxvii.** Insert the optical post into a 3″ post holder and tighten thumbscrew.

**xxviii.** Attach the post holder to a mounting base using a 3/8″ long cap screw.

**xxix.** Secure the post holder to the 12″ × 24″ breadboard using a 5/8″ long cap screw.

**xxx.** *Check if Kőhler illumination setup is properly installed.* Turn the EMCCD on and open HCImage software.

**xxxi.** Start live acquisition mode.

**xxxii.** Turn on the epifluorescence illumination source.

**xxxiii.** Place grid target on the stage and focus on the grid.

**xxxiv.** Open and close the condenser diaphragm (lower iris diaphragm), and confirm that the brightness of the image changes.

**xxxv.** Open and close the field diaphragm (upper iris diaphragm), and confirm that the circular field of view changes.

? TROUBLESHOOTING

**xxxvi.** Leave the upper diaphragm in the open position. Adjust the lower diaphragm to achieve the desired LED excitation intensity.

**Microscope magnifying power calibration** •TIMING **1-3 h—** ▲ CRITICAL It is highly recommended to repeat this procedure whenever changes are made in the optical train or if the image magnification appears unnatural. An example of the procedure with screen captures can be found in Supplementary Fig. 14.

**28** Position the camera using the micrometer so that the flange is ~2.48mm away from the edge of the tube lens.

**29** Place calibration grid slide on the microscope stage.

**30** Image a suitable section of the calibration grid (eg. 100 μm × 100 μm squares).

**31** Open grid image with ImageJ.

**32** Measure average number of pixels along square edge (take at least 10 measurements).

**33** Calculate physical length of grid image using camera pixel size (eg. 16 μm/px for Hamamatsu ImagEM C9100-13)

▲ CRITICAL STEP Check that there is no significant human error by estimating error ~ 2*(standard deviation) / (average measured length) < 0.01 (1%).

**34** Calculate magnification factor using the formula (detector pixel size [μm/px]) * (measured grid size [px]) / (known grid size [μm]).

**35** Compare the magnification value by the theoretical magnification in Supplementary Table 1. If the difference is greater than 1%, adjust the vertical position of the camera and repeat Steps 28-34.

**Low-light imaging—** ▲ CRITICAL The remainder of the Procedure section deals with radioluminescence imaging. See Box 1 for instructions for fluorescence and Box 2 for bioluminescence imaging. If bioluminescence and/or fluorescence imaging is performed in conjunction with radioluminescence imaging, image processing (Steps 52-70) and

scintillator cleaning (Steps 80-87) should be done after the imaging experiments are completed. While this protocol is demonstrated using radiolabeled glucose analog ($^{18}$F-FDG) to quantify cell metabolism of human breast cancer cells, any alpha- or beta-emitting radiotracer can be used to perform radioluminescence microscopy on cells or tissue specimens.

**36** Perform radioactive labeling for adherent cells (option A), suspension cells (option B) or tissue specimen (option C).

! CAUTION Always follow best safety practices when handling radioactive materials. Most beta-emitting radionuclides also emit potentially harmful gamma rays. Before using radioactive substances, contact environmental health and safety and inquire about radiation safety training. Wear personal radiation monitors (finger and whole-body). Avoid prolonged exposure to radioactivity and use lead shielding when possible. Plastic shields do not protect against gamma radiation.

### A Radiolabeling adherent cells •TIMING 1-1.5 h

**i.** Plate approximately 50,000 cells in a 35 mm glass bottom dish.

**ii.** Incubate for 1 day in normal cell culture media and check that cell density is sparse enough for single cell imaging. Cells should be separated by at least 25 μm (membrane-to-membrane).

**iii.** Incubate in glucose-free media for 30-45 min to starve the cells.

**iv.** Using a dose calibrator ($^{18}$F setting), prepare 1 ml solution of $^{18}$F-FDG (20 MBq/ml) by diluting $^{18}$F-FDG with glucose-free cell culture media.

▲ CRITICAL STEP The effective concentration of the radiotracer is reduced by half with each half-life. Thus it is important to prepare the experiments in advance to maximize the quality of the collected data.

**v.** Replace glucose-free media from the glass bottom dish with 200 - 400 μl of diluted $^{18}$F-FDG solution (just enough to cover the glass surface). Preventing the $^{18}$F-FDG solution from overflowing beyond the cover slip area will help minimize the amount of radioactivity during incubation.

**vi.** Incubate cells for 30-45 min at 37°C in 5% $CO_2$.

**vii.** Wash cells three times with PBS or fresh media and fill with 2 ml media (phenol-red-free media recommended for fluorescence imaging).

! CAUTION Regard all waste generated during the washing process as radioactive. Follow proper radioactive handling procedures.

**viii.** Gently place a $CdWO_4$ scintillator on top of the cells, using tweezers if necessary.

▲ CRITICAL STEP The square scintillator should fit entirely within the 35 mm round glass area of the dish and sink to the bottom of the dish.

ix. Load the dish on the microscope stage.

### B Radiolabeling suspension cells •TIMING 1.5-2 h

i. Incubate cells with the desired radioisotope under the desired conditions.

ii. Wash cells three times with PBS by centrifuging at 300g for 5 min, removing solution and re-suspending the cells.

iii. Adjust the cell concentration to $20 \times 10^6$ cells/ml and place the cells on ice.

! CAUTION Regard all waste generated during the washing process as radioactive. Follow proper radioactive handling procedures.

iv. Prepare a 1× Matrigel solution from the stock solution according to the manufacture's recommendations and keep it on ice.

▲ CRITICAL STEP Mix 100μl of cell solution with 100 μl of Matrigel solution in an Eppendorf tube. Immediately dispense 10 μl of this mixture onto the center of the imaging dish and place a scintillator on top of the drop. The cells and Matrigel will spread out due to the weight of the scintillator.

v. Load the dish on the microscope stage.

### C Preparing radiolabeled tissue specimen •TIMING 1.5-2 h

i. Obtain radioactive tissue specimen.

ii. Wash tissue specimen with PBS three times.

iii. Place tissue in a crytotome mold with OCT compound and freeze the sample in a -20 °C freezer or on dry ice.

iv. Using a microtome (cutting temperature -18°C), cut tissue into 8-10 μm thick sections.

v. Place a single section of interest onto an imaging dish, using tweezers or paintbrush if necessary

vi. Once the tissue section is dry, gently place $CdWO_4$ scintillator on top of tissue section, using tweezers if necessary.

vii. Load the dish on the microscope stage.

### Acquiring radioluminescence image •TIMING 30 min

37 Remove dichroic mirror from the mount, if epifluorescence illumination module is installed. This is to enhance the photon capture efficiency.

38 Using HCImage, turn on live acquisition (‘Live’) with the following settings: no EM gain, no pixel binning, exposure time of 100 ms. Start live acquisition mode.

39 Turn on white light source and adjust intensity. Pixel value should utilize the entire range of grayscale values, i.e. the brightest spot in the image should be below 65,535 for a 16-bit camera.

**40** Using the Z stage controller, adjust focus to maximize contrast.

**41** Move the dish using the XY stage actuator until a suitable area is found. A cell confluency of 10-20% is ideal for radioluminescence microscopy.

**42** Turn off live acquisition, and acquire and save the brightfield image (‘Capture1′).

**43** Turn on live acquisition and adjust focus until the cells have minimum contrast or if they seem to disappear. This indicates that the focal plane is centered on the cells.

**44** Turn off light source, set EM gain to maximum setting and set pixel binning to 4 × 4.

! CAUTION Ensure that the light is turned off prior to increasing the EM gain, as overexposure may easily damage the detector.

**45** Turn on live imaging and visualize ionization flashes. Adjust exposure time to increase or decrease the number of flashes in the image. The goal is to have approximately 5-10 flashes per frame.

▲ CRITICAL STEP Using Z-stage controller, move objective lens to focus on the edge of the scintillator. First move the objective lens down and away from the edge of the scintillator until the scintillation tracks are clearly blurry. Then, in small increments, move the objective back towards the scintillator until sharp scintillation flashes start to appear. Some flashes will appear as small bright dots; other will display more complex shapes. The goal is to find the first focal position where the flashes are in focus. If needed, repeat this step several times until the microscope is consistently focused on the same location. Be careful not to move the Z-stage too rapidly, or else the focusing plane will be deep in the $CdWO_4$ scintillator, resulting in inaccurate localization. Scintillation flashes should be substantially brighter than the background noise.

