

Video Article

In vivo Imaging Method to Distinguish Acute and Chronic InflammationJen-Chieh Tseng¹, Andrew L. Kung²¹Lurie Family Imaging Center, Dana-Farber Cancer Institute, Harvard Medical School²Division of Pediatric Hematology/Oncology/Stem Cell Transplantation, Columbia University Medical CenterCorrespondence to: Jen-Chieh Tseng at jen-chieh_tseng@dfci.harvard.eduURL: <http://www.jove.com/video/50690>DOI: [doi:10.3791/50690](https://doi.org/10.3791/50690)

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Abstract

Inflammation is a fundamental aspect of many human diseases. In this video report, we demonstrate non-invasive bioluminescence imaging techniques that distinguish acute and chronic inflammation in mouse models. With tissue damage or pathogen invasion, neutrophils are the first line of defense, playing a major role in mediating the acute inflammatory response. As the inflammatory reaction progresses, circulating monocytes gradually migrate into the site of injury and differentiate into mature macrophages, which mediate chronic inflammation and promote tissue repair by removing tissue debris and producing anti-inflammatory cytokines. Intraperitoneal injection of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, sodium salt) enables detection of acute inflammation largely mediated by tissue-infiltrating neutrophils. Luminol specifically reacts with the superoxide generated within the phagosomes of neutrophils since bioluminescence results from a myeloperoxidase (MPO) mediated reaction. Lucigenin (bis-N-methylacridinium nitrate) also reacts with superoxide in order to generate bioluminescence. However, lucigenin bioluminescence is independent of MPO and it solely relies on phagocyte NADPH oxidase (Phox) in macrophages during chronic inflammation. Together, luminol and lucigenin allow non-invasive visualization and longitudinal assessment of different phagocyte populations across both acute and chronic inflammatory phases. Given the important role of inflammation in a variety of human diseases, we believe this non-invasive imaging method can help investigate the differential roles of neutrophils and macrophages in a variety of pathological conditions.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50690/>

Introduction

Inflammation is a highly regulated biological response involved in a variety of human diseases, including microbial infection¹, wound healing², diabetes³, cancer⁴, cardiovascular⁵, neurodegenerative⁶, and autoimmune diseases⁷. Tissue inflammation requires proper coordination of various immune cells in order to achieve pathogen clearance, tissue repair, and disease resolution. Neutrophils and macrophages are key immune mediators of tissue inflammation. In the acute phase of inflammation, neutrophils are the first responders to various harmful stimuli and tissue damage⁸. The neutrophils rapidly extravasate from circulation to the site of injury, where the cells inactivate invading microbes by releasing anti-microbial granules and phagocytosis. During phagocytosis, neutrophils engulf invading microbes into phagosomes, within which the cells produce high levels of superoxide ($O_2^{\cdot-}$). Phagosomal superoxide is the primary source of many downstream reactive oxygen species (ROS). For example, superoxide can be dismutated to hydrogen peroxide (H_2O_2) by spontaneous dismutation or by superoxide dismutase (SOD)^{9,10}. In neutrophils, myeloperoxidase (MPO) further converts hydrogen peroxide to anti-microbial hypochlorous acid (HOCl)¹¹. As the inflammatory responses continue, circulating monocytes gradually migrate into the site of injury and differentiate into mature macrophages², whose phagocytic function help remove inactivated pathogens and cell debris. In addition, as a key regulator in the later phase of inflammation, macrophage promotes tissue repair by producing anti-inflammatory cytokines¹² and by generating extracellular ROS at a lower level⁹. The ROS generated at this later stage regulate tissue remodeling, new vessel formation, and reepithelialization¹³.

