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Real-time *in vivo* Imaging of LPS-induced Local Inflammation and Drug Deposition in NF-κB Reporter Mice

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[Abstract] Wound, biomaterial, and surgical infections are all characterized by a localized and excessive inflammation, motivating the development of *in vivo* methods focused on the analysis of local immune events. However, current inflammation models, such as the commonly used *in vivo* models of endotoxin-induced inflammation are based on systemic, usually intraperitoneal, administration of lipopolysaccharide (LPS), causing endotoxin shock. Here, we describe a model of LPS-induced local inflammation in NF-κB-RE-Luc reporter mice. LPS, alone or with added therapeutic substances, is delivered locally via a hydrogel which is deposited subcutaneously, providing a spatially defined environment, enabling *in vivo* bioimaging analyses of local NF-κB activation. Evaluation of drug efficacy can be analyzed longitudinally in the same mouse, and using fluorescently labeled drugs, local drug deposition can be simultaneously analyzed, and correlated to the site of inflammation. Finally, the protocol can also be used to study retention and systemic release of the drug from locally deposited gels and other biomaterials.

Keywords: Inflammation, NF-κB, *In vivo*, Mouse model, Bioimaging, Therapy

[Background] An excessive TLR response causes localized and sometimes disproportionate inflammation, as observed in different types of wound and biomaterial infections. These wound complications delay proper healing and increase the risk of severe infections and sepsis. Considering the latter, several experimental models of sepsis and endotoxin shock have been developed which study the development of systemic inflammation (Lewis *et al.*, 2016). There is however a need for models that address localized inflammatory events from a mechanistic and therapeutic perspective. Activation of the transcription factor NF-κB is a key component of various inflammatory conditions, and hence, NF-κB is considered an important therapeutic target (Liu *et al.*, 2017). Real-time, longitudinal *in vivo* imaging of NF-κB activation in NFκB-RE-Luc reporter mice is an important tool in studies on inflammatory disease and efficacy of drug treatments. NFκB-RE-Luc reporter mice carry a transgene containing NFκB-responsive elements from the CMVα promoter placed upstream of a basal SV40 promoter, and a modified firefly luciferase cDNA (Carlsen *et al.*, 2002). This reporter element can be induced by LPS and TNF-α (Carlsen *et al.*, 2002), and provides an excellent *in vivo* tool to monitor transcriptional responses of NF-κB. To achieve visualization of gene expression, luciferin, a substrate for luciferase, is

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administrated intraperitoneally to mice which in turn generate luminescent signals. Thereafter, *in vivo* imaging using IVIS spectrum is used for acquisition and analysis of the recorded signals.

Here, we describe a model of LPS-induced local inflammation in NFκB-RE-Luc reporter mice. LPS, mixed in a hydroxyethyl cellulose hydrogel, is injected subcutaneously on the back of mouse. Subcutaneous deposition of hydrogel provides a defined and controlled environment for studies of local NF-κB activation. In addition to LPS, therapeutic agents can also be included in the same hydrogel and their efficacy can be analyzed longitudinally in the same mouse. Moreover, drug deposition and release can also be imaged by using fluorescently labeled drugs. To evaluate local anti-inflammatory efficacy of a peptide drug and for imaging its local distribution, this *in vivo* imaging protocol has successfully been used by us (Puthia *et al.*, 2020).

Materials and Reagents

1. 5 ml polypropylene tube
2. 50 ml tube
3. Syringes, 1 ml (Soft-Ject, Henke-Sass Wolf, catalog number: 5010-200V0)
4. Needle 23 G × 1” (BD, catalog number: 300800)
5. Syringe filter, 0.2 µm (Filtropur S 0.2, Sarstedt, catalog number: 83.1826.001)
6. Alcohol wipes (Cutisoft wipes, BSN Medical, catalog number: 204364)
7. BALB/c tg(NFκB-RE-Luc)-Xen reporter mice (Taconic Biosciences, catalog number: 10499) (8-12 weeks old; male or female)
   Note: BALB/c tg(NFκB-RE-Luc)-Xen reporter mice are used for inflammation imaging. If the purpose is to image drug deposition only, other laboratory mouse strains can be used.
8. LPS from *Escherichia coli* O111:B4 (Sigma-Aldrich, catalog number: L3024)
9. TCP-25 (GKYGFYTHVFRLKKWIQKVIDQFGE)
   As a drug, we used a thrombin-derived peptide TCP-25. For *in vivo* imaging, TCP-25 was mixed with 2% (w/w) Cy5 labelled TCP-25 (Biopeptide, San Diego, CA, USA).
10. Hydroxyethyl cellulose (HEC, Natrosol 250 HX, MW 1,000,000; Ashland Industries Europe GmbH, catalog number: 431292)
11. XenoLight D-luciferin, Potassium salt (PerkinElmer, catalog number: 122799)
12. DPBS, without calcium and magnesium (Thermo Fischer Scientific, catalog number: 14190250)
13. Endotoxin free water (Sigma, catalog number: TMS-011-A)
14. Isoflurane (Forane, Baxter, catalog number: CA2L9100)
15. 1.5% HEC hydrogel (see Recipes)
16. D-luciferin solution (see Recipes)
17. LPS solution (see Recipes)