? TROUBLESHOOTING

**46** Adjust the camera exposure time until each frame contains an average of ~10 flashes for the 20× lens and ~5 flashes for 40× lens.

**47** Acquire at least 10,000 images under the ‘Sequence’ tab in HCImage. Disable ‘Enable Maximum’ and enable ‘Review Images’ to achieve increased frame rate.

**48** Save the radioluminescence images into a dedicated folder containing no other images. Saving the file to a solid-state drive improves frame rate performance.

**49** Capture analog scintillation image by adjusting exposure time between 0.5 and 5 min. Imaging parameters such as binning, EM gain, and acquisition time may be changed depending on the signal intensity.

**50** After imaging is completed, remove imaging dish.

**51** Collect a sequence of 1,000 dark images (no sample present) with the same imaging parameters used for the digital acquisition.

! CAUTION Use caution when running the EMCCD camera with high gain, as it may be damaged by overexposure to light. Make sure that the gain is turned off before opening the light tight enclosure or turning on the illumination light.

**Reconstructing and processing radioluminescence image** •TIMING **15-30 min**

— ▲ CRITICAL Image reconstruction should be performed after bioluminescence and/or fluorescence imaging has been completed, as the procedure can be time consuming. If applicable, process the calibration image with ORBIT. A software manual with detailed figures is included in the downloadable ORBIT toolbox.

**52** Launch the ORBIT software by typing ‘ORBIT’ in MATLAB. The graphical screen will pop up with a blank area on the left.

**53** Open brightfield image and apply approximate flat-field correction (‘Normalize Brightness’).

**54** Open scintillation images (‘Open LUM Images’ button). If queried, select dark image collected in Step 51.

▲ CRITICAL STEP ORBIT is compatible with both single- and multi-page TIF images. The target folder to be opened should only contain scintillation images (no brightfield or dark images).

**55** Check error messages and address them if needed.

? TROUBLESHOOTING

**56** Initiate the interactive set-up mode by pressing ‘Refresh’ button.

**57** Determine optimal processing parameters (‘H value’ and ‘binary threshold’).

▲ CRITICAL STEP The first frame in the sequence will be processed with the default parameters. The goal of this step is to find the optimal ‘H value’ and ‘binary threshold’. First, visually identify scintillation flashes. Next, increase the ‘H value’ starting from 0 until background noise disappears but scintillation flashes remain. Next, increase the ‘binary threshold’, starting from the gray value of background pixels, until the scintillation flashes are clearly segmented.

**58** If needed, adjust the ‘Track Sharpness’ and ‘Track Length’ to reject tracks that are too blurry or too long to be included in the reconstruction. Rejected tracks are identified in red font.

**59** Using the arrow buttons, toggle between image frames to check that all the scintillation tracks are properly identified and included. If not, repeat Steps 57-58.

**60** Press the ‘Reconstruct’ button to initiate the image reconstruction process. This process will take up to 15 minutes, depending on the computer. The ‘busy’ indicator will disappear when the reconstruction is complete.

**61** After reconstruction, two figures will be displayed:

**62** The first image represents the radionuclide distribution in units of counts/pixel.

**63** The second image is an overlay of the radioluminescence and brightfield images.

**64** Inspect the images to check that the reconstructed image display sharp radiotracer uptake, consistent with the brightfield image.

? TROUBLESHOOTING

**65** Four PNG image files are created in the working folder: a brightfield image (BF.png), a radioluminescence image in pseudo-color (DecayMap.png) and in black and white (DecayMap_BW.png), an overlay of the radioluminescence and brightfield image (Fusion.png), and the entire set of workspace variables (ScintData.mat), including the processing parameters.

### Quantifying reconstructed radioluminescence image •TIMING 15 min

**66** Launch the ROI analysis software by typing 'CellROI' in MATLAB. Select the previously reconstructed file (ScintData.mat).

**67** Toggle between brightfield and radioluminescence windows to select individual cells with localized radioactivity.

**68** To measure background noise level, repeat previous steps but select regions of the image devoid of cells

**69** A set of workspace variables with the ROI coordinates and scintillation count of individual cells and background noise will be created (PlotCoordinates.mat).

**70** Data can be displayed with available plotting tools (eg. BoxPlotR[26])

### (Optional) Sensitivity calibration for radioluminescence microscopy •TIMING 1-1.5 h

**71** Thoroughly mix 3-5 MBq of $^{18}$F-FDG with OCT compound and pour into a cryotome mold. Food dye may be used to verify uniform mixing.

**72** Freeze the mixture on dry ice for 15 min and then unmold frozen mixture.

**73** Using a microtome (cutting temperature -18C), cut a 10 μm slice from the OCT block.

**74** Using paintbrush and/or tweezers, gently drop it on $CdWO_4$ scintillator and allow it to dry.

**75** Estimate the radioactivity per surface area $A_0$ for the OCT slice using the known concentration of the radioactivity $A$ and the known thickness $T$ of the slice ($A_0 = A$ [MBq/mm$^3$] × $T$[mm]).

**76** Compute the surface density of radiolabeled molecules in the tissue section, $N_0 = A_0 / \lambda$, where $\lambda$ is the decay constant.

**77** Acquire radioluminescence image of the OCT slice using the procedure described in Steps 37-51.

**78** Using one large region of interest, calculate the number of recorded counts per surface area $D$ [counts/mm$^2$] on the radioluminescence image.

**79** Using $N_0$ and $D$ computed from Steps 76 and 78, apply equation (3) in Supplemental Note 1 to solve for the unknown sensitivity $S$ of the imaging system.

### Cleaning used scintillators •TIMING 15 min

**80** ! CAUTION Use extra care when handling the Piranha solution. Do not leave hot Piranha solution unattended and clearly label contents. Dispose the solution in accordance to the Environmental Health and Safety Department.

**81** Allow radioactivity to decay fully (<74 kBq) prior to cleaning the scintillators.

**82** Prepare ~20 ml of Piranha solution in a graduated glass beaker.

**83** Submerge the used scintillators in the Piranha solution for 10 min.

**84** Transfer scintillators to a beaker filled with deionized water and rinse several times with deionized water.

**85** Transfer scintillators to a beaker filled with ethanol.

**86** Remove scintillators from ethanol one by one and blow dry.

**87** Store scintillators in sterile environment.

## Troubleshooting

Troubleshooting guidelines can be found in Table 1.

**Table 1**
**Troubleshooting**

| Step | Problem | Possible reasons | Solution |
|---|---|---|---|
| **9A(vi), 9B(vi)** | Significantly off-center field of view | Difficulty in centering the tube lens during assembly | Place a small flashlight (preferably one that fits SM1 diameter) on top of the tube lens mount, facing downwards, and observe the beam emerging from the Nikon 4× λ to the camera cap. Use this light as a visual guide to center the dichroic cube. |
| **27A(vi), 27B(xiv)** | Uncollimated light source (non-circular beam with uneven brightness) | Distance between the LED source and the aspheric condenser lens is incorrect | Place the light source on a flat surface and point to a surface that is ~2″ away from the edge of the aspheric condenser lens. Rotate the retainer ring to make sure that the condenser lens has sufficient space to move. Slide the condenser back and forth until an image of the LED array can be seen. Slowly shift the lens until the LED array disappears. The image of the LED should have a circular shape. Point between 2″-4″ to check if the beam does not significantly diverge. Use the two retainer rings to tighten the lens and locked onto that position. |
| **27B(xxxv)** | Both beam shape and brightness changes when | Incorrect optical train setup | Start from Step 27B(ix) and repeat the procedure since any part of the lens |

