Noninvasive monitoring of lesion size in a heterologous mouse model of endometriosis
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1   **TITLE:**

2   Noninvasive Monitoring of Lesion Size in a Heterologous Mouse Model of Endometriosis

3

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21   **KEYWORDS:**

22   Endometriosis, heterologous, mouse model, mCherry, in vivo, monitoring

23

24   **SHORT ABSTRACT:**

25   Here, we present a protocol for live-imaging of fluorescently labeled human endometrial

26   fragments grafted in mice. The method allows studying the effects of drugs of choice on

27   endometriotic lesion size through monitoring and quantification of fluorescence emitted by the

28   fluorescent reporter on real time

29

30   **LONG ABSTRACT:**

31   Here, we describe a protocol for the implementation of a heterologous mouse model in which

32   progression of endometriosis can be assessed in real time through noninvasive monitoring of

33   fluorescence emitted by implanted ectopic human endometrial tissue. For this purpose,

34   biopsies of human endometrium are obtained from donor women ongoing oocyte donation.

35   Human endometrial fragments are cultured in the presence of adenoviruses engineered to

36   express cDNA for the reporter fluorescent protein mCherry. Upon visualization, labeled tissues

37   with an optimal rate of fluorescence after infection are subsequently chosen for the

38   implantation in recipient mice. One week prior to the implantation surgery, recipient mice are

39   oophorectomized, and estradiol pellets are placed subcutaneously to sustain the survival and

40   growth of lesions. On the day of surgery mice are anesthetized, and peritoneal cavity accessed

41   through a small (1.5 cm) incision by the *linea-alba.* Fluorescently labeled implants are tweezed,

42   briefly soaked in glue and attached to the peritoneal layer. Incisions are sutured, and animals

43   left to recover for a couple of days. Fluorescence emitted by endometriotic implants is usually

44   non-invasively monitored every 3 days for 4 weeks with an *in vivo* imaging system. Variations in

45 the size of endometriotic implants can be estimated in real time by quantification of the
46 mCherry signal and normalization against the initial time-point showing maximal fluorescence
47 intensity.
48
49 Traditional preclinical rodents of models of endometriosis do not allow non-invasive monitoring
50 of lesion in real time but rather allow evaluation of the effects of drugs assayed at the end
51 point. This protocol allows one to track lesions in real time and is more useful to explore the
52 therapeutic potential of drugs in preclinical models of endometriosis. The main limitation of the
53 model thus generated is that non-invasive monitoring is not possible over long periods of time
54 due to the episomal expression of Ad-virus.
55
56 **INTRODUCTION:**
57 Endometriosis is a chronic gynecologic disorder initiated by the implantation of the functional
58 endometrium outside the uterine cavity. Ectopic lesions grow and induce inflammatory
59 processes leading to chronic pelvic pain and infertility[1]. It is estimated that up to 10%–15% of
60 women of reproductive age are affected by endometriosis2, and it is present in approximately
61 40-50% of infertile women[3]. Current pharmacological treatments for endometriosis are unable
62 to completely eradicate lesions and not free of side effects[4,5]. The research for more efficient
63 therapies requires of the refinement of the existing animal models of endometriosis in such a
64 way that human lesions can be appropriately mimicked, and the effects of compounds on lesion
65 size among others can be closely assessed.
66
67 Primate models have been used to mimic endometriosis by implanting ectopic lesions
68 histologically identical and at similar sites as in humans [6-8]; however, ethical concerns and the
69 high economic costs related to experimentation with primates limit their use[9]. Consequently,
70 the use of small animals, especially rodents, for the implementation of in-vivo models of
71 endometriosis continues to be favored as it allows studies with larger numbers of
72 individuals[10,11]. Endometriosis can be induced in these animals by transplanting either pieces of
73 rodent uterine horns ("homologous models")[12,13] or human endometrial/endometriotic tissue
74 to ectopic sites (heterologous models)[14]. In contrast to humans, rodents do not shed their
75 endometrial tissue and thereby endometriosis can not be developed spontaneously in these
76 species. Therefore, homologous mouse models of endometriosis have been criticized due to
77 the fact that implanted ectopic mouse uterine tissue does not reflect the characteristics of
78 human endometriotic lesions[15].
79
80 Appropriate physiology of endometriosis can be mimicked in the heterologous models of
81 endometriosis where fresh human endometrial fragments are implanted into immunodeficient
82 animals. In conventional heterologous models, the therapeutic effects of compounds of interest
83 are commonly assessed at the end point by the assessment of lesion size with the use of
84 calipers[16]. An obvious limitation is that, as such, endpoint animal models do not allow studying
85 implantation dynamics or endometriotic lesion development over time. An additional limitation
86 is that the use of calipers does not allow accurate measurements of lesion size. Indeed, the
87 standard error provided by calipers is in the same range (*i.e.*, millimeters) as the size of the
88 lesions implanted in mice, thus restricting the capacity of these tools to detect actual variations

89   in size.

90

91   In order to overcome such limitations, herein, we describe the generation of a heterologous
92   mouse model of endometriosis in which implanted human tissue is engineered to express a
93   reporter m-Cherry fluorescent protein. Detection of the fluorescent signal with an appropriate
94   image system enables non-invasive monitoring of lesion status with simultaneous
95   quantification of its size in real time. Thus, our model provides clear advantages when
96   compared to conventional endpoint models as it brings the opportunity of real-time non-
97   invasive monitoring and the possibility to perform more objective and accurate estimation of
98   variations in lesion size.

99

100   **PROTOCOL:**

101

102   The use of human tissue specimens was approved by the Institutional Review Board and Ethics
103   Committee of the Hospital Universitario La Fe. All patients provided written informed consent.
104   The study involving animals was approved by the Institutional Animal Care Committee at the
105   Centro de Investigacion Principe Felipe de Valencia, and all procedures were performed
106   following the guidelines for the care and use of mammals from the National Institutes of
107   Health.

108

109   **1)   Endometrial Tissue Collection and Pre-Processing**

110

111   1.1)   Obtain a good quality biopsy of human endometrial aspirate by using a cannula
112   attached to a suction device. Pour the biopsy into a flask containing 10 mL of sterile saline and
113   wash with gentle manual agitation.

114

115   Note: Procedures on how to obtain good quality biopsies have been previously described[17].

116

117   1.2)   Wash the biopsy of any remaining blood or mucus. Repeat the process by pouring the
118   tissue to flasks containing fresh saline as many tines as required until tissue is observed clean.

119

120   1.3)   Transfer biopsy fragments with a healthy appearance into a solution containing 10 mL of
121   complete Dulbecco's Modified Eagle's medium (DMEM) medium with 10% Fetal Bovine Serum
122   (FBS) and 1% antibiotic-antimycotic solution.

123

124   1.4)   Pour the content in a 10 cm Petri dish and proceed to chop the tissue into 5-10 mm^3
125   pieces with a pair of scalpels.

126

127   **2)   Adenoviral Transfection of Endometrial Fragments.**

128

129   Note: All the materials that are going to be employed in the process should be introduced in
130   the hood in advance. Take out everything that is not going to be used in the process and place a
131   flask with bleach. All material that comes into contact with the adenoviral vector must be
132   disinfected with the bleach before discarding it in the biohazard container.

133

2.1)   Once the biopsy has been chopped, take a new Petri dish. Pipette multiple 30-50 µL DMEM drops of spread throughout the whole dish. Leave enough space between drops so they do not come into contact.

2.2)   Aided with a needle attached to syringe, place a single piece of fragment inside each of the medium drops.

Note: Each drop should contain a single piece of endometrium.

2.3)   Prepare the Ad-mCherry working solution by diluting 1:20 of the mCherry adenoviral stock solution [$1 \cdot 10^{10}$ ifu/mL]) into DMEM medium WITHOUT antibiotics (DMEM + 10% filtered FBS).

2.4)   Dispense 100-200 µL of the Ad-mCherry solution per well on a 96-well plate, filling as many wells as fragments are available in the Petri dish drops (step 2.2). In addition, fill at least 3 wells with adenovirus free DMEM medium as a negative control.

2.5)   Aided with a needle attached to syringe, transfer each of the fragments in the Petri dish (step 2.2) to each of the wells containing ad-mCherry solution in the 96-well plate.

2.6)   Place the 96 well-plate containing the endometrial fragments with Ad-mCherry solution into an incubator at 37 °C with 5% CO_2 for 16 h.

2.7)   Wash out the remaining adenoviruses in the medium by transferring tissues into a new 96-well plate filled with complete DMEM medium (WITH antibiotics-antimycotics, free of adenovirus) and incubate for 37 °C with 5% CO_2 for 24-48 h.

NOTE: Remember to cover with bleach all well plates and tips that came in contact with Ad-virus before discarding into the biohazard container.

2.8)   Take out the plate from the incubator and place it under a fluorescence microscope at 568 nm (red channel) to test for optimal labeling.

2.9)   Choose the most brilliant fragments and place them into a new 96-well plate with fresh complete DMEM medium (WITH antibiotics-antimycotics, free of adenovirus).

2.10)   Seal the plate well with a plastic paraffin film and transport it to into the specific-pathogen-free animal area for implantation of endometrial fragments into recipient animals.

