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Subcutaneous Injection of Human Colorectal Cancer Cells in Athymic Nude Mice to Evaluate Antitumor Efficacy

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Abstract

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide, highlighting the urgent need for improved preclinical models to evaluate potential therapies. Animal models are essential for elucidating tumor biology and assessing novel therapeutic interventions. Mouse models offer key advantages over existing models, including genetic manipulability, rapid reproduction, cost-effectiveness, and the availability of immunocompetent and immunodeficient strains, enabling precise modeling of human cancers.

This protocol focuses on the subcutaneous induction of the bioluminescent colorectal cancer cell line HCT-116 in an immunodeficient murine model, followed by non-invasive bioluminescence imaging using the IVIS system and caliper to monitor tumor progression and growth. Using luminescent cells provides a real-time, quantifiable approach for tracking tumor development and response to therapeutic interventions, reducing the need for invasive procedures. A cohort of mice (n = 12) is required for tumor cell implantation, and luminescence intensity is measured to establish a standardized model for therapeutic testing. Tumor volume is typically calculated using only length and width measurements or width.

The current methodology ensures consistent tumor establishment while minimizing° variability, ultimately enhancing the reliability of preclinical assessment. The findings of this study will contribute to refining tumor models for drug testing and improving the translational potential of preclinical research in clinical applications. By employing advanced imaging techniques and a reproducible experimental design, this study aims

to improve the accuracy and efficiency of *in vivo* drug evaluation for colorectal cancer treatment.

Introduction

Colorectal cancer (CRC) is a major global health burden, ranking among the most prevalent and deadly malignancies[1]. Developing reliable preclinical models is essential for evaluating new therapeutic approaches, as traditional *in vitro* assays fail to fully capture the complexity of tumor growth and drug response in a living system. *In vivo* models, particularly murine models, have been used to provide valuable insights into tumor biology, allowing researchers to assess the efficacy of potential treatments in a controlled environment.

The use of luminescent CRC cells in murine models represents a significant advancement in preclinical research, enabling real-time, noninvasive monitoring of tumor progression[2]. This study aims to establish a standardized protocol for subcutaneous tumor induction and IVIS imaging to track tumor growth dynamically. A subcutaneous injection model was selected because of its technical simplicity, reproducibility, and ease of longitudinal tumor monitoring. By leveraging bioluminescence imaging, researchers can obtain precise, quantifiable data on tumor development and therapeutic responses without the need to euthanize animals at multiple time points[3].

The IVIS imaging system is a powerful tool for assessing tumor burden with high sensitivity and specificity, enhancing the reproducibility and accuracy of *in vivo* studies. This study will serve as a foundation for future therapeutic evaluations, providing a standardized and efficient approach to preclinical drug testing for colorectal cancer treatment. By refining tumor induction methods and implementing advanced imaging techniques, this research contributes to bridging the gap between preclinical and clinical applications in oncology[4].

Protocol

Animals were maintained under specific pathogen-free conditions according to the guidelines established by the *Instituto Murciano de Investigación Biosanitaria* (IMIB, Murcia) ethical committee (No A13240502). Inspections were conducted weekly by qualified personnel in compliance with R. D. 1201/2005.

1. Animals

NOTE: All animals must be securely and safely restrained before injection. If working with immunodeficient mice (i.e., nude athymic mice such as Crl: NU(NCr)-Foxn1nu), it is essential to maintain sterility. The current study utilized 12 athymic nude mice, 5 weeks old (20-22 g for females and 22-26 g for males) at the beginning of the experiment (50% males). All anesthesia and experimental procedures were conducted in the *Guía de Ética y Buena Práctica en Investigación* of the center, with approval from the institutional ethics committee (Protocol No. A13240502) and in compliance with Spanish legislation (R.D. 1201/2005).

1. Before initiating the assay, acclimate the mice, aged 4 weeks, to the animal center for approximately 1 week. Subsequently, carry out the following procedures.