| Step | Problem | Possible reasons | Solution |
|---|---|---|---|
| | operating either aperture diaphragm | | may have shifted during the installation process. |
| **45** | High background noise | Efflux of the radiotracer | Ensure that the experiment is performed as soon as the radiotracer incubation procedure is complete. |
| | | Insufficient washing | Ensure that the cells treated with the radiotracers are thoroughly washed and filled with fresh DMEM prior to imaging. |
| | | Stray light | Capture dark image with maximum EM gain (if using EMCCD) or long exposure time with and without camera lid. If different, check for any sources of light inside dark box (eg. LED from motor controller) or for leaks in light-enclosure. Deep-cooled CCD sensors can also trap charge after exposure to bright light. If so, wait a few minutes after exposing the camera to bright light. |
| **55** | ORBIT warning message | dark.mat file is based on approximate dark image values | Create a dark.mat file by acquiring a dark image. |
| **55** | ORBIT error message | Dark image size different from scintillation image | Make sure that the dark image and the scintillation images were captured using the same binning. |
| | | Image bit depth consistency | All scintillation images in the folder must be saved using the same bit depth settings (eg. 16-bit in HCImage). |
| | | Image width consistency, Image height consistency | All scintillation images in the folder must be saved with the same pixel size. |
| **64** | Blurry reconstructed image | Image plane out of focus from the scintillator edge | Make sure that the focal plane is on the edge of the scintillator plate during the experiment. |
| | | Image reconstruction parameters are incorrect | Repeat Steps 57-60, making sure that the segmentation of the scintillation tracks is correct. |
| **64** | No radiotracer uptake | Cells are no longer alive | Check cell viability. |
| | | Radiotracer issues | Check the quality of the radiotracer. Radiotracer specific activity may be too low. |
| Box 2 – **step 1** | Cells shifted between two acquisitions | Accidental contact during removal of scintillator or addition of reagents. | Try securing the dish to the stage, using superglue for instance. Gridded glass bottom dishes can also be used to easily move the stage back into position. |
| Box 2 – **step 9** | Unusually bright dark image | Stray light leaking into the system or originating from within the system | Check the enclosure for light leaks, as explained in troubleshooting procedure for preventing stray light (Step 46). |

## •TIMING

Steps 1-8, assembling low-light microscope base and camera mount: 2-4 h

Step 9A, assembling simplified tube lens module: 1 h

Step 9B, assembling tube lens module with dichroic mirror mount for epifluorescence imaging: 1-2 h

Steps 10-26, assembling objective and specimen stage: 4-6 h

Step 27A, assembling transillumination module: 1-2 h

Step 27B, adding epifluorescence illumination module: 1-2 d

Steps 28-35, microscope magnifying power calibration: 1-3 h

Step 36A, radiolabeling adherent cells: 1-1.5 h

Step 36B, radiolabeling suspension cells: 1.5-2 h

Step 36C, preparing radiolabeled tissue specimen: 1.5-2 h

Steps 37-51, acquiring radioluminescence image: 30 min

Steps 52-65, reconstructing and processing radioluminescence image: 15-30 min

Steps 66-70, quantifying reconstructed radioluminescence image: 15 min

Steps 71-79, (optional) sensitivity calibration for radioluminescence microscopy: 1-1.5 h

Steps 80-87, cleaning used scintillators: 15 min

Box 1, fluorescence imaging using the modular low-light microscope: 15 min

Box 2, bioluminescence imaging using the modular low-light microscope: 30 min

## Anticipated Results

To illustrate the LLM's capabilities, we characterized cell metabolism by sequentially performing radioluminescence, fluorescence and bioluminescence microscopy on the same cells using the LLM setup with epifluorescence illumination features. If larger imaging area is desired, the epifluorescence module can be removed to increase the imaging area by ~20% (Supplementary Fig. 15).

In the experiment demonstrated here, radionuclide imaging was performed first to minimize decay of the radiotracer and to limit the cytotoxic effects of ionizing radiation. $^{18}$F-FDG uptake in MDA-MB-231/Luc cells was quantified using radioluminescence microscopy. For optimal image quality, the microscope was focused on the bottom edge of the $CdWO_4$ scintillating crystal. Accurate focus can be difficult to achieve since scintillation flashes caused by long-range ionizing photons occur everywhere within the scintillator. Therefore, the scintillator must first be brought out of focus (Fig. 4a), then slowly be brought back into focus until the first sharp scintillation flashes appear (Fig. 4b). Moving the focal plane deeper into the scintillator does not affect the focus of the visible ionization events but would yield poor image quality in the final reconstructed image (Fig. 4c and 4d).

To confirm that the microscope is in focus, an analog image was acquired with a 30 sec acquisition time (Fig. 5a). A digital image was then acquired using a total exposure time of 10 min, split into 10,000 individual frames (Fig. 5b). To localize the cells, we acquired a brightfield image (Fig. 5c) and created an overlay showing both the brightfield and the digital radioluminescence image (Fig. 5d). One thing to note is that if the scintillation images are captured under the same binning conditions, the analog image is visually blurrier than its digital counterpart (Supplementary Fig. 16). Furthermore, hot spots caused by ionizing radiation (these are likely to be γ-rays or Bremsstrahlung X-rays) hitting the EMCCD sensor directly are generally observed in analog images due to the long exposure (red arrows). The analog image is proportional to the optical fluence [**COPYEDITOR: Please allow fluence.** ] reaching the EMCCD sensor, which is only indirectly related to the actual number of radioactive decays in the sample. In contrast, the digital image represents the number of radioactive decays that were detected by the microscope. This quantity can be related to the actual number of radioactive decays through a simple sensitivity calibration.

To demonstrate multimodal imaging (Fig. 6), we acquired brightfield, fluorescence, bioluminescence, and radioluminescence image of the same cells (MDA-MB-231/Luc). For fluorescence, we imaged a live-cell nuclear stain. For bioluminescence, we imaged the constitutive expression of a firefly luciferase (Luc) reporter. Since the images were acquired on the same cells, a composite image representing the three imaging modalities are demonstrated in the last column.

Radioluminescence images reconstructed with ORBIT can be further analyzed to estimate single-cell radionuclide uptake. Radionuclide measurements are quantitative and sensitive, thus well suited for this purpose. Using circular regions of interest, the number of counts per cell was estimated. The total number of detected radioactive decays per cell was then displayed and compared to background radioactivity (Fig. 7). While radioluminescence imaging requires the cells to be spaced apart by at least 35 μm, fluorescence and bioluminescence imaging can be performed on more confluent samples (Supplementary Fig. 17). Moreover, since bioluminescence signals can be captured in less than 1 s (Supplementary Fig. 18), high-resolution real-time bioluminescence imaging can be performed.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by funding from the National Institutes of Health (NIH) under grants 5R01CA186275 and 1R21CA193001. T.J.K. was supported in part by NCI training grant T32 CA118681. The authors gratefully acknowledge the support of Olympus for its loan of the LV200 microscope.

## References

1. Welsh DK, Noguchi T. Cellular bioluminescence imaging. Cold Spring Harb Protoc. 2012
2. Badr CE, Tannous BA. Bioluminescence imaging: progress and applications. Trends Biotechnol. 2011; 29:624–633. [PubMed: 21788092]

