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Supplementary information

Inverse electron demand Diels–Alder click chemistry for pretargeted PET imaging and radioimmunotherapy

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Supplementary Methods:
Cell Culture and Xenograft Implantation

Leveraging Inverse Electron Demand Diels-Alder Click Chemistry for Pretargeted PET Imaging and Radioimmunotherapy

*Samantha M. Sarrett[1,2,§], Outi Keinänen[1,3,4,§], Eric J. Dayts[1], Guillaume Dewaele-Le Roi[1,5], Cindy Rodriguez[1,5], Kathryn E. Carnazza[6], Brian M. Zeglis[1,2,3,5,7]**

[1]*Department of Chemistry, Hunter College, City University of New York*
[2]*Ph.D. Program in Biochemistry, Graduate Center of the City University of New York*
[3]*Department of Radiology, Memorial Sloan Kettering Cancer Center*
[4]*Department of Chemistry, Radiochemistry, University of Helsinki, Helsinki, Finland*
[5]*Ph.D. Program in Chemistry, Graduate Center of the City University of New York*
[6]*Brain and Mind Research Institute & Appel Institute for Alzheimer's Disease Research, Weill Cornell Medical College*
[7]*Department of Radiology, Weill Cornell Medical College*

[§]These authors contributed equally

Corresponding author: *Brian M. Zeglis. 413 East 69th Street, New York, NY, 10021. Phone: 212-896-0433. Fax: 212-772-5332. E-mail: bz102@hunter.cuny.edu" bz102@hunter.cuny.edu

CELL CULTURE

MATERIALS

Reagents

- 0.25% Trypsin solution (0.25% trypsin/0.53 mM EDTA in HBSS without calcium or magnesium, 30-2101™, ATCC®, Manassas, Virginia, USA)
- Ethanol (reagent alcohol, A962P-4, ThermoFisher Scientific, Waltham, MA, USA)
- Sterile PBS (DPBS, no calcium, no magnesium, 14190144, ThermoFisher Scientific, Waltham, MA, USA)
- Cell media: Iscove's Modified Dulbecco's Medium (IMDM, 12-440-053, ThermoFisher Scientific, Waltham, MA, USA), supplemented with 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 units/mL streptomycin

Equipment

- Cell incubator (5.0% CO_2, 37 °C, HERACell 150i, ThermoFisher Scientific, Waltham, MA, USA *or equivalent*)
- Cell culture treated flasks (Corning™ Cell Culture Treated Flasks, 150 cm^2, 10-126-34, ThermoFisher Scientific, Waltham, MA, USA *or equivalent*)
- Cell counter (Vi-Cell XR, Beckman Coulter, Brea, CA, USA *or equivalent*)
- Cell counting vials (Vi-Cell™ XR Sample Vials, 383721, Beckman Coulter, Brea, CA, USA *or equivalent*)
- Serological pipettes (Fisherbrand™ Sterile Polystyrene Disposable Serological Pipets with Magnifier Stripe; 13-675-2C (2 mL), 13-676-10C (5 mL), 13-676-10F (10 mL), 13-676-10M (25 mL), ThermoFisher Scientific, Waltham, MA, USA *or equivalent*)
- Serological pipetter (Fisherbrand™ Pipet Controller, FB14955202, ThermoFisher Scientific, Waltham, MA, USA *or equivalent*)
- Water bath (Polyscience 5 L general purpose water bath, 50-60 Hz, 3.3 A, Model: WB05, Polyscience, Niles, IL, USA *or equivalent*)
- Centrifuge (5810 R, Eppendorf, Hauppauge, New York, USA *or equivalent*)
- Biosafety hood (model: NU-S477-600, series: SP5, NuAire Laboratory Equipment, Plymouth, MN, USA *or equivalent*)

Reagent Setup

<u>70% Ethanol in H_2O (10.0 L)</u>

Mix 7.0 L of ethanol with 3.0 L of ultrapure water. Store this solution at room temperature for up to 1 month.