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Equipment

1. Magnetic stirrer
2. IVIS spectrum (PerkinElmer, model: catalog number: 124262)
3. Ultrasonic bath (Elma Schmidbauer GmbH, model: Elmasonic S30H)
4. Cordless hair clipper (Aesculap, catalog number: GT416)
5. Magnetic stirrer (Fisher Scientific, catalog number: 11715704)

Software

1. Living Image 4.5.5 Software (PerkinElmer)
2. Prism, version 8.3.0 (GraphPad Software, LLC.)

Procedure

A. Preparation of hydrogel-LPS mixture

1. To make hydrogel containing LPS, add 5 µg LPS (1 mg/ml solution) to 95 µl of 1.5% HEC gel. Vortex the mixture vigorously for 5 min and centrifuge for 3 min (2,575 *x g*, room temperature) to remove air bubbles. A range of 5-50 µg LPS can be used to obtain different grades of inflammation.
   Note: Gel LPS mixture should be prepared prior to shaving the mouse dorsum (i.e., before Procedure C).
2. Slowly, take 200 µl of LPS gel mixture in a 1 ml syringe. Avoid taking in air bubbles. Attach a 23-gauge needle to the syringe and adjust the gel volume to 100 µl by slowly removing excess gel from the syringe. Move to Procedure C.
   Note: Hydrogel is quite thick, do not aspirate using needle as it will be difficult and will produce air bubbles.

B. Preparation of hydrogel-Cy5-labeled drug mixture

1. In a 5 ml polypropylene tube, add the required amount of fluorescently labeled drug to the gel and vortex vigorously for 5-10 min. Centrifuge for 3 min (2,575 *x g*, room temperature) to remove air bubbles. In our study, for *in vivo* imaging, TCP-25 mixed with 2% (w/w) Cy5 labeled TCP-25 was added to the gel. We used a final concentration of 0.1% TCP-25 in the gel. To study combined effects, drug/drugs can be added along with LPS in the same hydrogel.
   Note: The hydrogel mixture should be prepared prior to shaving of mouse dorsum (i.e., before Procedure C).
2. Slowly, take 200 µl of drug gel mixture in a 1 ml syringe. Avoid taking in air bubbles. Attach a 23-gauge needle to the syringe and adjust gel volume to 100 µl by slowly removing excess gel from the syringe. Move to Procedure C.

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Note: Hydrogel is quite thick, do not aspirate using needle as it will be difficult and will produce air bubbles.

C. Preparation of mice for subcutaneous injection

1. After receiving BALB/c tg(NFκB-RE-Luc)-Xen reporter mice from supplier, keep them in quarantine for at least one week (or depending on animal facility's rules). A brief description of mouse preparation for subcutaneous gel deposition and imaging is illustrated in Figure 1.