3. Coulon V, et al. Subcellular imaging of dynamic protein interactions by bioluminescence resonance energy transfer. Biophys J. 2008; 94:1001–1009. [PubMed: 17921204]
4. Luker KE, et al. Kinetics of regulated protein–protein interactions revealed with firefly luciferase complementation imaging in cells and living animals. Proc Natl Acad Sci USA. 2004; 101:12288–12293. [PubMed: 15284440]
5. Shimomura O, Johnson FH. Calcium binding, quantum yield, and emitting molecule in aequorin bioluminescence. Nature. 1970; 227:1356–1357. [PubMed: 4393938]
6. Welsh DK, Yoo SH, Liu AC, Takahashi JS, Kay SA. Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Curr Biol. 2004; 14:2289–2295. [PubMed: 15620658]
7. Gambhir SS. Molecular imaging of cancer with positron emission tomography. Nature Reviews Cancer. 2002; 2:683–693. [PubMed: 12209157]
8. Weber MJ. Inorganic scintillators: today and tomorrow. Journal of Luminescence. 2002; 100:35–45.
9. Pratx G, et al. Radioluminescence microscopy: measuring the heterogeneous uptake of radiotracers in single living cells. PloS one. 2012; 7:e46285. [PubMed: 23056276]
10. Sengupta D, Pratx G. Single-cell characterization of FLT uptake with radioluminescence microscopy. J Nucl Med. 2016 jnumed. 115.167734.
11. Natarajan A, Türkcan S, Gambhir SS, Pratx G. Multiscale framework for imaging radiolabeled therapeutics. Mol Pharm. 2015; 12:4554–4560. [PubMed: 26460685]
12. Puncher MR, Blower PJ. Radionuclide targeting and dosimetry at the microscopic level: the role of microautoradiography. European Journal of Nuclear Medicine. 1994; 21:1347–1365. [PubMed: 7875174]
13. Miller BW, et al. The iQID camera: An ionizing-radiation quantum imaging detector. Nucl Instr Meth Phys Res A. 2014; 767:146–152.
14. Barthe N, Chatti K, Coulon P, Maıtrejean S, Basse-Cathalinat B. Recent technologic developments on high-resolution beta imaging systems for quantitative autoradiography and double labeling applications. Nucl Instr Meth Phys Res A. 2004; 527:41–45.
15. Ogoh K, et al. Bioluminescence microscopy using a short focal-length imaging lens. J Microsc. 2014; 253:191–197. [PubMed: 24386879]
16. Goda K, et al. Combining fluorescence and bioluminescence microscopy. Microsc Res Tech. 2015; 78:715–722. [PubMed: 26096873]
17. Hoshino H, Nakajima Y, Ohmiya Y. Luciferase-YFP fusion tag with enhanced emission for single-cell luminescence imaging. Nat Methods. 2007; 4:637–639. [PubMed: 17618293]
18. Kim TJ, Tuerkcan S, Ceballos A, Pratx G. Modular platform for low-light microscopy. Biomed Opt Express. 2015; 6:4585–4598. [PubMed: 26601020]
19. Türkcan S, et al. Single-cell analysis of [18F] Fluorodeoxyglucose uptake by droplet radiofluidics. Anal Chem. 2015; 87:6667–6673. [PubMed: 26035453]
20. Nakatsu T, et al. Structural basis for the spectral difference in luciferase bioluminescence. Nature. 2006; 440:372–376. [PubMed: 16541080]
21. Greer LF, Szalay AA. Imaging of light emission from the expression of luciferases in living cells and organisms: a review. Luminescence. 2002; 17:43–74. [PubMed: 11816060]
22. Contag CH, Bachmann MH. Advances in in vivo bioluminescence imaging of gene expression. Annu Rev Biomed Eng. 2002; 4:235–260. [PubMed: 12117758]
23. Lambrechts D, et al. A causal relation between bioluminescence and oxygen to quantify the cell niche. PLoS One. 2014; 9:e97572. [PubMed: 24840204]
24. Pratx G, et al. High-resolution radioluminescence microscopy of 18F-FDG uptake by reconstructing the β-ionization track. J Nucl Med. 2013; 54:1841–1846. [PubMed: 24003077]
25. Sengupta D, et al. Bright Lu2O3: Eu thin-film scintillators for high-resolution radioluminescence microscopy. Adv Healthc Mater. 2015; 4:2064–2070.
26. Spitzer M, Wildenhain J, Rappsilber J, Tyers M. BoxPlotR: a web tool for generation of box plots. Nat Methods. 2014; 11:121–122. [PubMed: 24481215]

**Box 1**

### Fluorescence imaging using the modular low-light microscope •TIMING 15 min

Here we describe how to perform fluorescence imaging using the modular low-light microscope. While various fluorescent probes can be used, the example below uses a commercial nuclear staining reagent to demonstrate the epifluorescence capability of the system.

1. Apply two drops of nuclear staining reagent (NucBlue Live ReadyProbes) in the culture dish and wait for 15 min. If performing in conjunction with radioluminescence microscopy, staining can be performed prior to loading the scintillator in the glass bottom dish.
2. In HCImage, change camera mode to ‘Normal’.
3. Insert dichroic mirror into the cube mount, if previously removed.
4. Turn on epifluorescence light source and set power to low.
5. Adjust the light power or the camera exposure time to achieve ~50% of maximum bit depth ($2^{16}$= 65536 for 16-bit detector sensors)
6. Capture blank image (no sample) with the same imaging parameters.

   ▲ CRITICAL STEP Background subtraction should be performed for quantitative results. Flat-field correction may also be required for some applications. The user may use ImageJ or MATLAB to perform background subtraction.

End of Box 1

**Box 2**

### Bioluminescence imaging using the modular low-light microscope •TIMING 30 min

Here we describe how to perform bioluminescence imaging using the modular low-light microscope. The cells must express a bioluminescent reporter such as firefly luciferase.

1. In brightfield mode, focus on the cells until they become barely visible. Acquire a brightfield image if needed.

   ▲ CRITICAL STEP If direct correlation between radioluminescence and bioluminescence is desired, keep the glass bottom dish in the same position during this procedure.

   ? TROUBLESHOOTING

2. Turn off the brightfield light source.
3. Remove dichroic mirror from the mount, if present.
4. Dispense 17-50 μl D-Luciferin stock solution in glass-bottom dish filled with 2 ml media (target concentration of 0.5-1.5 mM). Wait for 10 minutes to allow the reaction to reach steady-state.
5. Check if cells are properly focused by running live acquisition mode with exposure time of 2 s and maximum EM gain. No binning is necessary.
6. Capture bioluminescence image.
7. Reduce EM gain to reduce excess factor noise.
8. Adjust exposure time to achieve ~50% of maximum bit depth.
9. Collect dark image (no sample) using the same camera settings. The circular imaging window should not be visible.

? TROUBLESHOOTING

End of Box 2

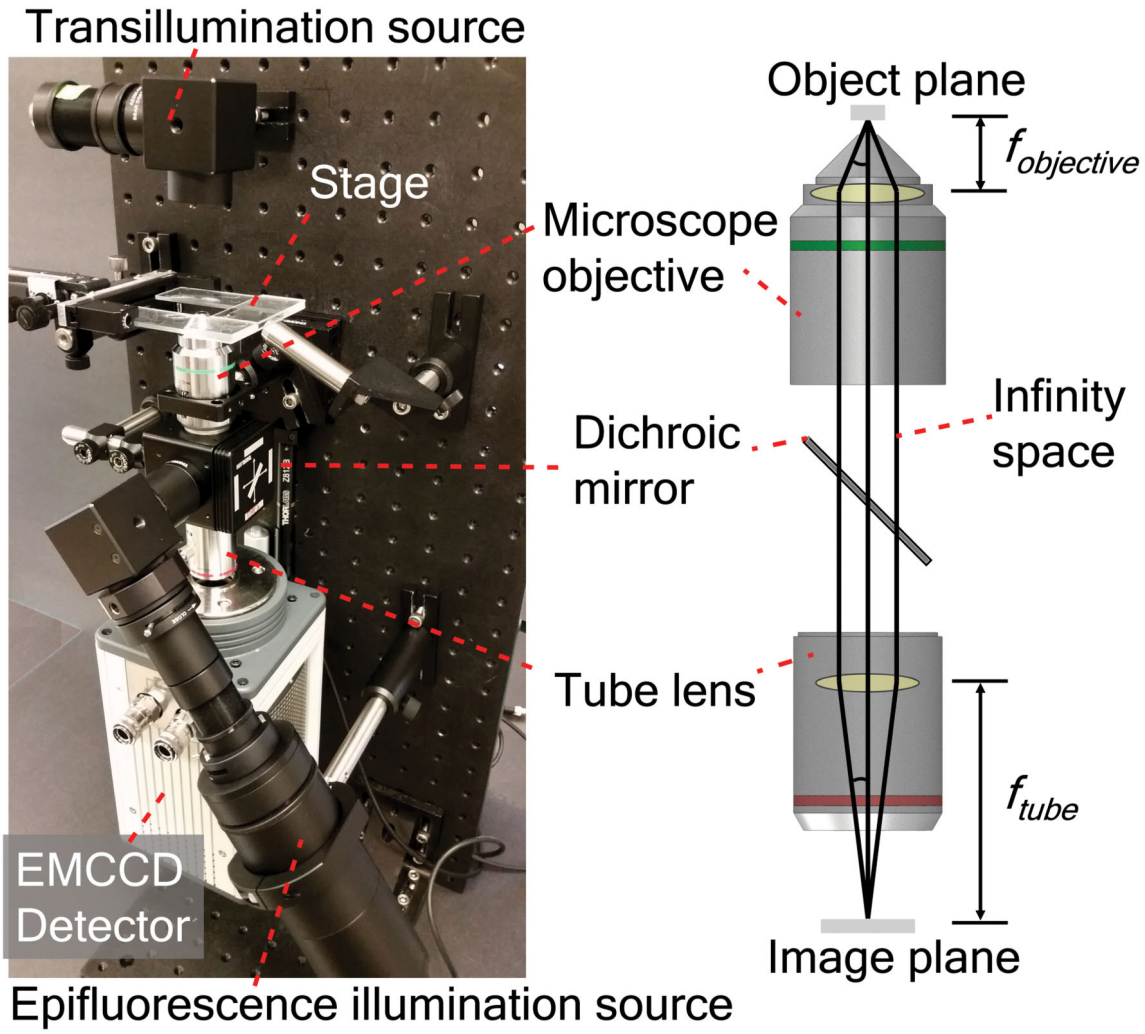


**Figure 1.**

Overview of the LLM. *Left*, photograph of the fully assembled LLM showing optical train component, epifluorescence illumination module and camera detector. *Right*, schematic diagram of the optical train. Black solid lines represent the imaging path of the infinity-corrected system, composed of two microscope objective lenses aligned back-to-back. A 50 mm-focal-length lens (Nikon CFI Plan Apochromat λ 4×) is used in place of the standard *f* = 200 mm tube lens.