2. D-luciferin stock preparation

1. Prepare a 15 mg/mL working solution of D-Luciferin Potassium Salt Bioluminescent Substrate in prewarmed Phosphate Buffered Saline (PBS) in a conical tube inside a biosafety cabinet. Invert the tube several times to homogenize the solution well.
2. Transfer the solution through a 0.2 µm syringe filter for sterilization.
3. Aliquot the sterilized solution in 1 mL opaque microtubes and store at -20 °C.
   NOTE: Luciferin is light-sensitive.

3. Extracellular matrix preparation

1. Thaw phenol-red-free commercial extracellular matrix (ECM) hydrogel overnight on ice inside a refrigerator between 2 °C and 8 °C[5].
2. On the day of injection, keep the ECM on ice and, using a prechilled P1000 micropipette, aliquot 200 µL of ECM into sterile tubes.
   NOTE: It is recommended to use wide-bore microtubes. Minimize temperature changes and avoid repeated freeze-thaw cycles.
3. Prechill the syringes and needles on ice before preparing injections to prevent sudden temperature changes of the ECM.

4. Cell detachment and counting

1. Plate HCT-116 bioluminescent cells (ATCC cell line CCL-247-LUC2) in T75 flasks a few days before tumor induction to ensure sufficient cell number. Maintain cell culture at 37 °C, 5% CO_2, and replace medium if necessary[6].
2. On the day of the experiment, aspirate the culture medium carefully to avoid disturbing adherent cells.
3. Wash the cells with 8 mL of sterile PBS (prewarmed at 30 °C in a water bath) to remove residual serum.
4. Add 2 mL per flask of warm Trypsin-EDTA (0.05%) and incubate at 37 °C for 4-5 min. Monitor cell detachment under an inverted microscope. If cells remain attached, gently tap the side of the flask to aid detachment.
5. Neutralize trypsin by adding 10 mL of complete growth medium containing fetal bovine serum (FBS).
6. Transfer cells into a 15 mL conical tube using a 10 mL serological pipette. Rinse flasks with an additional 2 mL of fresh medium to collect any remaining cells and add the washes into the 15 mL tube.
7. Centrifuge the cell suspension at 200 × *g* for 5 min at room temperature. Carefully aspirate the supernatant without disturbing the cell pellet.
8. Gently resuspend the cell pellet in 3 mL of seeding medium to achieve a homogeneous suspension.
9. Count the cells in a Neubauer chamber or an automatic cell counter. Determine cell viability by mixing 10 µL of the cell suspension with 10 µL of Trypan blue and count viable cells under an inverted microscope.
   NOTE: Prepare a sufficient number of cells for the study: at least 3×10^6 viable cells per mouse.
10. Resuspend the cells in sterile PBS (prewarmed at 30 °C in a water bath) and modify the concentration of viable cells to a final concentration of 2×10^7 cells/mL with PBS. Prepare aliquots of 200 µL.

5. Set up equipment items and anesthetize animals.

1. Turn on the imaging system at least 4 h before imaging.
   NOTE: Follow the system's instructions to allow the initialization step, including adjusting settings for light intensity and camera exposure time depending on signal-to-noise ratio. This setting must be set manually based on the experimental conditions.
2. Weigh the animals and calculate the volume of D-luciferin that must be administered (200 µL of a 15 mg/mL solution per 20 g of the animal).
3. Clean and disinfect the imaging chambers and cabinet.
4. Identify and safely restrain each mouse for anesthesia.
   NOTE: Wear two pairs of gloves to protect hands from accidental needle sticks during the subcutaneous injection.
5. Anesthetize mice with 2% isoflurane in oxygen at 1.8-2.0 L/min flow. Turn on the suction pump simultaneously with the anesthesia pump.