3)   **Generation of the Endometriosis Mouse Model**

NOTE: Use 6-8-week-old athymic nude (or similar immunocompromised strains) female mice housed in specific pathogen–free conditions, as recipient animals. To avoid hormonal cycle-

177 dependent variations and simultaneously fuel lesion growth with estradiol, animals are
178 ovariectomized and placed with 60-day release capsules containing 18 mg of 17 βeta-Estradiol
179 (17β-E$_2$). Oophorectomy and pellet placement have to be performed at least one week in
180 advance of grafting endometrial fragments into the recipient animals.
181
182 **3.1)   Oophorectomy**
183
184 Note: Prepare surgical sterilized material in a hood. Prepare anesthesia equipment and a post-
185 surgical recovery zone ready in the same room.
186
187 3.1.1) Perform a subcutaneous injection of morphine derivative at a dose of 5 mg/kg per
188 mouse. Let the mice rest for 30 min after injection so as analgesics effects of drug can be
189 manifested.
190
191 3.1.2) Connect the inhalation anesthesia equipment and let oxygen and isoflurane (2% mg/kg)
192 flow for a few min into a sealed anesthesia chamber.
193
194 3.1.3) Introduce the animal into the isoflurane anesthesia chamber. Wait for 3-5 min and
195 check that animals are fully anesthetized by pressing one of its paws. Transfer the animal to the
196 surgery area and maintain anesthesia by placing a mask with continuous flow of isoflurane gas
197 covering the respiratory airways.
198
199 3.1.4) After disinfecting the area with chlorohexidine, perform a transverse 0.5 cm costal
200 incision approximately at the height of the hip with sharp scissors.
201
202 3.1.5) Separate the skin from the muscle to get access to the abdominal cavity, identify the
203 white fat pad that surrounds the ovary and retract the ovary with dissection forceps.
204
205 3.1.6) Tie a knot around the oviduct with absorbable suture and tighten it to ensure
206 appropriate hemostasis before excising the ovary.
207
208 3.1.7) Close the muscular layer with 6-0 absorbable suture, and then close the skin with 6-0
209 non-absorbable suture. Clean the area again with antiseptic solution.
210
211 3.1.8) Repeat the procedure to remove the contra lateral ovary.
212
213 **3.2)   Estradiol pellet implant**
214
215 Note: Take care that the animal is anesthetized during the oophorectomy surgery to place
216 pellets at that point.
217
218 3.2.1) Immediately after the completion of the oophorectomy procedure, clean the skin with
219 antiseptic solution surrounding the neck and make a transverse subcutaneous small (0.5 cm)
220 incision with sharp scissors in the nape.

221

222 3.2.2) Use the scissors to dissect the skin from the muscle, making a pocket large enough to
223 allow placing the pellets.

224

225 3.2.3) Insert the pellet containing 18 mg of 17β-E$_2$ and suture the skin with a 6-0 non-
226 absorbable suture. Clean the area again with antiseptic solution.

227

228 3.2.4) Place the animal in the recovery zone and administer an optimal dose of long-lasting
229 analgesia to ease the recovery period.

230

231 **3.3)**   **Endometrial implant surgery**

232

233 NOTE: Allow at least a period of seven days quarantine to allow full recovery of animals after
234 oophorectomy before starting endometrial fragment implantation surgery. For optimal
235 synchronization with labeling of tissue, collect the biopsy 2-3 days before implantation surgery
236 so as to avoid long-term culture of explants.

237

238 3.3.1) Get the surgical room in the specific pathogen free zone ready in advance. Prepare the
239 hood with all required surgical material, the anesthesia equipment and the post-surgical
240 recovery zone also.

241

242 3.3.2) Bring the animals to the room, perform a subcutaneous injection of a morphine
243 derivative at a dose of 5 (mg/kg) in each mouse. Let the mice rest for 30 min after injection so
244 analgesics effects of drug can be manifested.

245

246 3.3.3) Connect the inhalation anesthesia equipment and let oxygen and isoflurane (2% mg/kg)
247 flow for a few min into a sealed anesthesia chamber.

248

249 3.3.4) Before starting surgery, move the plate containing fluorescently labeled fragments
250 (from step 2.10) into the hood, unseal it and pour the fragments into a Petri dish for easier
251 handling.

252

253 3.3.5) Introduce the animal into the isoflurane anesthesia chamber. Wait for 3-5 min and
254 check that animals are fully anesthetized by pressing one of its paws. Transfer the animal to the
255 surgery area and maintain anesthesia by placing a mask with continuous flow of isoflurane gas
256 covering the respiratory airways.

257

258 3.3.5) Place the anesthetized animal face up. Disinfect the ventral area. Perform a longitudinal
259 1.5 cm incision in abdomen with sharp scissors and separate the skin from the muscle. Then,
260 perform a longitudinal 1.5 cm incision in the muscle to access the peritoneal cavity.

261

262 3.3.6) Hold the left edge of the abdomen muscular wall with mini-forceps and fold it trying to
263 expose the inner face of the peritoneum on the outside.

264

265 3.3.7) Take an endometrial implant with mini tweezers, soak it briefly in an n-butyl-ester
266 cyanoacrylate adhesive and place it to the peritoneum where it will get attached. Let it dry for a
267 few seconds. Repeat steps 3.3.6 and 3.3.7 to place an implant on the contralateral side of the
268 peritoneum.
269
270 3.3.8) Close the muscular layer with an absorbable 6-0 suture, and then close the skin with a
271 non-absorbable 6-0 suture. Clean the area again with antiseptic solution.
272
273 3.3.9) Place the animal in the recovery zone and administer an optimal dose of long-lasting
274 analgesia.
275
276 **4)** ***In Vivo* Fluorescent Imaging with an *in vivo* imaging system**
277
278 4.1) Turn on the *in vivo* imaging system device, initialize the program and allow the CCD
279 camera to cool down for a few min.
280
281 4.2) Prepare the inhalation anesthesia equipment. Open the isoflurane flow at 2% for a
282 couple of min to fill the anesthetic chamber. Prepare the post-anesthesia recovery zone.
283
284 4.3) Once the program has started and the CCD camera have cooled down, click the **Imaging**
285 **Wizard** tool: A tutorial starts with a series of consecutive windows displayed, each one
286 corresponding to a parameter of interest with several options available to be chosen by clicking
287 in the corresponding box. Move forward through the tutorial by selecting the appropriate boxes
288 in each window and click the **OK** button to move to the next set of parameters with the
289 following sequence.
290
291 4.3.1.) Select the **Epiluminescence box** for the fluorescence parameter, select the **mCherry box**
292 for the filter pairs parameter. Check the **Photograph mode** and **Confirm Focus** boxes and select
293 the Automatic box for the exposure parameter.
294
295 4.4) Once the instrument has been set up, move one animal inside the anesthesia chamber.
296 When fully anesthetized, transfer the animal inside the *in vivo* imaging system cage and place it
297 side up with its head inside a tubule connected to the anesthesia machine. Close the lid and
298 click **Acquire** for monitoring.
299
300 4.5) Acquire the images appearing (a total of five images, one image for each pair of filters
301 selected) and save data by clicking the **Save As** button. Move the mouse to the area of post-
302 anesthesia recovery. Repeat the process with the remaining animals.
303
304 4.6) Repeat monitoring two or three times a week to follow-up the signal appropriately
305 during the time course.
306
307 4.7) Proceed to sacrifice the animal at the end of the time course by CO_2 asphyxiation.
308

309   **5)   Quantification of *In Vivo* Fluorescence Images**

310

311   **5.1)   Segregation of actual fluorescence through image unmixing:**

312

313   5.1.1) Open the ***In Vivo* Imaging Analysis Coupled Software Program**.

314

315   5.1.2) Choose the "Sequenceinfo" file to start the analysis. Two windows will appear:
316   "Sequence view" and "Tool Palette". Choose **Tool Palette** and once the menu has been
317   displayed select the following options.

318

319   5.1.3) Select **Corrections** and click on the box **Adaptive FL Background Subtraction** to remove
320   the undesirable fluorescent signals from the luminescent image data. Choose the threshold of
321   greatest interest and click **Set**.

322

323   5.1.4.) Click **Spectral Unmixing**, select the wavelengths of interest, the method chosen for
324   unmixing (library, guided, automatic or manual) and then click **Start Unmix**.

325

326   5.1.5) Select the **Unmixed** image corresponding to mCherry signal and double click. A new
327   window will appear with the final image of the signal of interest.

328

329   5.1.6) Repeat step 5.1.3.

330

331   5.1.7) OPTIONAL: If a representative image (JPEG) of the unmix result is needed, choose the
332   desired settings in **Tool Palette | Image Adjust** (color table, binning, contrast, *etc*.), and then
333   click on **Export Graphics** in the unmix window to export the current image view as an image.

334

335   5.1.8) Save the unmixed file: **File | Save As | Choose Folder** and **Ok**.

336

337   5.1.9) Repeat the process with the rest of the monitoring days and with all animals.

338

339   **5.2)   ROIs set up and signal quantification**

340

341   5.2.1) Click **Browse** and select the **Unmixed file** (see step 5.1.8) of interest to be analyzed. A
342   new window will appear.

343

344   5.2.2) Click **Add To List** to include all the unmixed files from each animal at different time
345   points and then click on **Load As A Group**. All images must appear as a single sequence.

346

347   5.2.3) Go to **Tool Palette** window: Click off the box I**ndividual** scale to obtain all images on the
348   same scale.

349

350   5.2.4) Double click in one image of the sequence and create a ROI on the zone of interest using
351   the following sequence. Go to **ROI Tools** and select **Countour** and **Auto 1** option, click on the
352   circle shape appearing, place it on the center the fluorescent signal and then click **Create** on the

353   <mark>displayed window.</mark>

354

355   Note: This automatically highlights pixels with an intensity of fluorescence above background
356   values (*i.e.*, lesion) and generates a shape whose area embraces the outlined pixels.