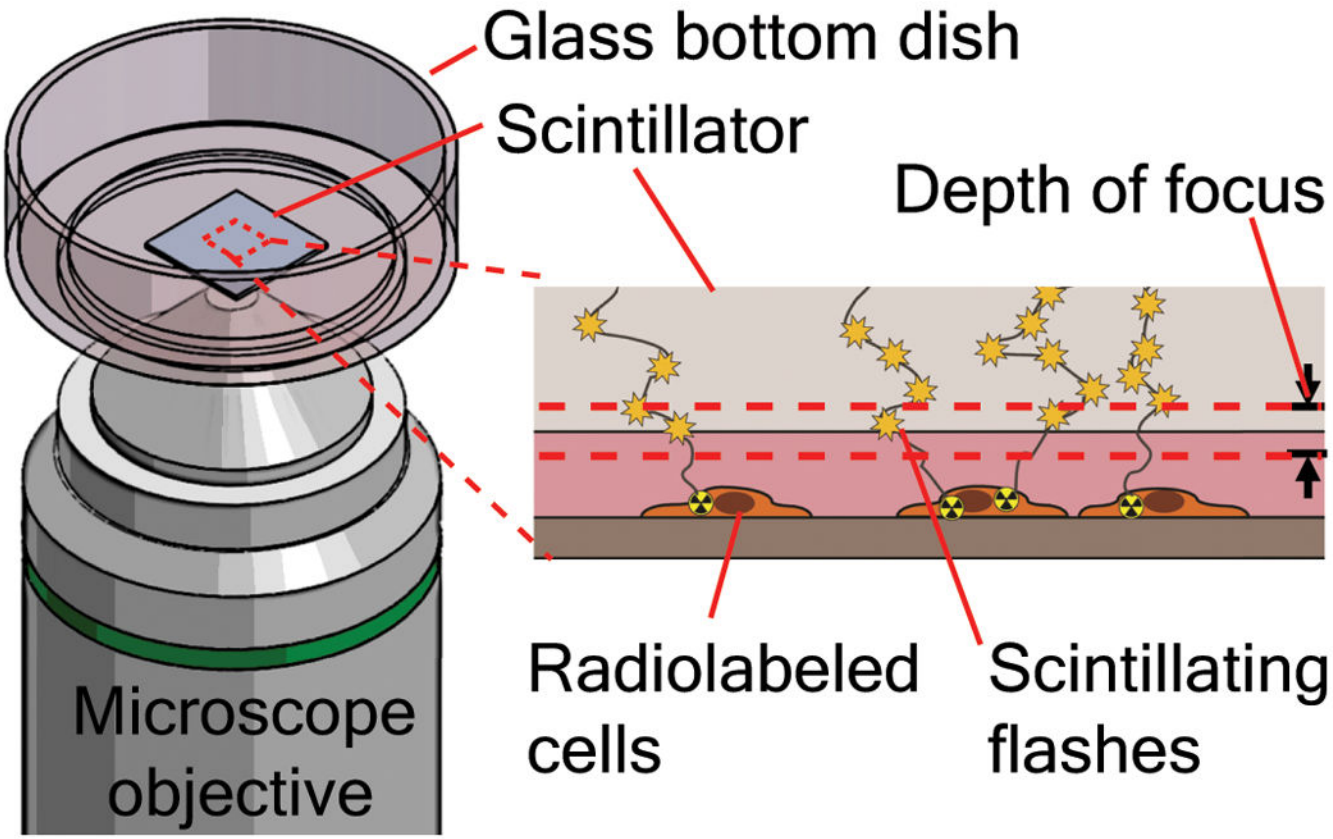


**Figure 2.**

Schematic diagram of radioluminescence microscopy. As the ionization track extends deep within the scintillator, the microscope must be focused on the bottom edge of the scintillator for the location of the emission to be accurately estimated. Alternatively, the scintillator can be placed at the bottom and the cells can be plated directly on the scintillator (not shown).

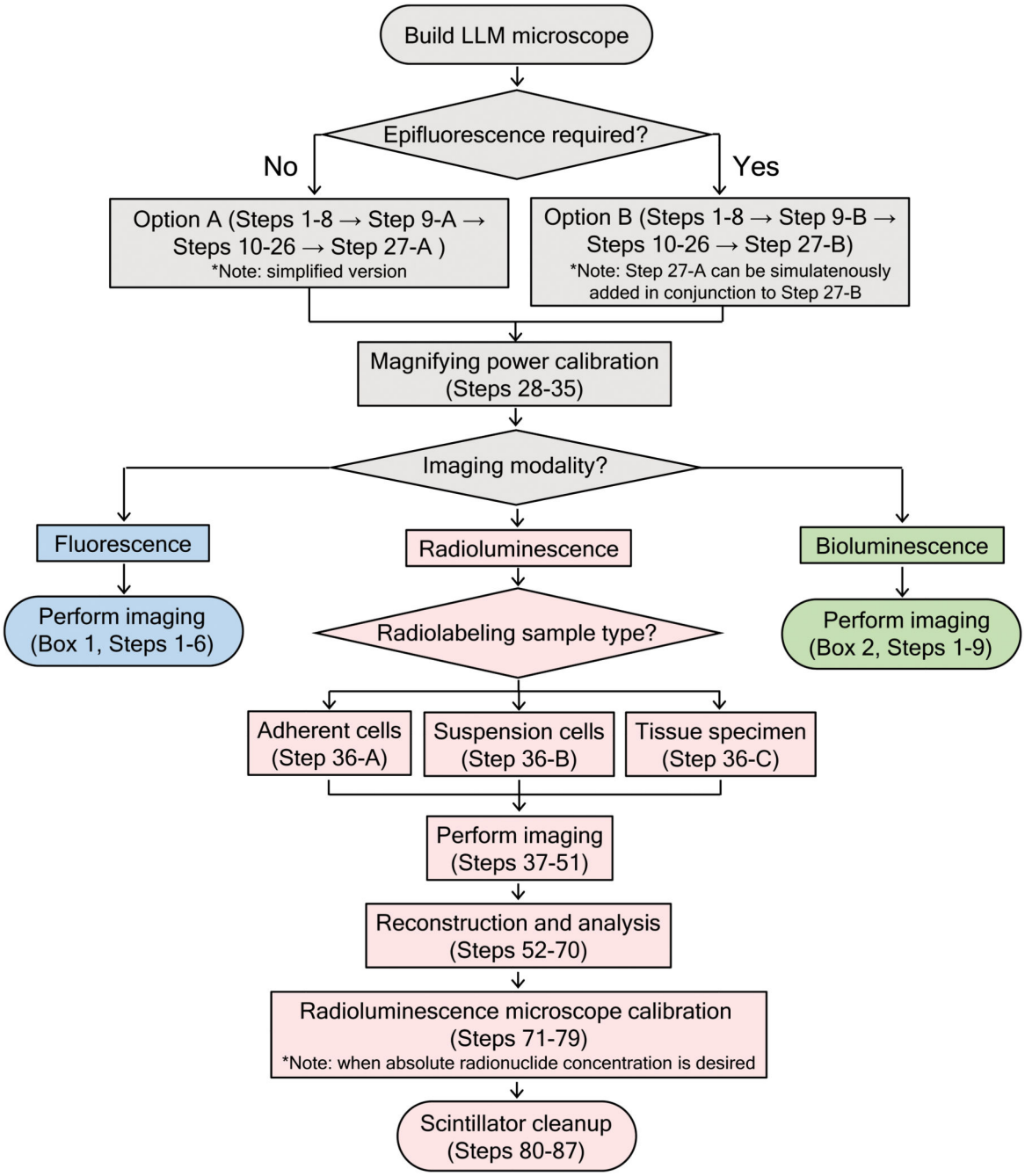


**Figure 3.**

Flow chart of the protocol. The top half of the flow chart (gray) explains the procedure to build the modular low-light microscope, and the bottom half explains the steps to perform multi-modal imaging of fluorescence (blue), radioluminescence (red) and bioluminescence (green).