6. Preparation of the cells for injection

1. Mix 200 µL of 2 × 10^7 bioluminescent CRC cells/mL with 200 µL of ECM in a cryovial inside a biosafety cabinet. Gently pipette with a P1000 pipette to avoid air bubble formation and dissociation.
2. Transfer the 400 µL mixture to a 1 mL syringe, remove air bubbles, and proceed immediately to injection.
3. Safely uncap the needle, always maintaining the sterility of the needle.
   NOTE: It is recommended to use individual needles for each animal to minimize contamination. A new needle is used for each mouse to prevent cross-mouse infection.
4. Attach a 25 G needle and adjust the syringe content to 200 µL, removing any air bubbles.
   NOTE: Injections are usually done with 200 µL, leading to 2 × 10^6 cells per injection.
5. Slide the needle back into the cap loosely without handling either the cap or the needle while the animal is restrained.

7. Subcutaneous tumor induction

1. Remove the mouse from the chamber once it is anesthetized and place the animal's snout in an outlet supplying the same anesthetic.
   NOTE: In our case, the nozzle was made using a glove.
2. Place the animal on its right side on a flat surface. Using the tips of the fingers of the non-dominant hand, gently pinch and lift the animal's skin in the lower left quadrant.
3. Before injecting, aspirate first to ensure the appropriate placement of the needle. Insert the needle bevel-up with the dominant hand, two-thirds into the lifted skin, parallel to the body, being very careful not to prick fingers or puncture through the double gloves.
   NOTE: Proper placement should yield negative pressure and no aspiration in the hub of the needle.
4. Inject the contents of the syringe (200 µL) into the lifted skin, right between the two fingers. Hold briefly before withdrawing the needle slowly. Remove the needle after the subcutaneous injection and discard it safely into a Sharps container.

8. Intraperitoneal injection of D-luciferin

1. Before returning the animal to the anesthesia chamber, administer D-luciferin intraperitoneally (IP) at a concentration of 150 mg/kg of body weight with the 15

mg/mL stock solution in a 30 G insulin syringe (200 µL per 20 g of the animal).

NOTE: Up to 0.5 mL injection is well tolerated.

2. Manually restrain, dorsal recumbence (abdomen side up), with the cranial (head) end of the animal pointed down. Allow the intestinal contents to move downward.
3. Keeping the needle bevel-side up and slightly angled at 15-20°, push the needle into the abdominal cavity so that the tip of the needle just penetrates the abdominal wall of the animal's left lower abdominal quadrant.
4. Slowly inject the D-luciferin into the intraperitoneal cavity, remove the needle, and discard it safely.
5. Place the mouse in the anesthesia chamber and allow it to stabilize for approximately 5 min. After 15 min, proceed with *in vivo* imaging.

   NOTE: Construct a D-luciferin kinetic curve for each new animal model to determine peak signal time. In our case, the light emission peaked at approximately 15 min in *in vivo* imaging.

9. *In vivo* imaging analysis

1. Place the fully anesthetized animal in the chamber of the imaging system, on the right flank. Ensure the animal is properly aligned with the camera and the light source to capture the tumor area.
2. Open the linked software. Choose the visual or **photographic imaging mode** to confirm the position of the animal. Save the data and change the folder´s name.
3. Choose the **bioluminescence imaging mode** and set it to **automatic**. Capture bioluminescence images and monitor the tumor light on the computer screen.
4. Once the imaging is complete, return the animal to its cage and observe any complications.
5. Perform postprocessing, first scale the bioluminescent light so that all the mice tumors show the same scale, usually between $1e^6$ and $1e^8$.
6. Set up a Region of Interest (ROI) around the tumor for analysis and copy it for further analyses. Measure the light of the ROI and export these results for further analysis. Paste previously created and copied ROI of the same size for subsequent tumors.
7. Shut down the system according to the manufacturer's instructions and clean the imaging chamber. Turn off the extraction and anesthesia pumps, together with the oxygen flow.

10. Tumor volume measurement

1. Anesthetize animals with isoflurane for tumor measurement. Place the animal in a stable position and cover its head. Quickly immobilize the animal.
2. Measure tumor length, width, and depth using a calibrated digital caliper.