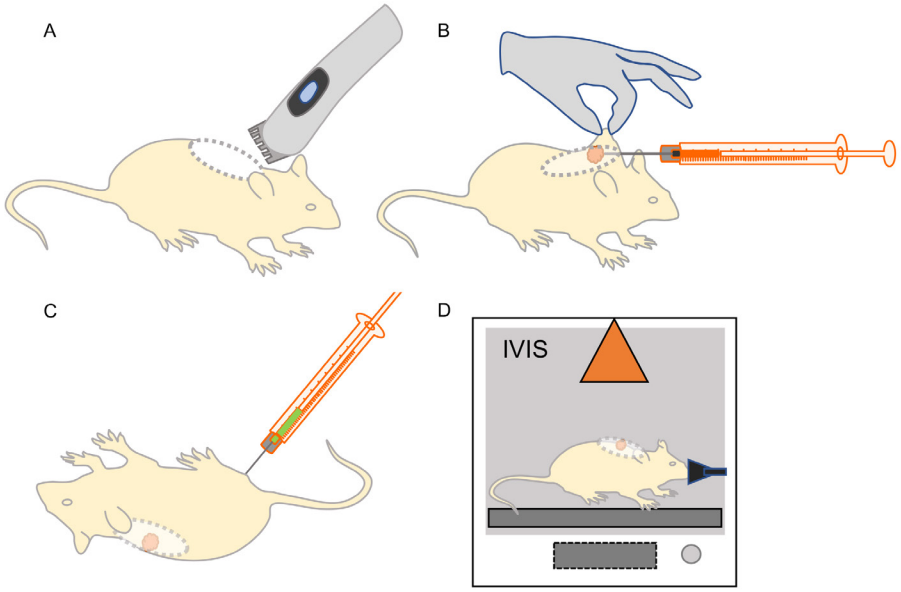

Figure 1. Preparation for subcutaneous gel deposition and IVIS imaging. A. Shaving of hair from dorsum of mice with a cordless clipper. B. Subcutaneous deposition of hydrogel. C. Intraperitoneal injection of the substrate D-luciferin. D. IVIS imaging.

2. Anesthetize mice with isoflurane-mixed oxygen (4% isoflurane for induction and 1.5-2% for maintenance). This can be achieved in an IVIS induction chamber or in any other isoflurane-oxygen delivery system. Shave hair from the dorsum (slightly below interscapular region) of mice with a cordless clipper. Depilation can also be achieved with depilatory creams. If depilatory creams are used, it should be performed at least 2 days prior to the subcutaneous injection.

   Note: Shaving or depilation is necessary as mouse hair can interfere with both luminescence and fluorescence imaging.
3. Wipe and clean the dorsum with alcohol wipe. Move immediately to next step.

D. Subcutaneous deposition of hydrogel

1. Manually restrain the anesthetized mice using thumb and forefinger, lift and fold the skin from interscapular area. Insert the needle under the skin and move forward at least 1 cm. Slowly,

inject 100 µl of LPS gel mixture, withdraw the needle and put your finger at the site of insertion to prevent any leakage from the site of deposition.

Note: As mouse skin is quite loose, ensure that you inject gel in the midline otherwise gel maybe placed to one side. For standard subcutaneous injection procedure please see Shimizu (2004).

2. Immediately after subcutaneous injection, transfer the mice to their respective cages.

E. IVIS imaging for NF-κB activity (luminescence imaging mode)

1. Fifteen min prior to IVIS imaging, administer D-luciferin solution intraperitoneally into the mice (150 mg/kg body weight). Put the mice back to the cages.

   Note: We image mice 3, 6 and 24 h post subcutaneous deposition of LPS gel.
2. Anesthetize mice in an induction chamber with 4% isoflurane-mixed oxygen. Complete anesthesia takes approximately 3-5 min.

   Note: Read IVIS spectrum user manual before using IVIS.
3. Once the mice are anesthetized, start the isoflurane supply (2%) for the IVIS imaging chamber. Immediately, transfer mice to the imaging chamber.

   Note: Turn on IVIS and start Living Image software at least 15 min prior to the imaging and initialize it. Initialization process cools down the camera to -90 °C. Also ensure that stage heating (37 °C) is working.
4. Place mice in prone position (backside up) on the stage. Position nose in nose cone for anesthesia.

   Note: Maximum five mice can be imaged at one time. If imaging more than one mouse at a time, ensure that there is minimum lag time between D-luciferin injections. There are other good sources for learning standard IVIS imaging in luminescence imaging mode (Cosette et al., 2016).
5. For imaging, set parameters in IVIS acquisition control panel. Select luminescent (imaging mode), auto (exposure), and D (field of view for 5 mice), or C (field of view for 3 mice).
6. Start imaging by clicking ‘acquire’ in IVIS acquisition control panel.
7. In case of weak signal, auto exposure can be changed to desired time with a maximum of 5 min of exposure. Field of view can be changed according to number of mice to be imaged at one time or size of the area of interest.

F. IVIS imaging for drug deposition (fluorescence imaging mode)

Note: Perform fluorescence imaging of the drug immediately after the luminescence imaging. If purpose of the experiment is only to visualize drug deposition in fluorescence imaging mode, there is no need to inject D-luciferin substrate.

1. In IVIS acquisition control panel, set imaging mode to fluorescence, exposure time to auto, excitation (650 nm) and emission (670 nm) for Cy5.
2. Start imaging by clicking ‘acquire’ in IVIS acquisition control panel.