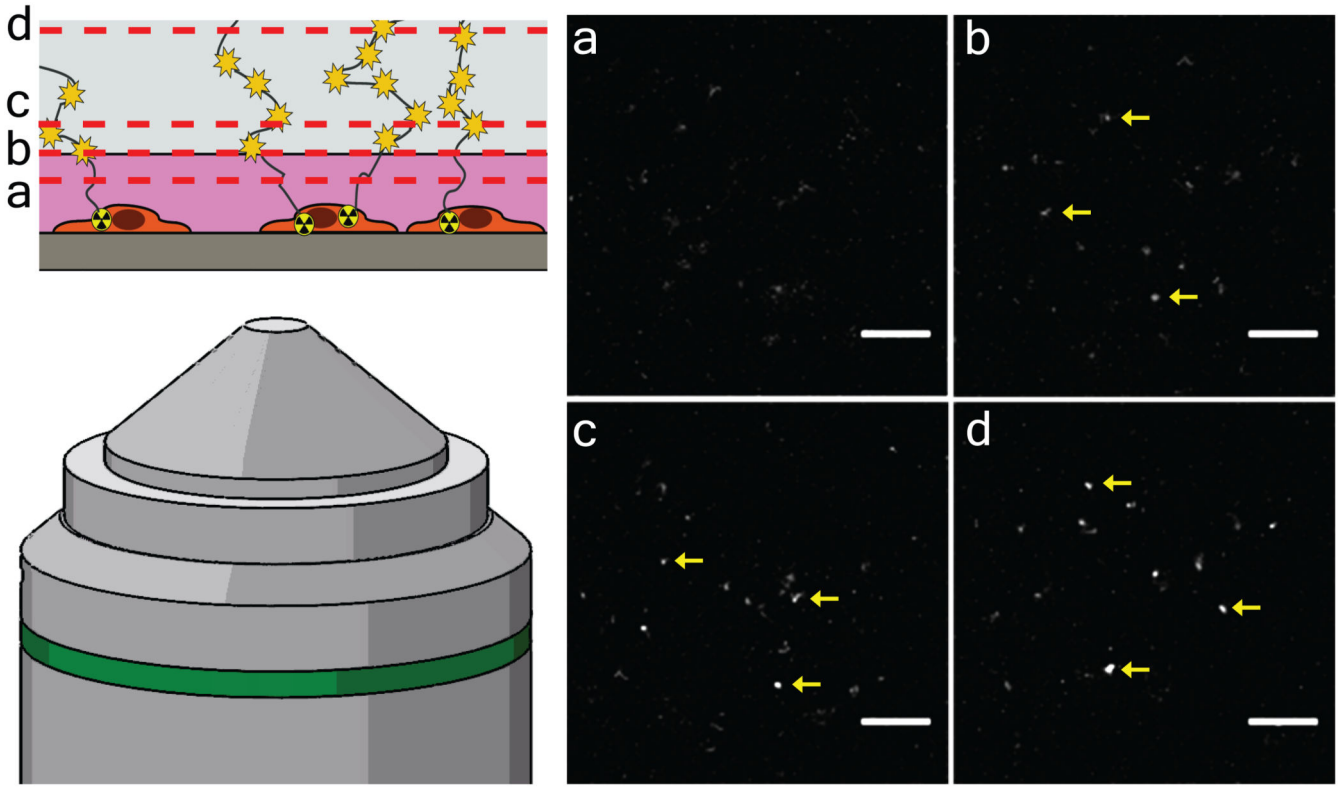


**Figure 4.**

Raw radioluminescence image of scintillation tracks. Images of the same sample are taken with the microscope objective focused (**a**) 10 μm below the bottom edge of the scintillator, (**b**) on the scintillator edge (0 μm), (**c**) 10 μm above the scintillator edge and (**d**) 50 μm above the scintillator edge. Scintillation tracks are sharp and focused only when the focal plane is positioned on or above the scintillator edge. Scale bar, 300 μm.

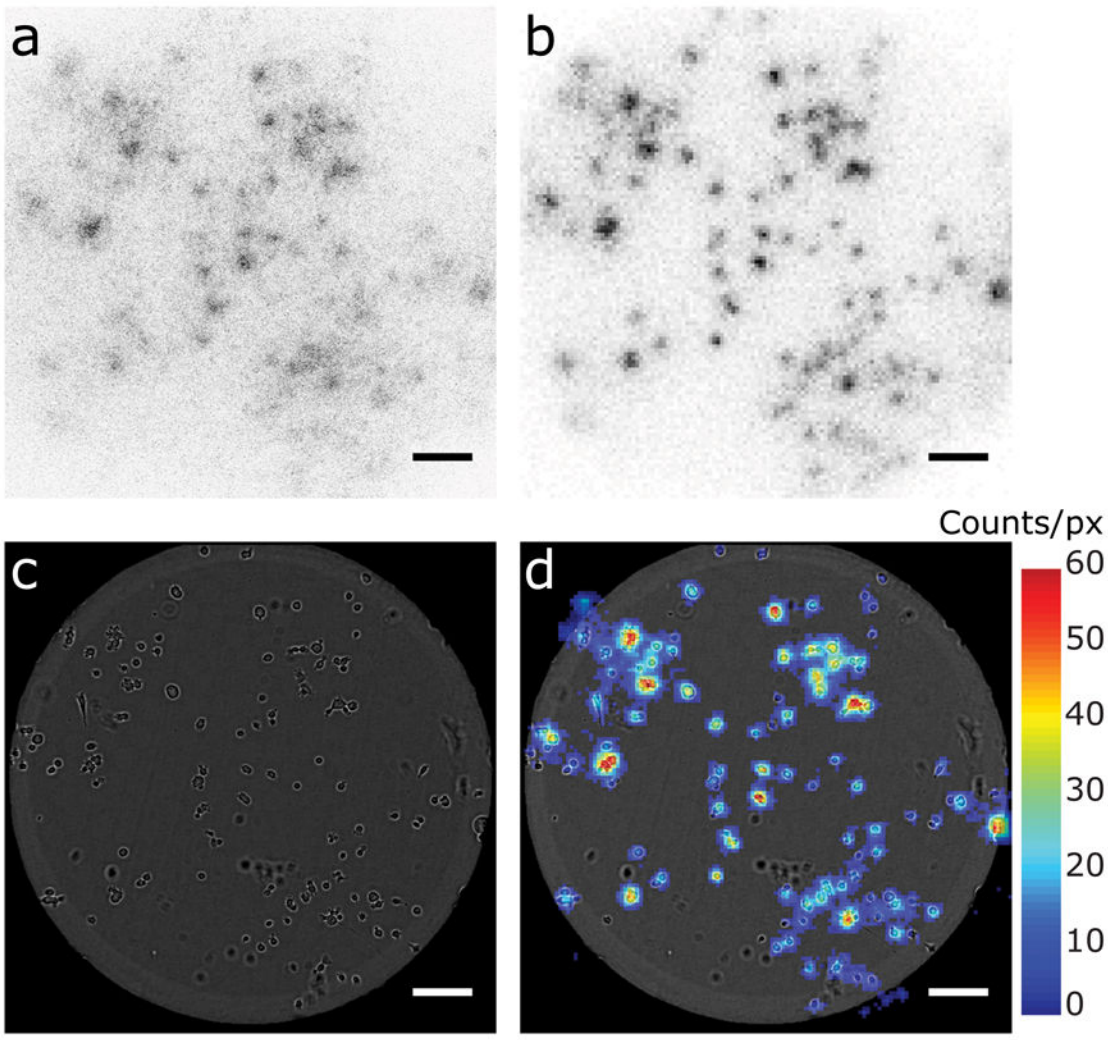


**Figure 5.**

Comparison of analog and digital radioluminescence imaging. (**a**) Analog image of acquired with no binning, 1200 EM gain and exposure time of 30 sec. (**b**) Reconstructed digital image of the same cells acquired with 4 × 4 binning, 1200 EM gain, exposure time of 50 ms per frame and 10,000 frames. (**c**) Brightfield image of the same cells. (**d**) Overlay image showing both the brightfield and the digital radioluminescence image, highlighting the localization of the radioactivity to individual cells. The false-color scale represents the physical number of detected counts per pixel. Scale bar, 200 μm.