   NOTE: Perform the measurements quickly, as the animal will begin to awaken approximately 1 min after removal from the anesthesia chamber.
3. Repeat measurements and calculate an average.
4. Calculate the volume according to the formula for spherical or elliptical tumors:

For spherical tumors

$$Tumor\ volume\ (cm^3) = \frac{1}{2}(length \times width^2) \times \frac{1}{1000} \quad (1)$$

For ellipsoid tumors

$$Tumor\ volume\ (cm^3) = \frac{4\pi}{3} \times \left(\frac{Length}{2}\right) \times \left(\frac{Width}{2}\right) \times \left(\frac{Depth}{2}\right) \times \frac{1}{1000} \quad (2)$$

NOTE: To minimize measurement bias, all tumor measurements within the study should be performed by the same operator. Additionally, it should be noted that on the day of tumor induction, accurate measurement is often challenging because the tumor mass is not yet fully solidified.

Representative Results

This protocol allows for the introduction of human cells into an immunodeficient mouse model, enabling the growth of these tumoral cells *in vivo*, which is always more physiologically relevant than *in vitro*. It is crucial to ensure that the experimental conditions are as reproducible as possible, that tumors contain a similar number of viable cells, and that they are implanted in a comparable and consistently accessible location.

To test the sensitivity of the bioluminescent HCT-116 cell line *in vivo*, 2×10^6 cells were subcutaneously injected into nude mice (n = 12). The optimized protocol facilitates the homogeneous injection of a viable number of cells, both in terms of emitted light and tumor volume ($P > 0.05$) (**Figure 1** and **Figure 2**). Bioluminescence imaging enables sensitive real-time imaging of small tumor lesions (**Figure 1A**) with homogeneous signals (**Figure 1B**). Bioluminescence measurements also showed a linear correlation between signal emission and tumor volume ($R = 0.81$, $P = 0.0013$) (**Figure 1C**).

To determine the most appropriate method for estimating tumor volume, we assessed two combinations of linear dimensions (d1, d2, d3) (**Equations 1** and **2**, **Figure 2A**). The theoretical volume of a spherical tumor may be calculated using **Eq. (1)**, based on the single longest diameter, whereas the volume of ellipsoidal tumors can be estimated employing **Eq. (2)** (**Figure 2B**). **Eq (1)** does cause an overestimation of tumor volume since it does not consider any changes in depth, which is smaller than the width (**Figure 2B**)[2]. In this regard, our optimization has been extensively performed in multiple studies, allowing us to monitor tumor light and growth over several weeks.