Phagocyte NADPH oxidase (Phox) is the primary source of superoxide production in both neutrophils and macrophages⁹. Phox is a multi-subunit complex whose assembly is tightly regulated⁹. The holoenzyme contains several cytosolic regulatory subunits (p67^{phox}, p47^{phox}, p40^{phox}, and RAC) and a membrane-bound heterodimer cytochrome b_{558} (consist of subunit CYBA and CYBB). Cytochrome b_{558} is the reaction core within which the CYBB subunit (also known as p91^{phox} and NOX2) carries out the primary redox chain reaction⁹. Interestingly, its assembly sites are different between neutrophils and macrophages. In resting neutrophils, cytochrome b_{558} is mostly present in the membrane of intracellular storage granules¹⁴. During phagocytosis, neutrophils assemble the holoenzymes at phagosomes⁹, where high levels of MPO activity are also present. The neutrophil Phox rapidly consumes oxygen and exerts its microbicidal power by ROS production, a phenomenon termed the respiratory burst¹¹. In contrast, macrophages have a lower level of MPO expression and cytochrome b_{558} is mostly found in the plasma

membrane^{15, 16}. Thus neutrophils produce high levels of superoxide for anti-microbial activity, while macrophages generate less superoxide for regulatory functions¹⁵.

Since inflammation is an intricate *in vivo* process, non-invasive imaging methods specific for different phases of inflammation would allow quantitative and longitudinal assessment of disease models. Using mechanistic studies, we have previously demonstrated the use of two chemiluminescent agents, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (bis-N-methylacridinium nitrate), for non-invasive imaging of acute and late (chronic) stages of inflammation, respectively¹⁷. Luminol enables visualization of neutrophil MPO activity in the acute phase of inflammation¹⁸⁻²⁰, whereas lucigenin bioluminescence can be used to assess macrophage activity in association with the late phase or chronic inflammation¹⁷. In this manuscript, we used two experimental inflammation models (s.c. PMA and s.c. LPS) to demonstrate these imaging techniques.

Protocol

Note: All animal studies were performed under approved institutional protocols and animal care guidelines.

1. Reagents and Solutions

1. PMA solution for s.c. inoculation: Prepare a stock solution of phorbol 12-myristate 13-acetate (PMA) at 5 mg/ml in DMSO. Store the stock solution at -20 °C. Before inoculation, thaw the stock solution and dilute to 1 mg/ml PMA in PBS.
2. LPS solution for s.c. inoculation: Dissolve lipopolysaccharide (LPS from *Salmonella enterica* serotype enteritidis) in sterile PBS at 1 mg/ml prior to s.c. inoculation.
3. Luminol solution for acute phase imaging: Dissolve luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, sodium salt) in sterile normal saline (0.9% NaCl) at 10 mg/ml. The solution can be stored at -20 °C before use.
4. Lucigenin solution for chronic phase imaging: Dissolve lucigenin (bis-N-methylacridinium nitrate) in sterile normal saline at 2.5 mg/ml. The solution can be stored at -20 °C before use.

2. Subcutaneous PMA Inflammation Model

1. Anesthetize NCr nude mice in an induction chamber with 1-2% isoflurane. Confirm general anesthesia by loss of movement and a constant respiratory rate. Transfer an animal to a nose cone supplied with isoflurane-mixed gas and keep the animal under anesthesia.
2. Use an isopropyl alcohol wipe to clean and disinfect the injection site on the left flank.
3. Using sterile technique, inject 50 µl of the PMA inoculation solution (containing 50 µg of PMA) into the subcutaneous space on the left flank. Remove excess fluid from the injection site by using an isopropyl alcohol pad. Avoid the use of analgesia as it may affect inflammatory responses.
4. Move the animal back to the housing cage and closely monitor its recovery from anesthesia. In order to assist in the recovery, use a heating pad to keep the animal warm.

3. Subcutaneous LPS Inflammation Model

1. Anesthetize C57BL/6J mice with 1-2% isoflurane in an induction chamber. Once establishing general anesthesia, transfer an animal to a nose cone supplied with isoflurane gas and keep the animal under anesthesia.
2. Use an isopropyl alcohol wipe to clean the injection site on the left footpad.
3. Using sterile technique, inject 50 µl of the LPS inoculation solution (containing 50 µg of LPS) into the left footpad. Remove excess fluid from the injection site by using an isopropyl alcohol pad. Avoid the use of analgesia as it may affect inflammatory responses.
4. Move the animal back to the housing cage and closely monitor its recovery from anesthesia. Use a heating pad to keep the animal warm during recovery.