   Note: For fluorescent imaging, IVIS spectrum is capable of using wavelength range from 415-850 nm. For in vivo and deep tissue imaging, it is important to note that wavelengths greater

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than 600 nm are preferred. Animal tissue absorbs significant amounts of light below wavelengths 600 nm. We have successfully used fluorescent labels such as Cy5, Alexa Fluor 660, Alexa Fluor 680, Alexa Fluor 750 and Cy7.

Data analysis

1. Bioluminescent or fluorescent signals acquired by IVIS can be quantified by Living Image Software. Figure 2 shows bioluminescence imaging of LPS-induced local inflammation in NF-κB reporter mice after subcutaneous injection of hydrogel-LPS mixture. Figure 3 shows fluorescence imaging of Cy5-labeled drug deposited subcutaneously in a hydrogel. Figure 4 shows simultaneous imaging of NF-κB activation and drug localization.

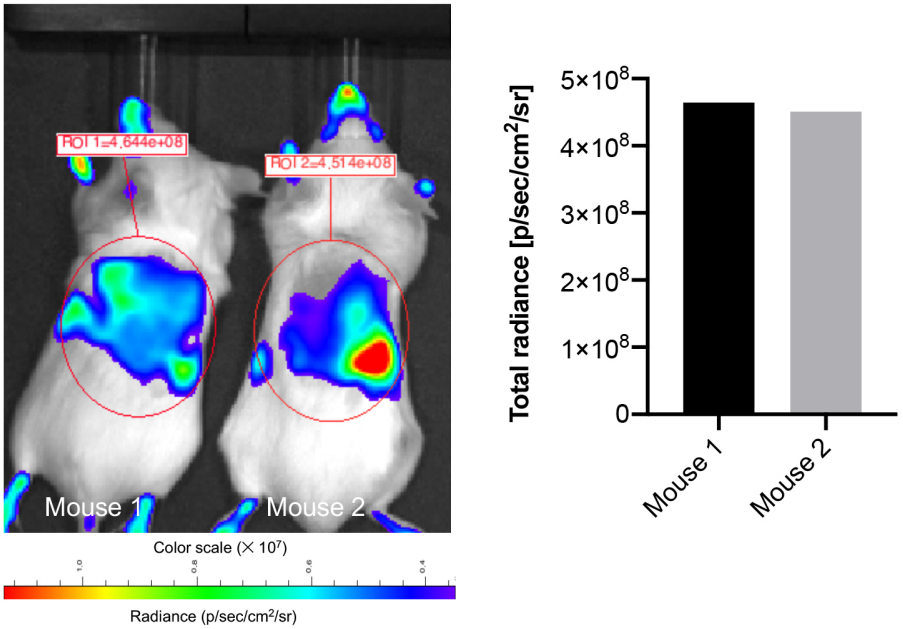

Figure 2. *In vivo* inflammation imaging by IVIS in NF-κB reporter mice. LPS in HEC hydrogel was subcutaneously deposited in transgenic BALB/c Tg(NF-κB-RE-luc)-Xen reporter mice. Imaging of NF-κB reporter gene expression was performed using the IVIS Spectrum. Image show bioluminescence at 3 h after subcutaneous deposition of the gel. Bar chart shows radiance measured locally around the gel deposition site. Minimal peripheral luminescent signal is also observed due to low systemic release of LPS from the gel.

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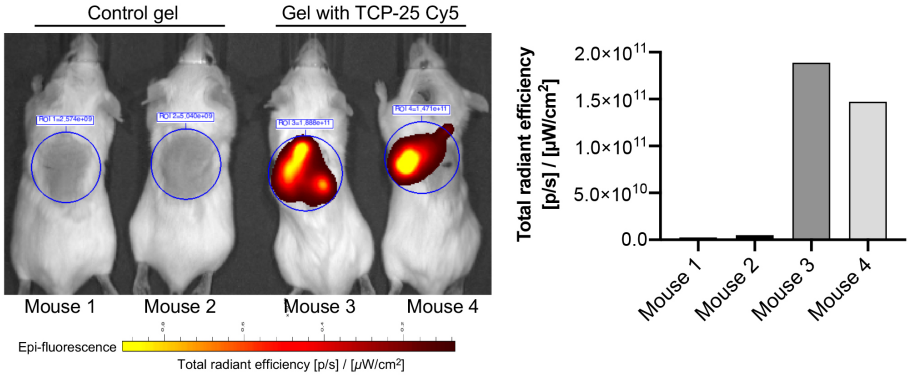

Figure 3. *In vivo* imaging of fluorescently-labeled drug subcutaneously deposited in mice. To image *in vivo* deposition, HEC hydrogel was mixed with Cy5-labeled TCP-25 and subcutaneously deposited on the back of mice. *In vivo* fluorescence imaging was performed using the IVIS spectrum. Image show distribution of TCP-25 Cy5 3 h after deposition of the gel. Bar chart shows fluorescence measured locally around the gel deposition site.