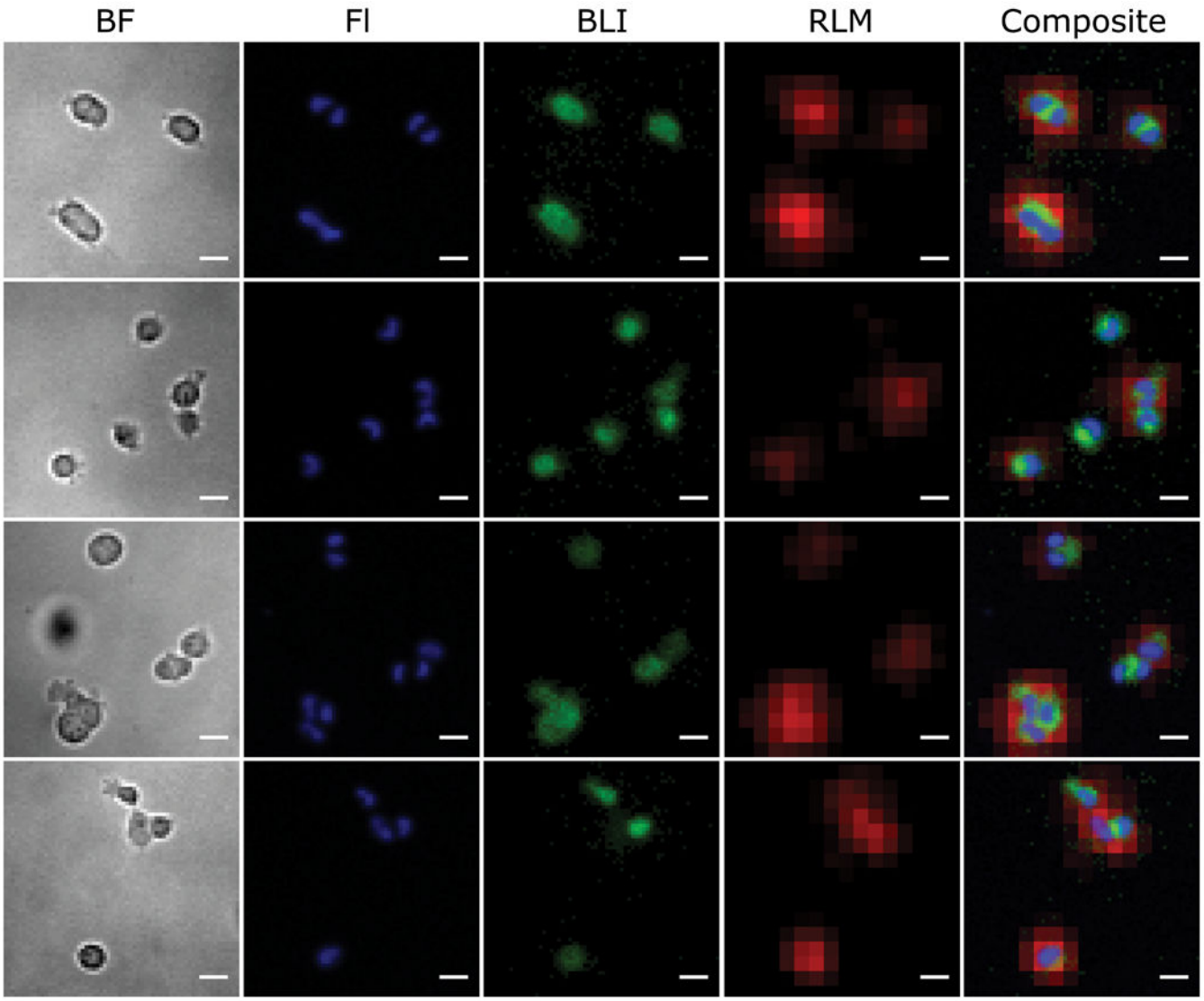


**Figure 6.**

Example of multimodal cell imaging of firefly-luciferase-expressing human breast cancer cells (MDA-MB-231/Luc). Each column represents brightfield, fluorescence (nuclear stain), bioluminescence (luciferase expression), radioluminescence ($^{18}$F-FDG uptake), and composite image of the three modalities, respectively. Each row represents cells from different regions of interest selected from Fig. 5. Scale bar, 25 μm.

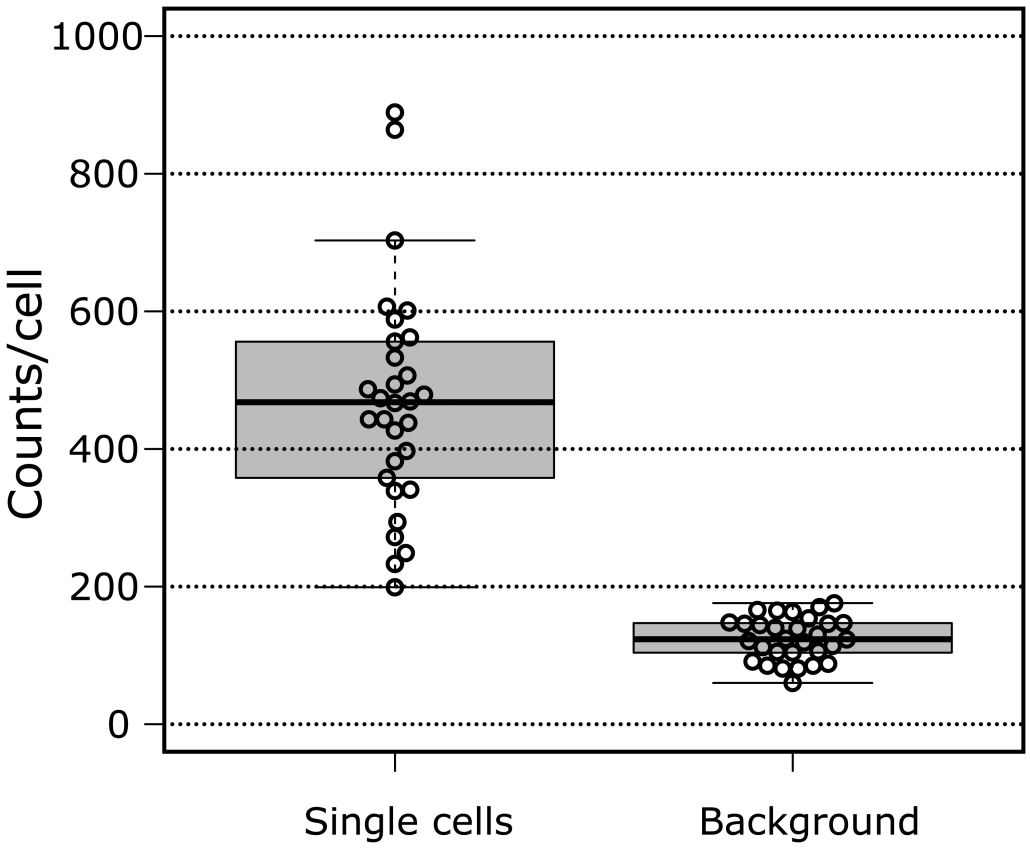


**Figure 7.**

Region-of-interest (ROI) quantitation of $^{18}$F-FDG uptake in single MDA-MB-231/Luc cells. Cells were seeded in a glass-bottom dish with concentration of 25,000 cells/ml. Prior to imaging, the cells were first starved under glucose-free medium for 30 min, then incubated with 20 MBq/ml of $^{18}$F-FDG for 30 min. Circular ROIs (96 μm diameter) were placed on cells that were sufficiently spaced from one another, and the total activity within the ROI was computed. *Left*, the heterogeneity of the cell population is observed showing that glucose uptake varies significantly from cell to cell. Black dots represent individual cells from the same dish (n=30). *Right*, as a control, background radioactivity values were measured in regions of the image devoid of cells (n=30).