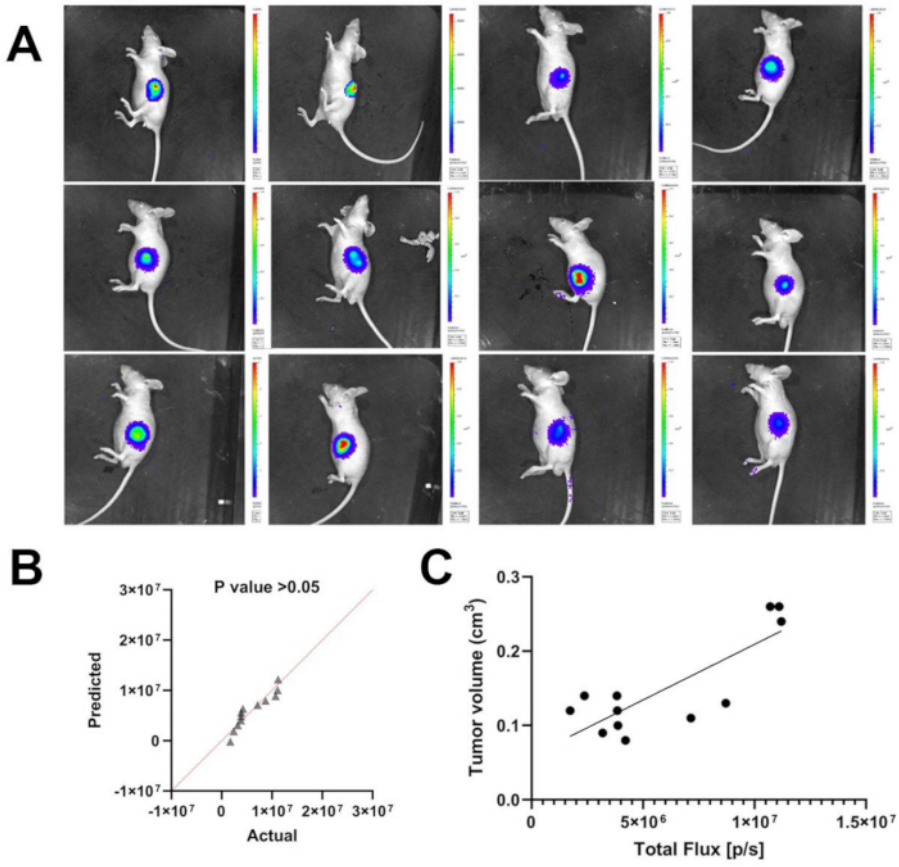

Figure 1: Title needed. (**A**) A total of 2 × 10^6 HCT-116 BioLuc cells were injected into the left flank, and mice were imaged for bioluminescence with the imaging system (n = 12). Bioluminescence values are indicated in photons/s (minimum, 1 × 10^5 p/s; maximum, 1 × 10^7 p/s). (**B**) Bioluminescence data passed the Kolmogorov-Smirnov normality test (P > 0.05). (**C**) Bioluminescence measurements are plotted against tumor volume measured by caliper with **Equation 1**. The line represents the ideal correlation between both measurements. Please click here to view a larger version of this figure.