4. Bioluminescence Imaging of Inflammation

1. Anesthetize the animal in an induction chamber with 1-2% isoflurane-mixed gas. Confirm general anesthesia by loss of movement and a constant respiratory rate.
2. While the animal is still under anesthesia, intraperitoneally (i.p.) inject the luminol solution (10 mg/ml), or the lucigenin solution (2.5 mg/ml) for acute or chronic inflammation imaging, respectively. The final dosage is 100 mg/kg for luminol and 25 mg/kg for lucigenin. A lower dose of lucigenin (10-15 mg/kg) can be used to avoid possible toxicity for some mouse strains. Signs of toxicity include shortness of breath and respiratory distress. The C57BL/6J strain has lower lucigenin tolerability than the NCr nude strain.
3. Transfer the animal into the imaging chamber of a bioluminescence imaging system.
4. Perform sequential bioluminescence imaging at 1 min intervals. Each imaging step is comprised of 1 min acquisition time, f/stop = 1, binning = 16 and 0 sec delay.
5. Within the image acquisition panel, to enable sequential multi-step imaging, click on the sequence setting button. Provide sufficient imaging steps in the acquisition profile to determine maximal luminescence output (usually 15 one-minute steps will suffice). The 15-min imaging section allows sufficient time for substrate absorption and systemic circulation.
6. Remove the animal from the imaging chamber and move it back to the housing cage. In order to assist in the recovery, use a heating pad to keep the animal warm.

- During post-acquisition analysis, use the imaging software package to calculate peak total bioluminescent signal through standardized regions of interest (ROI). The images are present as radiance in photons/sec/cm²/sr with minimal and maximal threshold indicated. Quantitative data are presented as total flux in photons per second per ROI.

Representative Results

We performed longitudinal bioluminescence imaging to assess acute and chronic inflammation in experimental inflammation models. Phorbol 12-myristate 13-acetate (PMA) is a potent protein kinase C (PKC) agonist that activates Phox for superoxide anion production and triggers intense acute inflammatory responses²¹. S.C. injection of 50 mg of PMA in NCr nude mice caused rapid skin irritation and acute inflammation at injection sites^{18, 22, 23}. We performed daily imaging for 4 days with the first images obtained as early as 3 hr after PMA injection. **Figure 1** demonstrated the longitudinal bioluminescence imaging using luminol and lucigenin as substrates. At early stages of inflammation (3 hr), we observed significant luminol bioluminescence indicating high levels of neutrophil MPO activity at the sites of PMA injection. No significant lucigenin bioluminescence was observed during these early stages of inflammation. Starting on day 2, we observed scab formation and therefore reduced luminescence intensity. As wound healing became apparent (**Figure 1A**, day 3 and 4), we observed steady increase in lucigenin bioluminescence (**Figure 1B**). These results illustrate that luminol bioluminescence is associated with the acute phase of inflammation, whereas lucigenin bioluminescence is closely associated with tissue repair in the late phases of inflammation.

Independently, we s.c. injected 50 µg of lipopolysaccharide (LPS) in the footpads of wild-type C57BL/6J mice, and performed longitudinal bioluminescence imaging for up to 10 days (**Figure 2A**). LPS is a membrane component of Gram-negative bacteria. S.C. inoculation of LPS is capable of triggering strong inflammatory responses by TLR4 receptor activation and subsequent inflammatory chemokine/cytokine production²⁴. Unlike PMA which directly activates Phox for superoxide production, LPS requires a longer period of time to fully activate tissue infiltrating phagocytes¹⁸. As neutrophils are the early responders to microbe infection, we observed elevated luminol bioluminescence during the first 4 days of acute inflammation, which declined after day 5 (**Figure 2B**). In contrast, lucigenin bioluminescence gradually increased in the late and chronic phase of inflammation (**Figure 2B**, days 5-10).