357

358   <mark>5.2.5)   Copy the created ROI shape and paste on a background zone where there is no signal.</mark>

359

360   <mark>5.2.6)   Click on **Measure ROIs | Select All** to display values of fluorescence intensity. Proceed to</mark>
361   <mark>select data values with the mouse, click right button, press copy and paste on a spreadsheet.</mark>

362

363   **6)   Data (fluorescent signal) Normalization**

364

365   6.1)   Select the initial time point at which signal intensity is maximal. Proceed to normalize
366   signal at each time point by using the formula:

367

368   Signal intensity at each time point / Maximal signal intensity observed during the time course) x
369   100.

370

371   **REPRESENTATIVE RESULTS:**
372   Here, we describe the process for creating a heterologous model of endometriosis in which the
373   architecture of lesions is preserved by implanting fluorescently labeled pieces of human
374   endometrium into immunocompromised mice, thus allowing non-invasive monitoring of lesion
375   progression. Labeling of endometrial fragments is achieved by infection with adenovirus
376   engineered to express mCherry, a protein emitting fluorescence in the near infrared region. In
377   **Figure 1,** we show representative images of human endometrial fragments infected with Ad-
378   mCherry observed under the fluorescence microscope. For illustrative purposes, both labeled
379   and non-labeled fragments are included so differences in fluorescence between infected and
380   non-infected tissues (autofluorescence) can be noted. During monitoring, in addition to the
381   reference wavelength for mCherry, fluorescent images are taken with different pairs of
382   excitation/emission wavelengths filters (**Figure 2**) to define the characteristic fluorescent
383   emission profile of tissues. The purpose of this action is to "unmix" actual fluorescence emitted
384   by lesions from background and autofluorescence emitted by host tissues and scar originated
385   during surgery respectively. An illustrative example of the unmix process is shown in **Figure 3**.
386   Estimations of variation in lesion size is performed by quantifying and normalizing fluorescent
387   signaling emitted by lesions during the time course. For this purpose, images of monitoring
388   containing raw fluorescence emitted by animals during each time point are first brought
389   together unnormalized (**Figure 4**) in a single file. Subsequently fluorescence is unmixed,
390   normalized and represented as a false color image (**Figure 5**). Finally, ROIs corresponding to
391   specific lesion and background signaling are automatically recognized by the program and
392   quantified (**Figure 6**). Background ROI signaling is subtracted from lesion ROI signaling and
393   results of intensity in each time point are normalized against the time point at which intensity is
394   maximal (**Figure 7**). At the end of the monitoring process, several weeks after surgery mice are
395   sacrificed and viable implant can be recovered attached to the mouse peritoneum (**Figure 8**).

396

Figure 1: Visualization of endometrial fragments with fluorescence microscope after Ad-mCherry infection A) Human endometrial fragment incubated with Ad-mCherry at 37 °C and 5% CO_2 during 24 h as a positive sample. **B)** Human endometrial fragment incubated at 37 °C and 5% CO_2 without Ad-mCherry as a negative control sample.

Figure 2: Raw imaging fluorescence emitted by labeled fragments implanted in mice. Picture shows representative imaging of raw fluorescence signal emitted by the same animal at a specific time point. Images correspond to screenshots obtained using software coupled to an *in vivo* imaging system device during a monitoring session. Each panel containing mice (numbered 1 - 5 in the left corner) corresponds to the fluorescence observed by using a different specific excitation/emission pair filter for acquiring images. Panel on the right (tool palette) show fluorescence parameters selected for acquisition of images

Figure 3: Unmixing of background vs specific fluorescence emitted by lesions. Picture shows representative images of the unmixing process performed to dissect actual fluorescence from lesions using the *in vivo* imaging system coupled software. Graph on the left panel denote normalized specific profiles of fluorescence emission by scar (green line, UMX1), lesions (red line, UMX2) and host tissue (blue line, UMX3 panel). Fluorescence intensity at different emission wavelengths (X-axis) is represented in units of radiant efficiency (Y-axis). Just note how each specific structure (*i.e.*, scar, labeled lesions and host tissue) emits a different fluorescence profile which allows identifying and segregating them specifically form each other. On the right panel, fluorescence arising from launching specific emission profiles for scar (UMX1), lesions (UMX2) and host tissue (UMX3) are shown superposed on photograph images of mice. A composite image (Composite) is also included for illustrative purposes to denote the segmentation of fluorescence emitted by lesions from that emitted by scar or host tissues. Middle panel shows parameters selected for unmixing with the image software coupled to the in-vivo imaging device

Figure 4. Time course monitoring of raw fluorescence emitted by lesions. Panel shows representative images of raw fluorescence emitted by a single mouse implanted with labeled human lesions (brilliant yellow spots) during the time course. Time points after surgery at which monitoring was performed are denoted as "Day (number)". Emission ad excitation pair filters used for monitoring are indicated in each panel/image. Each panel is identified by a specific code (BKG) in the upper part containing info related to the date at which fluorescence was acquired

Figure 5: Time course monitoring of normalized fluorescence emitted by lesions. Representative images corresponding to unmixed, normalized fluorescent signaling emitted by human lesions (spots with rainbow color) superposed in a single mouse during the time course (days after surgery). Time points after surgery at which monitoring was performed are denoted as "Day (number)". Each panel is identified by a specific code (BKG) in the lower part containing info related to the date at which fluorescence was acquired. Rainbow palette color on the right

441   side identifies fluorescence intensity (Radiant efficiency) emitted by lesions at each time point.
442   Note how strong fluorescence intensity during the initial time points (*i.e.*, red color in the
443   center of lesions on days 1,5 and 8) declines during the time course (*i.e.,* blue color in lesions on
444   days 20 and 25).
445
446   **Figure 6: Use of ROIs for quantification of fluorescence intensity in lesions during the time**
447   **course.** Figure shows panel of images of normalized fluorescence emitted by lesions in a single
448   mouse during the time course (*i.e.,* **Figure 5**) with the addition of ROIs (delineating lesions and
449   background) for quantification of fluorescence intensity. ROI 1 and ROI 2 identify the amount of
450   fluorescence emitted by each of the two lesions during the time course. BKG identify the
451   amount of fluorescence emitted by the host tissue (background fluorescence) during the time
452   course. Background fluorescence is subtracted from ROIs for quantification purposes. Time
453   points after surgery at which monitoring was performed are denoted as "Day (number)".
454   Images acquired at each time point are labeled with a specific code (BKG) at the bottom of each
455   one containing info related to the date at which fluorescence was acquired (first eight digits
456   following BKG detail data for year(2014)-month (10) and -day(10 to 31)), an individual
457   identification code (last 6 digits) and the specific profiles of fluorescence emission used for
458   unmixing (UMX2) Rainbow palette color on the right side provides a visual scale of fluorescence
459   intensity (Radiant efficiency) emitted by lesions at each time point. Note how fluorescence
460   intensity values in the two lesions (ROI1 and ROI2) are higher at the initial time points (days 1
461   and 5) and decays during the time course to reach the lowest values at the end time points
462   (days 20 and 25).
463
464   **Figure 7: Normalization of fluorescence intensity during the time course.** Table in the upper
465   part shows illustrative example of the values of fluorescence intensity (radiant efficiency)
466   emitted by two mCherry labeled lesions (ROI1 and ROI2) implanted in a mouse (R25).
467   Monitoring of fluorescence was performed at different days after surgery (D5-D25) during the
468   time course. D5- Graph at the bottom illustrates typical pattern of normalized fluorescence
469   emitted by lesions infected with mCherry decaying during the time course. Y-axis shows values
470   of fluorescence normalized to express the percentage of decay by using the formula (Signal
471   intensity at each time point / Maximal signal intensity observed during the time course) x 100.
472   Time points (Days (Dx) after implanting surgery) at which fluorescence was monitored are
473   indicated in the X-axis. Note initial increase of signaling during the first 24 hrs after surgery,
474   corresponding to stabilization of lesion, the peak in fluorescence intensity around D1-D5 and its
475   subsequent decay due to episomal expression of mCherry during the time course
476
477   **Figure 8: Macroscopic appearance of implanted endometriotic lesions.** Representative images
478   showing macroscopic appearance of endometriotic lesions implanted in mice at the end of the
479   monitoring process upon sacrifice
480
481   **DISCUSSION:**
482   The protocol herein detailed describes the implementation of an animal model of
483   endometriosis in which the architecture of implanting lesions architecture is preserved whilst
484   simultaneously allowing real time assessment of fluorescence emitted by mCherry labeled

485   endometrial tissue. In this protocol, we describe the use of a specific *in vivo* imaging system and
486   related software to non-invasively assess fluorescence emitted by the labeled lesion. Each user
487   should adapt the protocol depending on the specific imaging device and related software
488   available at their institution. Monitoring is performed in anesthetized animals through an
489   isoflurane gas anesthesia machine coupled to an *in vivo* imaging system. To avoid interference
490   with auto-fluorescence emitted by wounds, it is recommended to start monitoring lesions
491   fluorescence at least three days after implantation surgery.
492
493   Heterologous mouse models of endometriosis similar to the one herein shown have been
494   previously described consisting in the implantation endometrial fragments labeled with green
495   fluorescent protein (GFP)[18,19]. The use of mCherry as a reporter for tagging the human tissue
496   provides however an advantage over GFP because of the enhanced tissue penetration of the
497   former. Due to its larger emission spectrum and higher photostability mCherry emits a brighter
498   signal that is more appropriate for the visualization of intraperitoneal fragments.
499
500   Due to its small size adenoviruses are the vectors of choice for infection (*i.e.*, labeling) of whole
501   tissue pieces as those provide acceptable diffusion through 3D structures. Even with that the
502   percentage of tissue infected cells is not higher than 30-35%. Thus, the limitation of this model
503   relies on the inefficient labeling of tissue and additionally the transient expression achieved by
504   adenoviruses. Indeed, fluorescence cannot be monitored beyond 4–6 week as it fades
505   progressively due to the episomal transient expression of the Ad-virus. Efficiency of labeling
506   and the period of monitoring might be increased by disrupting the donor tissue and infecting
507   isolated single epithelial/stromal cells with ad-virus previous to being injected into recipient
508   mice[19,20]. Such an approach, however, reduces the extent at which the animal model mimics
509   the physiology of endometriosis provided that ectopic human lesions do not consist in a
510   disorganized accumulation of single epithelial/stromal cells but rather in well-structured
511   endometriotic tissue.
512
513   In this protocol, the most critical step is the labeling of the tissue and most specifically the
514   determination of the appropriate concentration of ad-virus required for optimal infection.
515   Indeed, the $1x10^{10}$ pfu/mL concentration pointed out in the protocol is mostly an
516   orientative/consensus figure based on our experience. The optimal concentration might differ
517   in each experiment depending on the type and quality of the biopsy and/or how quickly this is
518   processed. We thus suggest testing at least three different (two-fold) titers in each experiment
519   and choosing the one providing optimal labeling based on visualization under the fluorescence
520   microscope.
521
522   Our protocol/model is useful to study the mechanisms implicated in the establishment and
523   early development of the endometriotic lesions. In spite of the period of time of monitoring is
524   constrained, the model is still useful to study and detect the effects of pharmacological
525   compounds able to exert dramatic effects on lesion size in a short period of time such as
526   antiangiogenic or antiestrogenic drugs. The development of more efficient and durable
527   methods of labeling human tissue are expected to spread non-invasive monitoring as a
528   consolidated technique to explore the potential therapeutic of a wider range of drugs in