**Table 1**

Comparison chart between LLM and commercial microscope (LV200). The conventional microscope is based on a generic infinity-corrected system.

| | LLM | LV200 | Conventional |
|---|---|---|---|
| **Compatible objective lens** | Various | Olympus | Various |
| **Brightness**[18] | High | High | Low |
| **Distortion correction**[18] | Excellent | Excellent | Excellent |
| **Overall aberration correction** | Excellent | Good | Excellent |
| **Fluorescence illumination** | Epi or Trans | Trans | Epi |
| **Modularity** | High | Low | Medium |
| **Field of view (20× objective)** | 2 mm (Trans)<br>1.6 mm (Epi) | 2.8 mm (Trans) | 1.3 mm |
| **Cost (without camera)** | Low | High | Medium |

**Table 2**

Components required for building the LLM. Unless otherwise noted, all parts are purchased from Thorlabs, Inc.

| LLM Compartment | Component Name | Catalog Number | Quantity | Note |
|---|---|---|---|---|
| Microscope base | 8″ × 8″ Aluminum breadboard | MB8 | 1 | |
| | 12″ × 24″ Aluminum breadboard | MB1224 | 1 | |
| | Right-angle mounting plate | AP90 | 1 | |
| | 5/8″ length cap screws (1/4″-20) | | 8 | |
| Camera mount | 1″ translation stage | PT1 | 1 | Based on Hamamtsu C9100-13 camera with 1/4″-20 mounting thread. Components may vary depending on camera system. |
| | 0.75″ length optical post | TR075 | 1 | |
| | 1″ length optical post holder | PH1 | 1 | |
| | 3/8″ length cap screws (1/4″-20) | | 2 | |
| | 0.5″ length set screws (1/4″-20) | | 2 | |
| Tube lens mount | SM1 to M25 adapter | SM1A12 | 1 | If using epifluorescence illumination, prepare materials from the ‘Dichroic mirror and tube lens mount (optional)’ in Table 3 instead |
| | SM1 lens mount | SMR1 | 1 | |
| | Right-angle end clamp | RA180 | 1 | |
| | 3″ length optical post | TR3 | 1 | |
| | 2″ length optical post | TR2 | 1 | |
| | 2″ length post holder | PH2 | 1 | |
| | Mounting base | BA1S | 1 | |
| | 5/8″ length cap screw (1/4″-20) | | 1 | |
| | 3/8″ length cap screw (1/4″-20) | | 1 | |
| Objective mount | 8-32 nut | | 1 | Can be found in local hardware stores |
| | Nylon-tipped setscrew | SS8N013 | 1 | |
| | Cage plate for microscope mount | CP03 | 1 | |
| | 0.75″ length optical post | TR075 | 1 | |
| | 2″ length post holder | PH2 | 1 | |
| | Mounting base | BA1S | 1 | |
| | 1/2″ translation stage | MT1B | 1 | |
| | Servo motor actuator | Z812B | 1 | Make sure that the actuator has the 3/8″ barrel fitting instead of 1/4″-80 thread |
| | Motor controller | TDC001 | 1 | |
| | 5/8″ length cap screws (1/4″-20) | | 3 | |
| | 3/8″ length cap screw (1/4″-20) | | 1 | |

| LLM Compartment | Component Name | Catalog Number | Quantity | Note |
|---|---|---|---|---|
| Microscope stage | 1.5″ length optical post | TR1.5 | 1 | |
| | 3″ length optical post | TR3 | 1 | |
| | 2″ length post holder | PH2 | 1 | |
| | Mounting base | BA1S | 1 | |
| | Right-Angle Clamp | RA90 | 1 | |
| | XY translation mount | XYFM1 | 1 | |
| | 5/8″ length cap screw (1/4″-20) | | 1 | |
| | 3/8″ length cap screw (1/4″-20) | | 1 | |
| | 1″ × 3″ slide glass | (Fisher Scientific) 12-544-4 | 6 | |
| Trans-illumination brightfield source | White light source | MWWHL3 | 1 | |
| | LED driver | LEDD1B | 1 | |
| | Power supply | KPS101 | 1 | |
| | 2″ length SM1 coupler | SM1T20 | 1 | |
| | SM1 lens mount | SMR1 | 1 | |
| | SM1 locking ring | SM1NT | 1 | |
| | 2″ length optical post | TR2 | 1 | |
| | 2″ length post holder | PH2 | 1 | |
| | Mounting base | BA1 | 1 | |
| | $f$ = 40 mm doublet lens with mount | AC254-040-A-ML | 1 | |
| | 5/8″ length cap screw (1/4″-20) | | 2 | |
| | 3/8″ length cap screw (1/4″-20) | | 1 | |

**Table 3**

Components required for building the epifluorescence module for the LLM. Unless otherwise noted, all parts are purchased from Thorlabs, Inc.

| LLM Compartment | Component Name | Catalog Number | Quantity | Note |
|---|---|---|---|---|
| Dichroic mirror and tube lens mount | SM1 to M25 adapter | SM1A12 | 1 | |
| | Dichroic filter mount base | DFM1B | 1 | |
| | 2″ length optical posts | TR2 | 2 | 3″ length posts may be used if camera base to breadboard distance is large |
| | 3″ length optical post | TR3 | 1 | |
| | Right-angled end clamp | RA180 | 2 | |
| | 2″ length post holders | PH2 | 2 | |
| | Mounting base | BA1S | 2 | |
| | SM1 threaded cap | SM1CP2 | 1 | |
| | 5/8″ length cap screw (1/4″-20) | | 2 | |
| | 3/8″ length cap screw (1/4″-20) | | 2 | |
| Epifluorescence illumination module | Slip ring for SM2 tube | SM2RC | 1 | |
| | 4″ length optical post | TR4 | 1 | |
| | 3″ length post holder | PH3 | 1 | |
| | Mounting base | BA1 | 1 | |
| | SM1 coupler | SM1T2 | 5 | |
| | Translating lens mount | LM1XY | 2 | |
| | External SM1 thread to internal SM2 thread adapter | SM1A2 | 2 | |
| | 2″ length SM2 lens tube | SM2M20 | 1 | |
| | 1″ length SM2 lens tubes | SM2L10 | 2 | |
| | 0.5″ length SM2 lens tube | SM2L05 | 1 | |
| | SM2 adjustable lens tube | SM2V10 | 1 | |
| | SM2 coupler | SM2T2 | 1 | |
| | SM1 iris diaphragms | SM1D25 | 2 | |
| | 0.3″ length SM1 lens tubes | SM1L03 | 2 | |
| | SM1 adjustable lens tube | SM1V10 | 1 | |
| | 1″ length SM1 lens tubes | SM1L10 | 2 | |
| | Cube mounted turning mirror | CM1-P01 | 1 | |
| | Ø50 mm *f*=40 mm condenser lens | ACL5040U | 1 | |
| | Ø1″ *f*=40mm doublet lens | AC254-040-A | 1 | |
| | Ø1″ *f*=125mm lens | LA1986-A | 1 | |
| | 5/8″ length cap screw (1/4″-20) | | 1 | |
| | 3/8″ length cap screw (1/4″-20) | | 1 | |
| Light source and filter components | LED driver | LEDD1B | 1 | |

| LLM Compartment | Component Name | Catalog Number | Quantity | Note |
|---|---|---|---|---|
| | LED power supply | KPS101 | 1 | |
| | Dichroic filter cube top | DFM1T2 | 1 | One filter cube top required per each filter set |
| | 565nm LED | M565L3 | 1 | Texas Red set |
| | Texas Red filters | MDF-TXRED | 1 | |
| | 470nm LED | M470L3 | 1 | GFP set |
| | GFP filters | MDF-GFP | 1 | |
| | 385nm LED | M385L2 | 1 | DAPI set |
| | DAPI filters | (Semrock) DAPI-1160B-000 | 1 | |