Together, these results demonstrate the ability to use differential substrate specificity to distinguish acute and chronic inflammatory stages. Importantly, imaging is mediated by the endogenous enzymatic activities of the immune cells, and there is no need for ectopic expression of reporters.

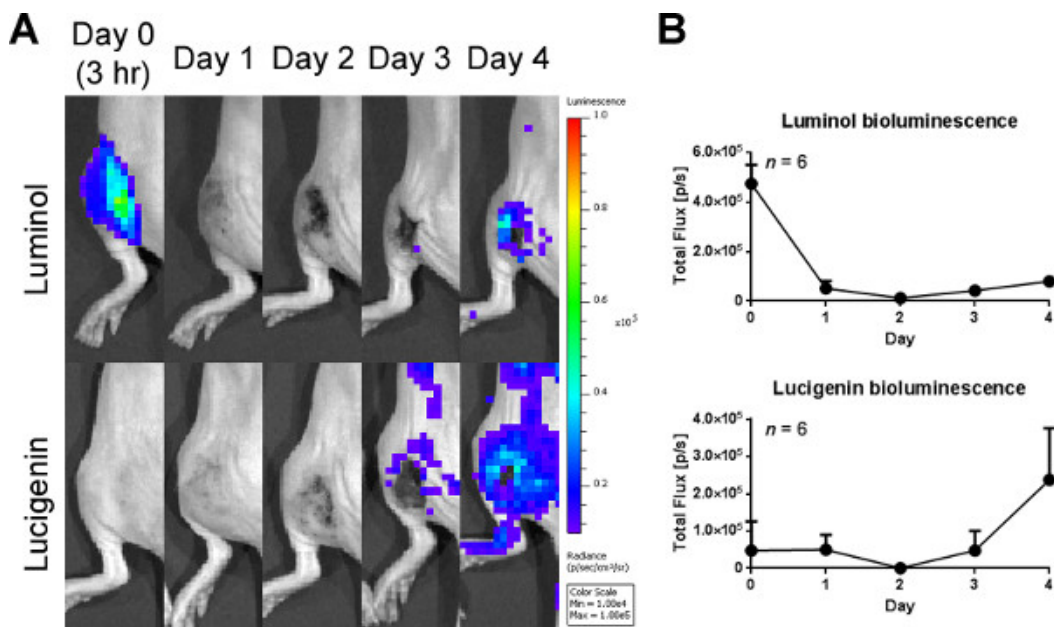


Figure 1. *In vivo* longitudinal bioluminescence imaging of acute and late phases of inflammation. (A) We induced local tissue inflammation by s.c. injection of 50 µg PMA, and performed daily luminol (100 mg/kg, i.p.) and lucigenin (25 mg/kg, i.p.) bioluminescence imaging starting at 3 hr after PMA injection. No significant background auto-luminescence was observed at sites of inflammation without chemiluminescent agents. (B) Quantitative representation of luminol and lucigenin bioluminescence at injection sites. In the acute phase of inflammation, luminol signals dominated the bioluminescence output (3 hr). In the later stages of inflammation, the sites of injection had higher lucigenin bioluminescence compared with luminol (day 4). Error bars represent the standard deviations of bioluminescence measurements at each imaging time point. [Click here to view larger figure.](#)