529 preclinical model endometriosis.
530

537 **DISCLOSURES:**
538 The authors have nothing to disclose.
539

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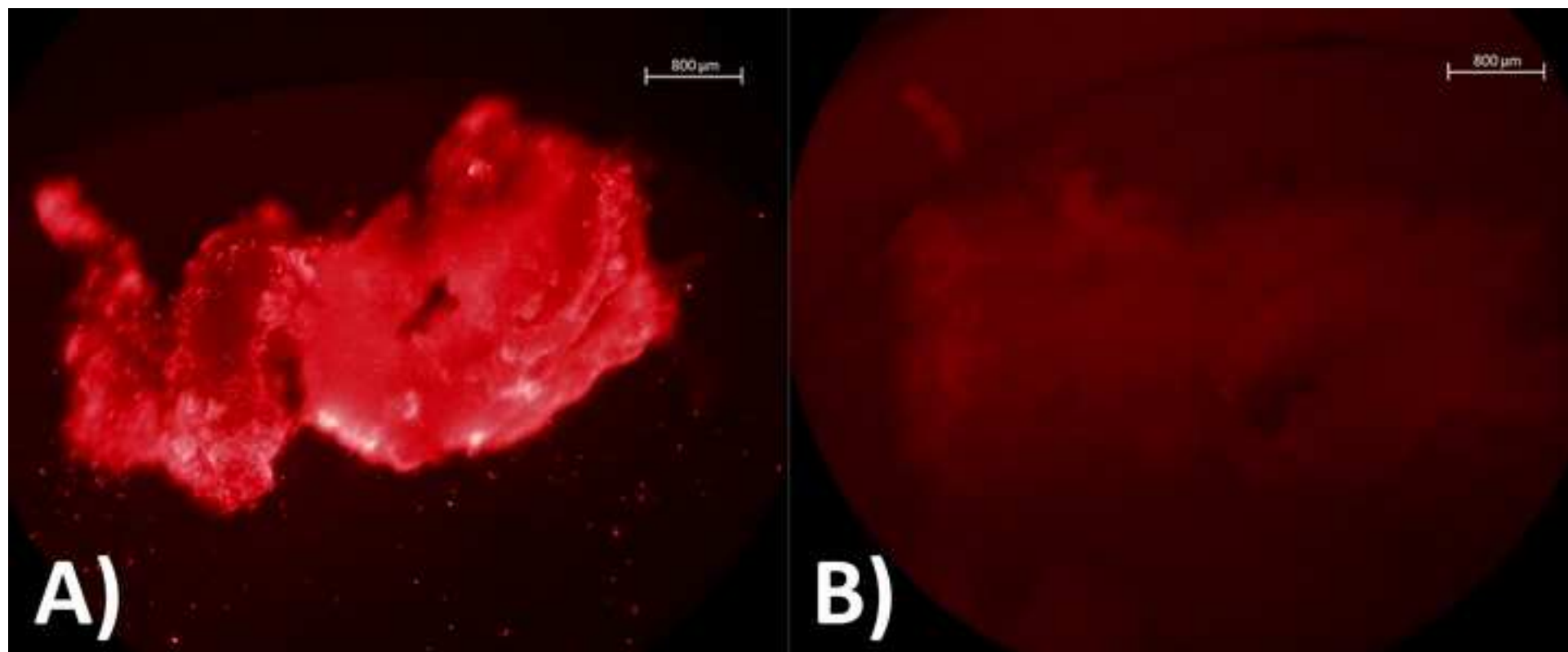588 *Reproductive Biology*; **176**, 25-30 (2014).
589

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

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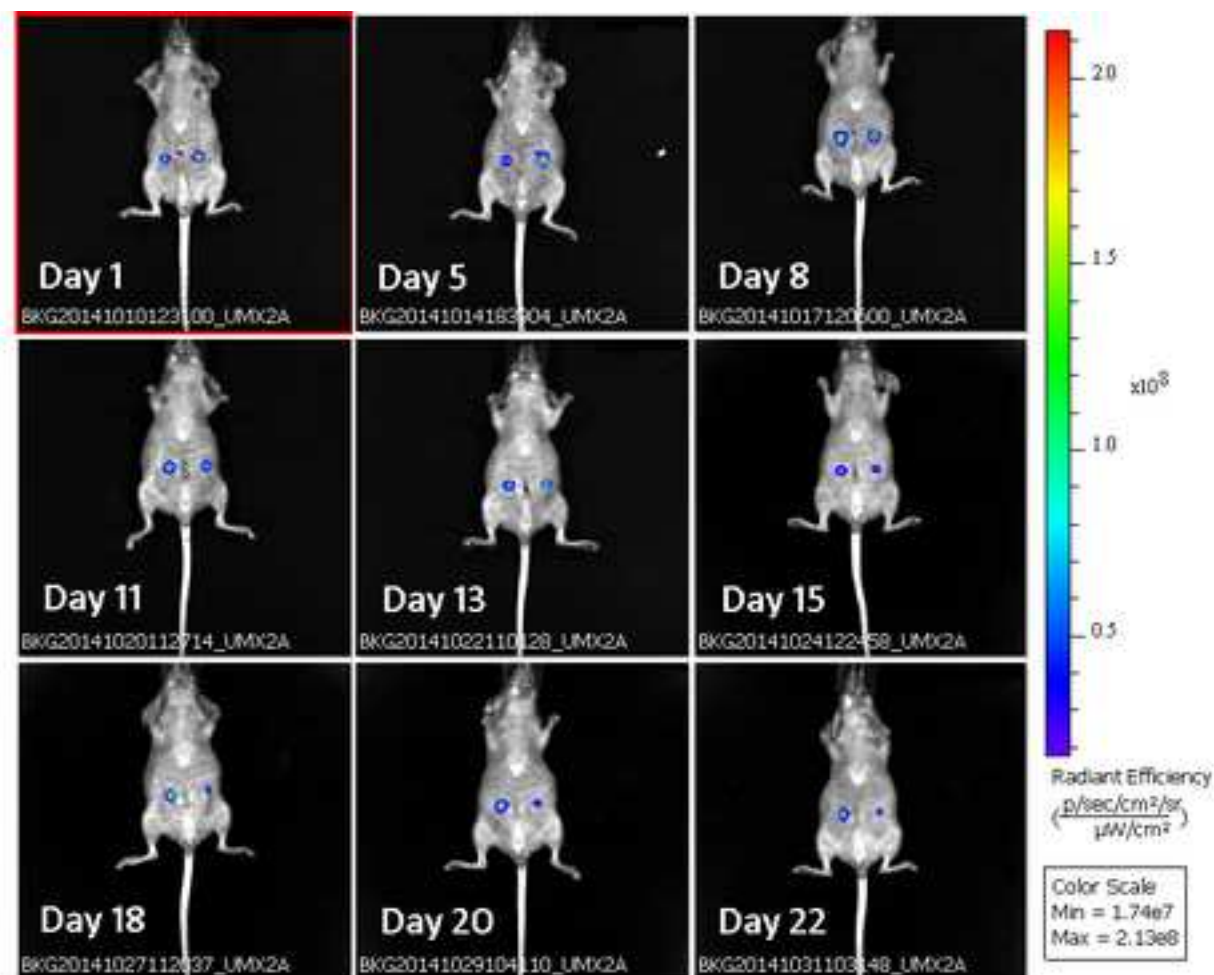
Figure 6

Figure 7

R25 - ROI	AVERAGE RADIANT EFFICIENCY							
	D5	D8	D11	D13	D15	D18	D20	D22
ROI1	8,35E+07	9,24E+07	5,11E+07	4,35E+07	3,97E+07	7,53E+07	7,80E+07	4,67E+07
ROI2	1,28E+08	1,07E+08	5,13E+07	4,80E+07	3,79E+07	3,52E+07	3,39E+07	3,60E+07

Avg Radiant Efficiency [p/s/cm²/sr] / [µW/cm²]Reduction % (D5)