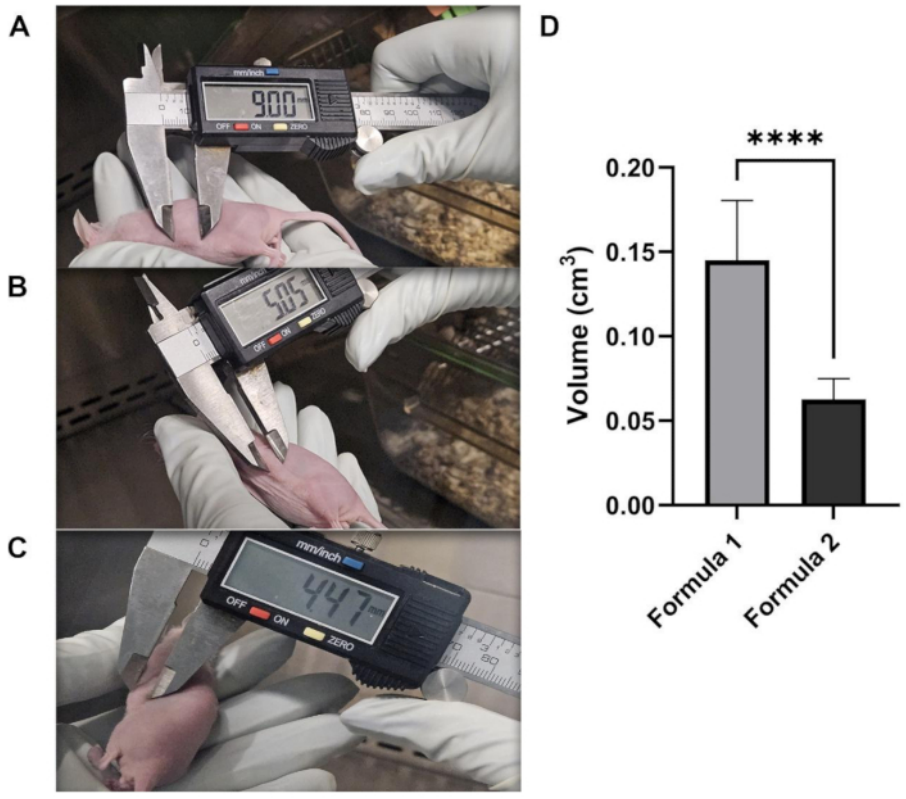

Figure 2: Subcutaneous injection of HCT-116 BioLuc cells into the left flank of nude mice (n = 12) to induce tumor growth. (**A-C**) Xenografts of HCT-116 BioLuc tumors in nude mice were measured by a digital caliper. (**B**) Tumor volume (cm^3) was calculated with a digital caliper using two mathematical equations. In **Eq. 1**, the tumor volume of the spheroid can be calculated by substituting the depth for the width. The second mathematical equation for ellipsoid volume estimation is (4/3 × π × (x × y × z)/2) / 1000, where length, width, and depth represent diameters in the x, y, and z axes. Please click here to view a larger version of this figure.

Discussion

The protocol for the subcutaneous induction of luminescent colorectal cancer cells in an athymic nude mouse model presents a standardized and reproducible approach for tumor formation and real-time monitoring. First, it is important to note that such studies are typically conducted over 28 days, with luminescence measurements performed on the day of tumor induction and subsequently weekly. Conversely, caliper measurements of tumor volume are not performed on the first day, as the cell suspension has not yet encapsulated; tumor measurements begin from the first week onwards and are then conducted weekly. Tumor growth was monitored using caliper measurements from the day of subcutaneous injection until tumors reached an approximate volume of 300 mm^3, as calculated using the formula described in previous studies[9].

One of the most critical steps in this method is the preparation of the tumor cell suspension in ECM, which enhances

tumor take rates by providing an ECM-like environment that supports cell viability and proliferation. Additionally, ensuring that luminescent cells are properly cultured and passaged before injection is essential to maintain cell viability and provide consistent tumor formation across experimental groups. Troubleshooting steps include ensuring that cells are at the optimal passage number to maintain viability and tumorigenicity.

Since immunodeficient mice are used, we recommend performing a pathogen test for *Corynebacterium bovis*, *Corynebacterium sp*. (HAC2) in the cell line before performing *in vivo* studies, as it could diminish the tumor size[10]. Another critical step is the administration of luciferin before imaging, as timing and dosage significantly impact bioluminescence signal intensity[11]. Luciferin must be injected intraperitoneally at 120 mg/kg, 15 min before imaging, to ensure accurate and reproducible measurements of tumor burden.

Several modifications can be applied to optimize the protocol based on experimental needs. For instance, alternative extracellular matrix components, such as collagen or fibrin, could be used instead of ECM if specific tumor microenvironment conditions are required. Additionally, adjusting the number of injected cells can help control tumor growth kinetics, depending on whether a rapid or slow tumor progression model is needed[12].

Despite these advantages, this protocol has several limitations. The use of athymic nude mice limits the ability to assess immune responses due to their lack of functional T cells. This makes the model less suitable for immunotherapy studies unless combined with humanized immune system approaches[13]. Another limitation is the reliance on ECM, which introduces variability due to its undefined composition and potential batch-to-batch differences[14]. Additionally, bioluminescence imaging, while highly sensitive, may not provide precise volumetric measurements compared to more advanced imaging modalities such as MRI or CT scans[15].

This protocol offers significant advantages over traditional tumor induction methods by providing a real-time and noninvasive approach for monitoring tumor progression. Additionally, the luminescent signal enables more precise monitoring of therapeutic responses compared to conventional endpoint measurements, such as histology or necropsy. Overall, this protocol enhances the reproducibility and reliability of preclinical evaluations, ultimately aiding in the development of more effective colorectal cancer treatments. By refining tumor induction techniques and leveraging advanced imaging methods, this model continues to play a crucial role in translational oncology research.

Disclosures

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this study.

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