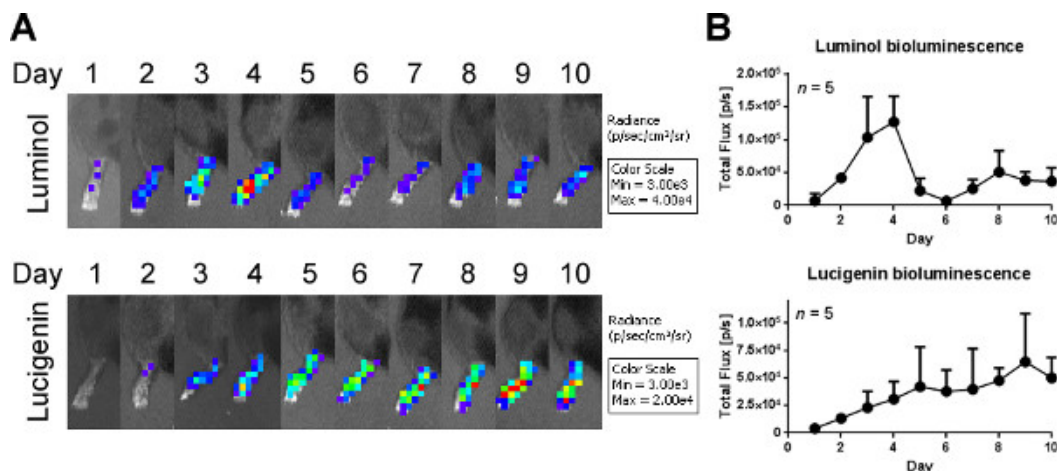


Figure 2. (A) 50 µg of LPS was injected s.c. into the footpads of C57BL/6J mice. Longitudinal bioluminescence imaging with luminol and lucigenin were performed daily for 10 days. **(B)** Quantitative representation of luminol and lucigenin bioluminescence at LPS injection sites. Luminol bioluminescence increased during the first 4 days of acute inflammation. However, in the late/chronic phases of inflammation, luminol signals rapidly declined and lucigenin bioluminescence gradually increased. Error bars represent the standard deviations of bioluminescence measurements at each imaging time point. [Click here to view larger figure.](#)

Discussion

In this report, we demonstrate a bioluminescence method for non-invasive imaging of inflammation in living animals. Taking advantage of two luminescent substrates, luminol and lucigenin, the method can distinguish different phases of inflammation. Luminol bioluminescence is associated with neutrophils in the acute phase of inflammation, whereas lucigenin bioluminescence is mediated by macrophages in the chronic phase. Relatively small (M.W. = 177.16 g/mol) and electrically uncharged, luminol can readily penetrate both plasma and phagosomal membrane²⁵⁻²⁹. In addition, luminol luminescence is specific to the intracellular MPO activity in neutrophils⁹. On the other hand, lucigenin is mostly membrane impermeable due to its larger size (M.W. = 510.5 g/mol) and two positive charges^{25, 27-30}.

Neutrophils and macrophages assemble the Phox holoenzyme at different subcellular locations. Although both reagents depend upon Phox to provide superoxide anion (O₂⁻), the disparity in cell permeability and MPO-dependency enables the substrates to differentiate inflammatory phagocytes in disease models. Neutrophils express most of the Phox in the membrane of intracellular vesicles (phagosomes)¹⁴, where high levels of MPO are also present. As a result, luminol bioluminescence is specifically associated with activated neutrophils during acute inflammation. In contrast, macrophages assemble Phox in the plasma membrane as they infiltrate and mature during chronic inflammation¹⁵. Non-permeable lucigenin can directly interact with the extracellular superoxide generated by macrophages to produce chronic inflammatory bioluminescence.

Inflammation is a complicated *in vivo* process that requires proper coordination of many cell types and various extracellular signaling molecules. In this video report, we demonstrate the synergistic use of both luminol and lucigenin, to monitor acute and late (chronic) phase inflammation in animal models. Since these substrates are commercially available and relatively inexpensive, we believe this relatively simple and robust imaging method can be readily translated to *in vivo* applications in many disease areas. Of note, compared with PET, MRI and near-infrared (NIR) fluorescent imaging techniques, the chemiluminescent method is not ideal for deep tissue imaging due to the substrates' blue emission spectra. To improve tissue penetration, it has been recently demonstrated that the blue chemiluminescence emission can be transferred to co-administrated nanoparticles for NIR emission³¹. Further modification of their chemical structures could potentially enhance their quantum efficiency for stronger light output and refine their emission spectra for deeper tissue penetration.

Disclosures

The authors declare no conflict of interest in this study.

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