Figure 8

Materials Table
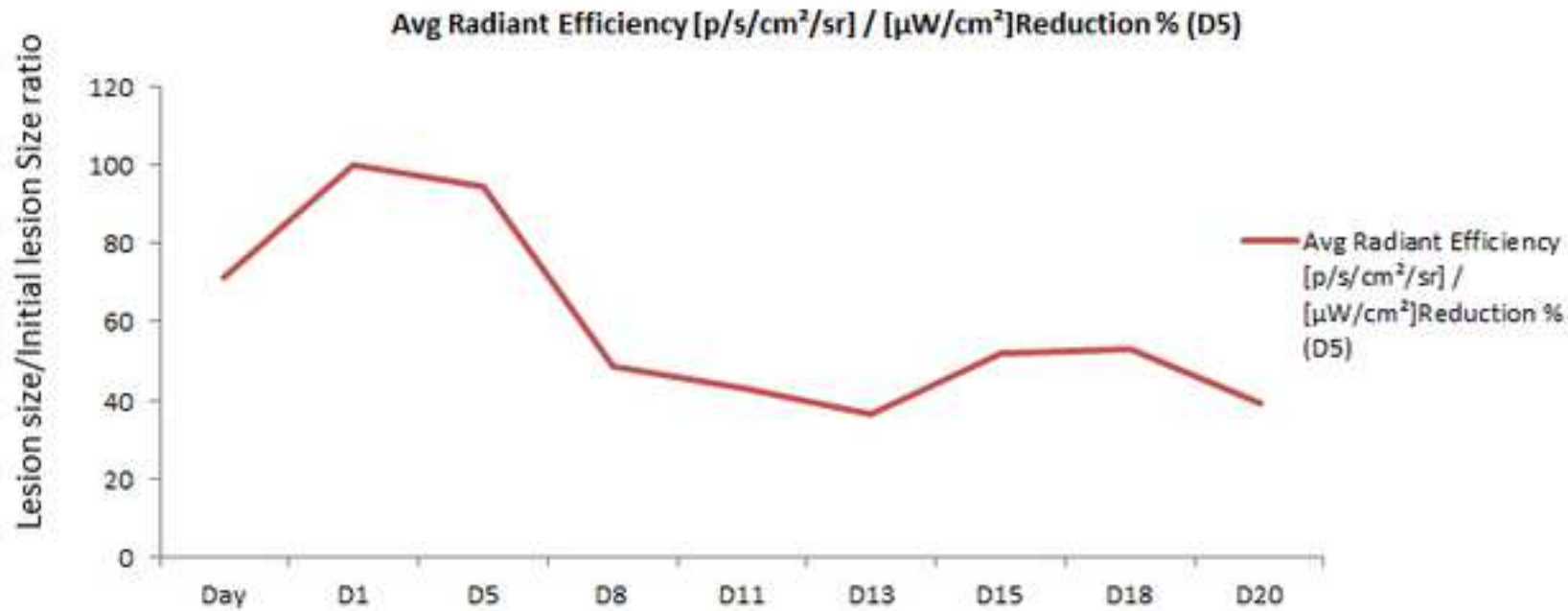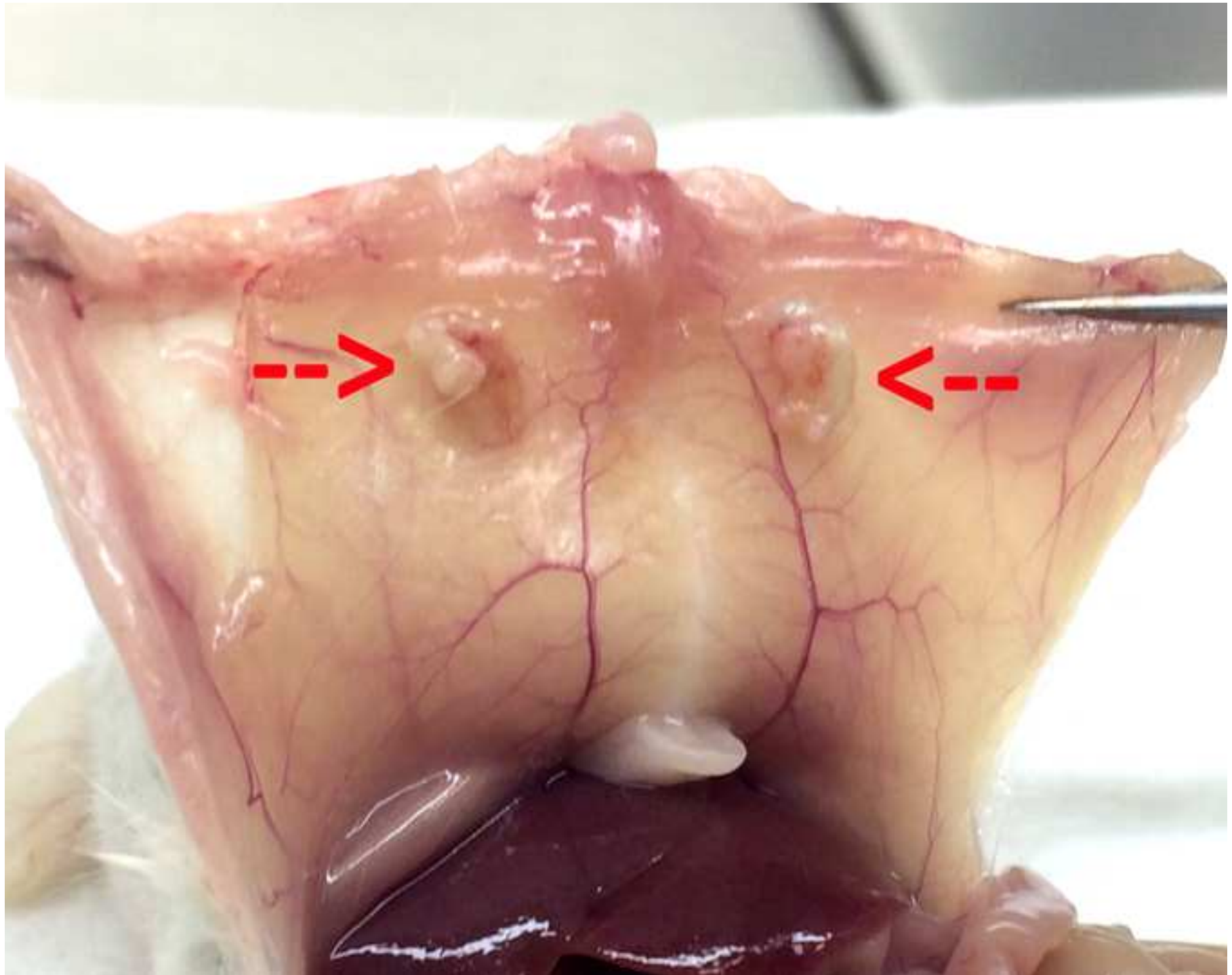
Name of Material/ Equipment	Company
Endosampler™	Medgyn
DMEM Medium	VWR
Ad-mCherry	Vector Biolabs
PBS, 1X solution, sterile, pH 7,4	VWR
Pellets 17-B-Estradiol 18 mg/ 60 days	Innovative Research of America
Vetbond™ Tissue Adhesive	3M
Petri dishes in polystyrene crystal	Levantina
Penicillin-Streptomicin	Sigma
Syringes, medical 10 ml 0,5 ml	VWR
Nitrile gloves, powder-free	VWR
Soft swiss nude mice	Charles River
Ivis Spectrum In vivo Imaging system	Perkin Elmer
Living Image® (Ivis software)	Perkin Elmer
Fetal Bovine Serum	Gibco
96-well cell culture treated plates	Life technologies
Urine flasks	Summedical
Sterile surgical blades	(Aesculap Division) Sanycare
Isovet 1000 mg/g	B-BRAUN
Buprex® 0.3 mg	Schering Plough S.A.
Injectable morphine solution 10 mg/mL	B BRAUN
Monofyl® Absorbable Sutures	COVIDIEN

Desinclor chlorhexidine	Promedic SA
Microscopy DMi8	Leica Mycrosystems
Hera Cell 150 Incubator	Thermo Scientific

Catalog Number	Comments/Description
22720	Cannula for sampling the uterine endometrium
HYCLSH30285.FS	Medium
1767	Adenoviral vector expressing mCherry
E504-500ML	Buffer for washes
SE-121	Hormone pellets for rodents
780-680	Tissue adhesive
367-P101VR20	Petri dishes
P4333-100ML	Antibiotics
CODA626616	Syringes
112-2754	Gloves
SNUSSFE05S	Mice for animal experiment
124262	In vivo Monitoring equipment
---	In vivo monitoring software
10082147	Enrichment serum
167008	Culture plates
4004-248-001	Flasks for washes
1609022-0008	Surgical blades
---	Isoflurane (Anesthetic)
---	Buprenorphine (Analgesic solution)
---	Morphine (Analgesic solution)
---	Sutures

---	Antiseptic solution
---	fluorescence microscope
51026282	Incubator

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1　**TITLE:**
2　Noninvasive Monitoring of Lesion Size in a Heterologous Mouse Model of Endometriosis
3
4　**AUTHORS & AFFILIATIONS:**
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21　**KEYWORDS:**
22　Endometriosis, heterologous, mouse model, mCherry, in vivo, monitoring
23
24　**SHORT ABSTRACT:**
25　Here, we present a protocol for live-imaging of fluorescently labeled human endometrial
26　fragments grafted in mice. The method allows studying the effects of drugs of choice on
27　endometriotic lesion size through monitoring and quantification of fluorescence emitted by the
28　fluorescent reporter on real time
29
30　**LONG ABSTRACT:**
31　Here, we describe a protocol for the implementation of a heterologous mouse model in which
32　progression of endometriosis can be assessed in real time through noninvasive monitoring of
33　fluorescence emitted by implanted ectopic human endometrial tissue. For this purpose,
34　biopsies of human endometrium are obtained from donor women ongoing oocyte donation.
35　Human endometrial fragments are cultured in the presence of adenoviruses engineered to
36　express cDNA for the reporter fluorescent protein mCherry. Upon visualization, labeled tissues
37　with an optimal rate of fluorescence after infection are subsequently chosen for the
38　implantation in recipient mice. One week prior to the implantation surgery, recipient mice are
39　oophorectomized, and estradiol pellets are placed subcutaneously to sustain the survival and
40　growth of lesions. On the day of surgery mice are anesthetized, and peritoneal cavity accessed
41　through a small (1.5 cm) incision by the *linea-alba.* Fluorescently labeled implants are tweezed,
42　briefly soaked in glue and attached to the peritoneal layer. Incisions are sutured, and animals
43　left to recover for a couple of days. Fluorescence emitted by endometriotic implants is usually
44　non-invasively monitored every 3 days for 4 weeks with an *in vivo* imaging system. Variations in

the size of endometriotic implants can be estimated in real time by quantification of the mCherry signal and normalization against the initial time-point showing maximal fluorescence intensity.