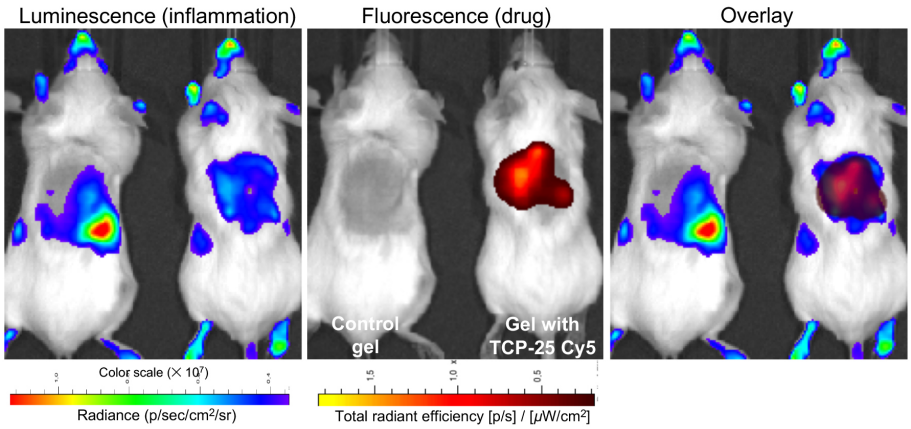

Figure 4. Simultaneous *in vivo* imaging of inflammation and drug subcutaneously deposited in NF-κB reporter mice. HEC hydrogel was mixed with LPS and Cy5-labeled TCP-25 and subcutaneously deposited on the back of mice. Simultaneous luminescence and fluorescence imaging was performed using the IVIS spectrum. Image show luminescence (inflammation), fluorescence (drug) and overlay at 3 h after deposition of the gel.

2. From tool palette, select region of interest (ROI) for an area where you observe/expect to observe bioluminescent signal. More than one ROI can be selected in one image. To get equal size, ROI from one mouse can be copied and applied to other mice too.
3. To measure bioluminescence, select radiance (photons) as units on the main image view. Click measure ROIs and a ROI measurements window displaying data will appear. Ensure that measurement types in ROI measurements window is also selected as radiance (photons). To measure fluorescence, select radiant efficiency.
4. Copy data to GraphPad Prism or Excel sheet for further analysis. Depending on the experimental question, data can be presented as total flux or average radiance.

5. To analyze differences in the means between two groups, a Mann-Whitney test can be used. To compare means between more than two groups, a Kruskal-Wallis test with post hoc (Dunn's) can be used.

Recipes

1. 1.5% hydrogel
   a. Preheat 10 mM Tris buffer (pH 7.4) to 56 °C in a 50 ml tube. Do not make more than 10 ml gel in a 50 ml tube as it otherwise will be difficult to stir
   b. While stirring with a magnetic stirrer, slowly add the required amount of HEC (1.5% w/v)
   c. Stir the mixture until a homogenous gel is formed. Remove air bubbles by centrifuging the gel formulation for 3 min (3.5 × 1,000 rpm, room temperature). A well-mixed gel should not have lumps
2. D-luciferin solution
   a. Prepare 15 mg/ml D-luciferin solution in DPBS
   b. Sterilize using a 0.2 µm syringe filter. Protect the solution from light
      Note: Small aliquots of the prepared solution can be stored in sterile tubes at -20 °C. Once the solution is thawed, use immediately and do not refreeze.
3. LPS solution
   a. Prepare a 1 mg/ml LPS solution in endotoxin-free water
   b. Sonicate for 1 min at highest setting in an ultrasonic bath (such as Elmasonic S30H)

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Competing interests

A.S. is a shareholder and board member of in2cure AB, a company developing anti-inflammatory peptides for therapeutic applications. M.P. has received consultancy fees from in2cure AB.

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Ethics

All animal experiments are performed according to Swedish Animal Welfare Act SFS 1988:534 and were approved by the Animal Ethics Committee of Malmö/Lund, Sweden (permit numbers M252-11, M88-91/14, M5934-19, 8871-19).

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