Traditional preclinical rodents of models of endometriosis do not allow non-invasive monitoring of lesion in real time but rather allow evaluation of the effects of drugs assayed at the end point. This protocol allows one to track lesions in real time and is more useful to explore the therapeutic potential of drugs in preclinical models of endometriosis. The main limitation of the model thus generated is that non-invasive monitoring is not possible over long periods of time due to the episomal expression of Ad-virus.

INTRODUCTION:

Endometriosis is a chronic gynecologic disorder initiated by ~~caused~~~~involving~~ the implantation of the functional endometrium outside the uterine cavity. Ectopic lesions grow and induce inflammatory processes ~~provoke~~~~grow causing inflammation of the host tissue maation which~~, ~~inflame~~ leading to chronic pelvic pain and infertility[1]. It is estimated that up to ~~Approximately~~ 10%–15% of women of reproductive age are affected by ~~develop~~ endometriosis[2], and it is present in approximately ~~up to~~ 40-50% ~~of infertile~~of infertile women[3]. Current pharmacological treatments for endometriosis are unable to completely eradicate lesions and not free of ~~ly, there is not an effective pharmacological treatments eradicating for endometriosis that completely obliterates the lesions without causing~~ side effects[4,5]. The research for more efficient therapies requires ~~the use of~~of the refinement of the existing ~~–~~animal models of ~~in~~endometriosis in such a way that ~~which~~ human lesions can be appropriately mimicked, and the effects of compounds on lesion size among others can be closely assessed.

Primate models have been used to mimic endometriosis by implanting ~~induce~~ ectopic lesions ~~that are~~ histologically identical and at similar sites as in human~~s~~ ~~endometriosis~~[6-8]; however, ethical ~~considerations~~concerns and the high economic cost~~s~~ related ~~to experimentation~~to experimentation ~~of~~with ~~experiments using~~ primates limit their use[9]. ~~For this reason~~Consequently, the use of ~~endometriosis models in~~ small animals, especially rodents, for the implementation of in-vivo models of endometriosis continue~~s~~ to be favored as it allows ~~to allow~~ studies with ~~in~~ larger numbers of ~~animals[10]~~individua~~k~~ls[10,11]. Endometriosis can be induced in these animals by transplanting either pieces of rodent uterine horns ("homologous models")[12,13] or human endometrial/endometriotic tissue to ectopic sites (heterologous models)[14]. In contrast to humans, rodents do not shed their endometrial tissue and thereby endometriosis can not be ~~do not~~ develop~~ed~~ ~~endometriosis~~spontaneously in these species. Therefore, homologous mouse models of endometriosis have been criticized due to the fact that implanted ectopic mouse uterine tissue does not reflect the characteristics of human endometriotic lesions[15].

Appropriate physiology of endometriosis can be mimicked in the heterologous models of endometriosis where fresh human endometrial fragments are implanted into immunodeficient animals. In conventional heterologous models, the therapeutic effects of compounds of interest are commonly assessed at the end point by the assessment of lesion size with the use of

89   calipers[16]. An obvious limitation is that, as such, endpoint animal models do not allow studying
90   implantation dynamics or endometriotic lesion development over time. An additional limitation
91   is that the use of calipers does not allow accurate measurements of lesion size. Indeed, the
92   standard error provided by calipers is in the same range (*i.e.*, millimeters) as the size of the
93   lesions implanted in mice, thus restricting the capacity of these tools to detect actual variations
94   in size.

96   In order to overcome such limitations, herein, we describe the generation of a heterologous
97   mouse model of endometriosis in which implanted human tissue is engineered to express a
98   reporter m-Cherry fluorescent protein. Detection of the fluorescent signal with an appropriate
99   image system enables non-invasive monitoring of lesion status with simultaneous
100   quantification of its size in real time. Thus, our model provides clear advantages when
101   compared to conventional endpoint models as it brings the opportunity of real-time non-
102   invasive monitoring and the possibility to perform more objective and accurate estimation of
103   variations in lesion size.

105   **PROTOCOL:**

107   The use of human tissue specimens was approved by the Institutional Review Board and Ethics
108   Committee of the Hospital Universitario La Fe. All patients provided written informed consent.
109   The study involving animals was approved by the Institutional Animal Care Committee at the
110   Centro de Investigacion Principe Felipe de Valencia, and all procedures were performed
111   following the guidelines for the care and use of mammals from the National Institutes of
112   Health.

114   **1)   Endometrial Tissue Collection and Pre-Processing**

116   1.1)   Obtain a good quality biopsy of human endometrial aspirate by using a cannula
117   attached to a suction device. Pour the biopsy into a flask containing 10 mL of sterile saline and
118   wash with gentle manual agitation.

120   Note: Procedures on how to obtain good quality biopsies have been previously described[17].

122   1.2)   Wash the biopsy of any remaining blood or mucus. Repeat the process by pouring the
123   tissue to flasks containing fresh saline as many tines as required until tissue is observed clean.

125   1.3)   Transfer biopsy fragments with a healthy appearance into a solution containing 10 mL of
126   complete Dulbecco's Modified Eagle's medium (DMEM) medium with 10% Fetal Bovine Serum
127   (FBS) and 1% antibiotic-antimycotic solution.

129   1.4)   Pour the content in a 10 cm Petri dish and proceed to chop the tissue into 5-10 mm^3
130   pieces with a pair of scalpels.

132   **2)   Adenoviral Transfection of Endometrial Fragments.**

133

134 Note: All the materials that are going to be employed in the process should be introduced in
135 the hood in advance. Take out everything that is not going to be used in the process and place a
136 flask with bleach. All material that comes into contact with the adenoviral vector must be
137 disinfected with the bleach before discarding it in the biohazard container.

138

139 2.1)   Once the biopsy has been chopped, take a new Petri dish. Pipette multiple 30-50 µL
140 DMEM drops of spread throughout the whole dish. Leave enough space between drops so they
141 do not come into contact.

142

143 2.2)   Aided with a needle attached to syringe, place a single piece of fragment inside each of
144 the medium drops.

145

146 Note: Each drop should contain a single piece of endometrium.

147

148 2.3)   Prepare the Ad-mCherry working solution by diluting 1:20 of the mCherry adenoviral
149 stock solution [1 ·10^{10} ifu/mL]) into DMEM medium WITHOUT antibiotics (DMEM + 10% filtered
150 FBS).

151

152 2.4)   Dispense 100-200 µL of the Ad-mCherry solution per well on a 96-well plate, filling as
153 many wells as fragments are available in the Petri dish drops (step 2.2). In addition, fill at least 3
154 wells with adenovirus free DMEM medium as a negative control.

155

156 2.5)   Aided with a needle attached to syringe, transfer each of the fragments in the Petri dish
157 (step 2.2) to each of the wells containing ad-mCherry solution in the 96-well plate.

158

159 2.6)   Place the 96 well-plate containing the endometrial fragments with Ad-mCherry solution
160 into an incubator at 37 °C with 5% CO_2 for 16 h.

161

162 2.7)   Wash out the remaining adenoviruses in the medium by transferring tissues into a new
163 96-well plate filled with complete DMEM medium (WITH antibiotics-antimycotics, free of
164 adenovirus) and incubate for 37 °C with 5% CO_2 for 24-48 h.

165

166 NOTE: Remember to cover with bleach all well plates and tips that came in contact with Ad-
167 virus before discarding into the biohazard container.

168

169 2.8)   Take out the plate from the incubator and place it under a fluorescence microscope at
170 568 nm (red channel) to test for optimal labeling.

171

172 2.9)   Choose the most brilliant fragments and place them into a new 96-well plate with fresh
173 complete DMEM medium (WITH antibiotics-antimycotics, free of adenovirus).

174

175 2.10)   Seal the plate well with a plastic paraffin film and transport it to into the specific-
176 pathogen-free animal area for implantation of endometrial fragments into recipient animals.

177
178 **3)   Generation of the Endometriosis Mouse Model**
179
180 NOTE: Use 6-8-week-old athymic nude (or similar immunocompromised strains) female mice
181 housed in specific pathogen–free conditions, as recipient animals. To avoid hormonal cycle-
182 dependent variations and simultaneously fuel lesion growth with estradiol, animals are
183 ovariectomized and placed with 60-day release capsules containing 18 mg of 17 βeta-Estradiol
184 (17β-E$_2$). Oophorectomy and pellet placement have to be performed at least one week in
185 advance of grafting endometrial fragments into the recipient animals.
186
187 **3.1)   Oophorectomy**
188
189 Note: Prepare surgical sterilized material in a hood. Prepare anesthesia equipment and a post-
190 surgical recovery zone ready in the same room.
191
192 3.1.1) Perform a subcutaneous injection of morphine derivative at a dose of 5 mg/kg per
193 mouse. Let the mice rest for 30 min after injection so as analgesics effects of drug can be
194 manifested.
195
196 3.1.2) Connect the inhalation anesthesia equipment and let oxygen and isoflurane (2% mg/kg)
197 flow for a few min into a sealed anesthesia chamber.
198
199 3.1.3) Introduce the animal into the isoflurane anesthesia chamber. Wait for 3-5 min and
200 check that animals are fully anesthetized by pressing one of its paws. Transfer the animal to the
201 surgery area and maintain anesthesia by placing a mask with continuous flow of isoflurane gas
202 covering the respiratory airways.
203
204 3.1.4) After disinfecting the area with chlorohexidine, perform a transverse 0.5 cm costal
205 incision approximately at the height of the hip with sharp scissors.
206
207 3.1.5) Separate the skin from the muscle to get access to the abdominal cavity, identify the
208 white fat pad that surrounds the ovary and retract the ovary with dissection forceps.
209
210 3.1.6) Tie a knot around the oviduct with absorbable suture and tighten it to ensure
211 appropriate hemostasis before excising the ovary.
212
213 3.1.7) Close the muscular layer with 6-0 absorbable suture, and then close the skin with 6-0
214 non-absorbable suture. Clean the area again with antiseptic solution.
215
216 3.1.8) Repeat the procedure to remove the contra lateral ovary.
217
218 **3.2)   Estradiol pellet implant**
219
220 Note: Take care that the animal is anesthetized during the oophorectomy surgery to place

221   pellets at that point.

222

223   3.2.1)   Immediately after the completion of the oophorectomy procedure, clean the skin with
224   antiseptic solution surrounding the neck and make a transverse subcutaneous small (0.5 cm)
225   incision with sharp scissors in the nape.

226

227   3.2.2)   Use the scissors to dissect the skin from the muscle, making a pocket large enough to
228   allow placing the pellets.

229

230   3.2.3)   Insert the pellet containing 18 mg of 17β-E$_2$ and suture the skin with a 6-0 non-
231   absorbable suture. Clean the area again with antiseptic solution.

232

233   3.2.4)   Place the animal in the recovery zone and administer an optimal dose of long-lasting
234   analgesia to ease the recovery period.

235

236   **3.3)** ==**Endometrial implant surgery**==

237

238   NOTE: Allow at least a period of seven days quarantine to allow full recovery of animals after
239   oophorectomy before starting endometrial fragment implantation surgery. For optimal
240   synchronization with labeling of tissue, collect the biopsy 2-3 days before implantation surgery
241   so as to avoid long-term culture of explants.

242

243   3.3.1)   Get the surgical room in the specific pathogen free zone ready in advance. Prepare the
244   hood with all required surgical material, the anesthesia equipment and the post-surgical
245   recovery zone also.

246

247   3.3.2)   Bring the animals to the room, perform a subcutaneous injection of a morphine
248   derivative at a dose of 5 (mg/kg) in each mouse. Let the mice rest for 30 min after injection so
249   analgesics effects of drug can be manifested.

250

251   3.3.3)   Connect the inhalation anesthesia equipment and let oxygen and isoflurane (2% mg/kg)
252   flow for a few min into a sealed anesthesia chamber.

253

254   3.3.4)   ==Before starting surgery, move the plate containing fluorescently labeled fragments==
255   ==(from step 2.10) into the hood, unseal it and pour the fragments into a Petri dish for easier==
256   ==handling==.

257

258   3.3.5)   Introduce the animal into the isoflurane anesthesia chamber. Wait for 3-5 min and
259   check that animals are fully anesthetized by pressing one of its paws. Transfer the animal to the
260   surgery area and maintain anesthesia by placing a mask with continuous flow of isoflurane gas
261   covering the respiratory airways.

262

263   3.3.5)   ==Place the anesthetized animal face up. Disinfect the ventral area. Perform a longitudinal==
264   ==1.5 cm incision in abdomen with sharp scissors and separate the skin from the muscle. Then,==

265 perform a longitudinal 1.5 cm incision in the muscle to access the peritoneal cavity.
266
267 3.3.6) Hold the left edge of the abdomen muscular wall with mini-forceps and fold it trying to
268 expose the inner face of the peritoneum on the outside.
269
270 3.3.7) Take an endometrial implant with mini tweezers, soak it briefly in an n-butyl-ester
271 cyanoacrylate adhesive and place it to the peritoneum where it will get attached. Let it dry for a
272 few seconds. Repeat steps 3.3.6 and 3.3.7 to place an implant on the contralateral side of the
273 peritoneum.
274
275 3.3.8) Close the muscular layer with an absorbable 6-0 suture, and then close the skin with a
276 non-absorbable 6-0 suture. Clean the area again with antiseptic solution.
277
278 3.3.9) Place the animal in the recovery zone and administer an optimal dose of long-lasting
279 analgesia.
280
281 **4)**   ***In Vivo* Fluorescent Imaging with an *in vivo* imaging system**
282
283 4.1)   Turn on the *in vivo* imaging system device, initialize the program and allow the CCD
284 camera to cool down for a few min.
285
286 4.2)   Prepare the inhalation anesthesia equipment. Open the isoflurane flow at 2% for a
287 couple of min to fill the anesthetic chamber. Prepare the post-anesthesia recovery zone.
288
289 4.3)   Once the program has started and the CCD camera have cooled down, click the **Imaging**
290 **Wizard** tool: A tutorial starts with a series of consecutive windows displayed, each one
291 corresponding to a parameter of interest with several options available to be chosen by clicking
292 in the corresponding box. Move forward through the tutorial by selecting the appropriate boxes
293 in each window and click the **OK** button to move to the next set of parameters with the
294 following sequence.
295
296 4.3.1.) Select the **Epiluminescence box** for the fluorescence parameter, select the **mCherry box**
297 for the filter pairs parameter. Check the **Photograph mode** and **Confirm Focus** boxes and select
298 the Automatic box for the exposure parameter.
299
300 4.4)   Once the instrument has been set up, move one animal inside the anesthesia chamber.
301 When fully anesthetized, transfer the animal inside the *in vivo* imaging system cage and place it
302 side up with its head inside a tubule connected to the anesthesia machine. Close the lid and
303 click **Acquire** for monitoring.
304
305 4.5)   Acquire the images appearing (a total of five images, one image for each pair of filters
306 selected) and save data by clicking the **Save As** button. Move the mouse to the area of post-
307 anesthesia recovery. Repeat the process with the remaining animals.
308

309   4.6)   Repeat monitoring two or three times a week to follow-up the signal appropriately
310   during the time course.
311
312   4.7) Proceed to sacrifice the animal at the end of the time course by CO_2 asphyxiation.
313

314   **5)**   ==**Quantification of *In Vivo* Fluorescence Images**==
315

316   **5.1)**   **Segregation of actual fluorescence through image unmixing:**
317

318   5.1.1)   Open the ***In Vivo* Imaging Analysis Coupled Software Program**.
319

320   5.1.2)   Choose the "Sequenceinfo" file to start the analysis. Two windows will appear:
321   "Sequence view" and "Tool Palette". Choose **Tool Palette** and once the menu has been
322   displayed select the following options.
323

324   5.1.3)   Select **Corrections** and click on the box **Adaptive FL Background Subtraction** to remove
325   the undesirable fluorescent signals from the luminescent image data. Choose the threshold of
326   greatest interest and click **Set**.
327

328   5.1.4.) Click **Spectral Unmixing**, select the wavelengths of interest, the method chosen for
329   unmixing (library, guided, automatic or manual) and then click **Start Unmix**.
330

331   5.1.5)   Select the **Unmixed** image corresponding to mCherry signal and double click. A new
332   window will appear with the final image of the signal of interest.
333

334   5.1.6)   Repeat step 5.1.3.
335

336   5.1.7)   OPTIONAL: If a representative image (JPEG) of the unmix result is needed, choose the
337   desired settings in **Tool Palette | Image Adjust** (color table, binning, contrast, *etc*.), and then
338   click on **Export Graphics** in the unmix window to export the current image view as an image.
339

340   5.1.8)   Save the unmixed file: **File | Save As | Choose Folder** and **Ok**.
341

342   5.1.9)   Repeat the process with the rest of the monitoring days and with all animals.
343

344   ==**5.2)**   **ROIs set up and signal quantification**==
345

346   ==5.2.1)   Click **Browse** and select the **Unmixed file** (see step 5.1.8) of interest to be analyzed. A==
347   ==new window will appear.==
348

349   ==5.2.2)   Click **Add To List** to include all the unmixed files from each animal at different time==
350   ==points and then click on **Load As A Group**. All images must appear as a single sequence.==
351

352   ==5.2.3)   Go to **Tool Palette** window: Click off the box **Individual** scale to obtain all images on the==

353 same scale.
354
355 5.2.4) Double click in one image of the sequence and create a ROI on the zone of interest using
356 the following sequence. Go to **ROI Tools** and select **Countour** and **Auto 1** option, click on the
357 circle shape appearing, place it on the center the fluorescent signal and then click **Create** on the
358 displayed window.
359
360 Note: This automatically highlights pixels with an intensity of fluorescence above background
361 values (*i.e.*, lesion) and generates a shape whose area embraces the outlined pixels.
362
363 5.2.5) Copy the created ROI shape and paste on a background zone where there is no signal.
364
365 5.2.6) Click on **Measure ROIs | Select All** to display values of fluorescence intensity. Proceed to
366 select data values with the mouse, click right button, press copy and paste on a spreadsheet.
367
368 **6)   Data (fluorescent signal) Normalization**
369
370 6.1)   Select the initial time point at which signal intensity is maximal. Proceed to normalize
371 signal at each time point by using the formula:
372
373 Signal intensity at each time point / Maximal signal intensity observed during the time course) x
374 100.
375
376 **REPRESENTATIVE RESULTS:**
377 Here, we describe the process for creating a heterologous model of endometriosis in which the
378 architecture of lesions is preserved by implanting fluorescently labeled pieces of human
379 endometrium into immunocompromised mice, thus allowing non-invasive monitoring of lesion
380 progression. Labeling of endometrial fragments is achieved by infection with adenovirus
381 engineered to express mCherry, a protein emitting fluorescence in the near infrared region. In
382 **Figure 1,** we show representative images of human endometrial fragments infected with Ad-
383 mCherry observed under the fluorescence microscope. For illustrative purposes, both labeled
384 and non-labeled fragments are included so differences in fluorescence between infected and
385 non-infected tissues (autofluorescence) can be noted. During monitoring, in addition to the
386 reference wavelength for mCherry, fluorescent images are taken with different pairs of
387 excitation/emission wavelengths filters (**Figure 2**) to define the characteristic fluorescent
388 emission profile of tissues. The purpose of this action is to "unmix" actual fluorescence emitted
389 by lesions from background and autofluorescence emitted by host tissues and scar originated
390 during surgery respectively. An illustrative example of the unmix process is shown in **Figure 3**.
391 Estimations of variation in lesion size is performed by quantifying and normalizing fluorescent
392 signaling emitted by lesions during the time course. For this purpose, images of monitoring
393 containing raw fluorescence emitted by animals during each time point are first brought
394 together unnormalized (**Figure 4**) in a single file. Subsequently fluorescence is unmixed,
395 normalized and represented as a false color image (**Figure 5**). Finally, ROIs corresponding to
396 specific lesion and background signaling are automatically recognized by the program and

397 quantified (**Figure 6**). Background ROI signaling is subtracted from lesion ROI signaling and
398 results of intensity in each time point are normalized against the time point at which intensity is
399 maximal (**Figure 7**). At the end of the monitoring process, several weeks after surgery mice are
400 sacrificed and viable implant can be recovered attached to the mouse peritoneum (**Figure 8**).
401
402 **FIGURE AND TABLE LEGENDS:**
403
404 **Figure 1: Visualization of endometrial fragments with fluorescence microscope after Ad-**
405 **mCherry infection A)** Human endometrial fragment incubated with Ad-mCherry at 37 °C and
406 5% CO_2 during 24 h as a positive sample. **B)** Human endometrial fragment incubated at 37 °C
407 and 5% CO_2 without Ad-mCherry as a negative control sample.
408
409 **Figure 2: Raw imaging fluorescence emitted by labeled fragments implanted in mice.** Picture
410 shows representative imaging of raw fluorescence signal emitted by the same animal at a
411 specific time point. Images correspond to screenshots obtained using software coupled to an *in*
412 *vivo* imaging system device during a monitoring session. Each panel containing mice (numbered
413 1 - 5 in the left corner) corresponds to the fluorescence observed by using a different specific
414 excitation/emission pair filter for acquiring images. Panel on the right (tool palette) show
415 fluorescence parameters selected for acquisition of images
416
417 **Figure 3: Unmixing of background vs specific fluorescence emitted by lesions.** Picture shows
418 representative images of the unmixing process performed to dissect actual fluorescence from
419 lesions using the *in vivo* imaging system coupled software. Graph on the left panel denote
420 normalized specific profiles of fluorescence emission by scar (green line, UMX1), lesions (red
421 line, UMX2) and host tissue (blue line, UMX3 panel). Fluorescence intensity at different
422 emission wavelengths (X-axis) is represented in units of radiant efficiency (Y-axis). Just note
423 how each specific structure (*i.e.*, scar, labeled lesions and host tissue) emits a different
424 fluorescence profile which allows identifying and segregating them specifically form each other.
425 On the right panel, fluorescence arising from launching specific emission profiles for scar
426 (UMX1), lesions (UMX2) and host tissue (UMX3) are shown superposed on photograph images
427 of mice. A composite image (Composite) is also included for illustrative purposes to denote the
428 segmentation of fluorescence emitted by lesions from that emitted by scar or host tissues.
429 Middle panel shows parameters selected for unmixing with the image software coupled to the
430 in-vivo imaging device
431
432 **Figure 4. Time course monitoring of raw fluorescence emitted by lesions.** Panel shows
433 representative images of raw fluorescence emitted by a single mouse implanted with labeled
434 human lesions (brilliant yellow spots) during the time course. Time points after surgery at which
435 monitoring was performed are denoted as "Day (number)". Emission ad excitation pair filters
436 used for monitoring are indicated in each panel/image. Each panel is identified by a specific
437 code (BKG) in the upper part containing info related to the date at which fluorescence was
438 acquired
439
440 **Figure 5: Time course monitoring of normalized fluorescence emitted by lesions.**

441 Representative images corresponding to unmixed, normalized fluorescent signaling emitted by
442 human lesions (spots with rainbow color) superposed in a single mouse during the time course
443 (days after surgery). Time points after surgery at which monitoring was performed are denoted
444 as "Day (number)". Each panel is identified by a specific code (BKG) in the lower part containing
445 info related to the date at which fluorescence was acquired. Rainbow palette color on the right
446 side identifies fluorescence intensity (Radiant efficiency) emitted by lesions at each time point.
447 Note how strong fluorescence intensity during the initial time points (*i.e.*, red color in the
448 center of lesions on days 1,5 and 8) declines during the time course (*i.e.,* blue color in lesions on
449 days 20 and 25).
450
451 **Figure 6: Use of ROIs for quantification of fluorescence intensity in lesions during the time**
452 **course.** Figure shows panel of images of normalized fluorescence emitted by lesions in a single
453 mouse during the time course (*i.e.,* **Figure 5**) with the addition of ROIs (delineating lesions and
454 background) for quantification of fluorescence intensity. ROI 1 and ROI 2 identify the amount of
455 fluorescence emitted by each of the two lesions during the time course. BKG identify the
456 amount of fluorescence emitted by the host tissue (background fluorescence) during the time
457 course. Background fluorescence is subtracted from ROIs for quantification purposes. Time
458 points after surgery at which monitoring was performed are denoted as "Day (number)".
459 Images acquired at each time point are labeled with a specific code (BKG) at the bottom of each
460 one containing info related to the date at which fluorescence was acquired (first eight digits
461 following BKG detail data for year(2014)-month (10) and -day(10 to 31)), an individual
462 identification code (last 6 digits) and the specific profiles of fluorescence emission used for
463 unmixing (UMX2) Rainbow palette color on the right side provides a visual scale of fluorescence
464 intensity (Radiant efficiency) emitted by lesions at each time point. Note how fluorescence
465 intensity values in the two lesions (ROI1 and ROI2) are higher at the initial time points (days 1
466 and 5) and decays during the time course to reach the lowest values at the end time points
467 (days 20 and 25).
468
469 **Figure 7: Normalization of fluorescence intensity during the time course.** Table in the upper
470 part shows illustrative example of the values of fluorescence intensity (radiant efficiency)
471 emitted by two mCherry labeled lesions (ROI1 and ROI2) implanted in a mouse (R25).
472 Monitoring of fluorescence was performed at different days after surgery (D5-D25) during the
473 time course. D5- Graph at the bottom illustrates typical pattern of normalized fluorescence
474 emitted by lesions infected with mCherry decaying during the time course. Y-axis shows values
475 of fluorescence normalized to express the percentage of decay by using the formula (Signal
476 intensity at each time point / Maximal signal intensity observed during the time course) x 100.
477 Time points (Days (Dx) after implanting surgery) at which fluorescence was monitored are
478 indicated in the X-axis. Note initial increase of signaling during the first 24 hrs after surgery,
479 corresponding to stabilization of lesion, the peak in fluorescence intensity around D1-D5 and its
480 subsequent decay due to episomal expression of mCherry during the time course
481
482 **Figure 8: Macroscopic appearance of implanted endometriotic lesions.** Representative images
483 showing macroscopic appearance of endometriotic lesions implanted in mice at the end of the
484 monitoring process upon sacrifice

485
486 **DISCUSSION:**
487 The protocol herein detailed describes the implementation of an animal model of
488 endometriosis in which the architecture of implanting lesions architecture is preserved whilst
489 simultaneously allowing real time assessment of fluorescence emitted by mCherry labeled
490 endometrial tissue. In this protocol, we describe the use of a specific *in vivo* imaging system and
491 related software to non-invasively assess fluorescence emitted by the labeled lesion. Each user
492 should adapt the protocol depending on the specific imaging device and related software
493 available at their institution. Monitoring is performed in anesthetized animals through an
494 isoflurane gas anesthesia machine coupled to an *in vivo* imaging system. To avoid interference
495 with auto-fluorescence emitted by wounds, it is recommended to start monitoring lesions
496 fluorescence at least three days after implantation surgery.
497
498 Heterologous mouse models of endometriosis similar to the one herein shown have been
499 previously described consisting in the implantation endometrial fragments labeled with green
500 fluorescent protein (GFP)[18,19]. The use of mCherry as a reporter for tagging the human tissue
501 provides however an advantage over GFP because of the enhanced tissue penetration of the
502 former. Due to its larger emission spectrum and higher photostability mCherry emits a brighter
503 signal that is more appropriate for the visualization of intraperitoneal fragments.
504
505 Due to its small size adenoviruses are the vectors of choice for infection (*i.e.*, labeling) of whole
506 tissue pieces as those provide acceptable diffusion through 3D structures. Even with that the
507 percentage of tissue infected cells is not higher than 30-35%. Thus, the limitation of this model
508 relies on the inefficient labeling of tissue and additionally the transient expression achieved by
509 adenoviruses. Indeed, fluorescence cannot be monitored beyond 4–6 week as it fades
510 progressively due to the episomal transient expression of the Ad-virus. Efficiency of labeling
511 and the period of monitoring might be increased by disrupting the donor tissue and infecting
512 isolated single epithelial/stromal cells with ad-virus previous to being injected into recipient
513 mice[19,20]. Such an approach, however, reduces the extent at which the animal model mimics
514 the physiology of endometriosis provided that ectopic human lesions do not consist in a
515 disorganized accumulation of single epithelial/stromal cells but rather in well-structured
516 endometriotic tissue.
517
518 In this protocol, the most critical step is the labeling of the tissue and most specifically the
519 determination of the appropriate concentration of ad-virus required for optimal infection.
520 Indeed, the $1x10^{10}$ pfu/mL concentration pointed out in the protocol is mostly an
521 orientative/consensus figure based on our experience. The optimal concentration might differ
522 in each experiment depending on the type and quality of the biopsy and/or how quickly this is
523 processed. We thus suggest testing at least three different (two-fold) titers in each experiment
524 and choosing the one providing optimal labeling based on visualization under the fluorescence
525 microscope.
526
527 Our protocol/model is useful to study the mechanisms implicated in the establishment and
528 early development of the endometriotic lesions. In spite of the period of time of monitoring is

constrained, the model is still useful to study and detect the effects of pharmacological compounds able to exert dramatic effects on lesion size in a short period of time such as antiangiogenic or antiestrogenic drugs. The development of more efficient and durable methods of labeling human tissue are expected to spread non-invasive monitoring as a consolidated technique to explore the potential therapeutic of a wider range of drugs in preclinical model endometriosis.

DISCLOSURES:
The authors have nothing to disclose.

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