Equipment Setup

Cell Counting

Add 0.2 mL of the cell suspension to a cell counting sample vial. Dilute the cell suspension with 0.8 mL of warm media. This will be a 1:5 dilution of the cell suspension. In the cell counting instrument, name the cells to be counted (*e.g.* SW1222), add the date, and mark the dilution (1:5, in this case). Allow the cell counting instrument to count the cells. It will calculate the number of cells per mL. Multiply this number by the total number of mL in the original tube to determine the total number of cells isolated.

PROCEDURE

!CRITICAL When performing cell culture, 70% ethanol must be used to disinfect hands, closed media bottles, and closed culture flasks as they enter the biosafety cabinet. The biosafety cabinet must be cleaned with 70% ethanol before and after use. Only sterile equipment and products are allowed within the biosafety cabinet after their packaging has been sprayed generously with 70% ethanol. Attentive care must be taken to ensure a disinfected and sterile environment for cell culture in order to reduce the risk of contamination and cell death. For adhesive cells, ensure that the flasks lay the correct way (refer to the manufacturer's instructions) in the incubator.

Seeding

1. Incubate the media at 37 °C for at least 30 minutes.
2. In the biosafety cabinet, add 27.0 mL of warm media to a clean cell culture flask.
3. Thaw the SW1222 cells in a 37 °C water bath.
4. Add a vial of thawed cells (~1 mL) to the cell culture flask.
5. Incubate the flask in cell incubator (5.0% CO_2, 37 °C) on its side until the cells reach 80% confluence. At this point, the cells must either be isolated for an experiment or split to a new flask (see below). Media changes must be performed often.

Media Change

!CRITICAL Most cells require media changes *every 2-3 days*. The color of the media is a good indicator of when to change the media. This will depend on both the type of cell and the type of media. The following procedure only works for adherent cells, not cells in suspension or cells that are both in suspension and adhesive. The model cell line for this protocol, SW1222, is adhesive.

1. Add the media bottle to a 37 °C water bath for at least 30 minutes.
2. Spray down the biosafety cabinet with 70% ethanol and wipe down.

3. Remove the cell flask from the incubator and spray it down with 70% ethanol before placing it inside the biosafety cabinet. Aspirate the media from the flask and discard it, being sure not to aspirate along the wall where the cells are located.
4. Add 27 mL of warm media to the flask containing the cells.
5. Incubate the flask in the cell incubator (5.0% CO_2, 37 °C) on its side until the cells reach 80% confluence. At this point, the cells must either be isolated for an experiment or split into a new flask (see below).

Cell Counting, Isolation, and Splitting

!CRITICAL Cells must be split once a culture flask becomes 80% confluent. Use a microscope to determine confluence visually.

1. Add the PBS, trypsin, and media bottles to a 37 °C water bath for at least 30 minutes.
2. Spray down the biosafety cabinet with 70% ethanol and wipe it down.
3. Remove the cell flask from the incubator and spray it down with 70% ethanol before placing it inside the biosafety cabinet.
4. Aspirate the media from the flask and discard it, being sure not to aspirate along the wall where the cells are located.
5. Run 10.0 mL of warm PBS along a wall away from the where the cells are growing. Gently rotate the liquid over the cells to wash any excess media off of them.
6. Aspirate the PBS from the flask and discard it, being sure not to aspirate along the wall where the cells are located.
7. Add 6.0 mL of 0.25% trypsin solution to the flask and incubate it in the cell incubator (5.0% CO_2, 37 °C) for 5 minutes.
8. Gently shake the cells off the wall and visually confirm that all the cells are suspended in the liquid (the clear media will now be cloudy). If the majority of cells are still attached to the wall, incubate for another 5 minutes. **!CRITICAL** If the cells are exposed to trypsin for too long, the trypsin can start to penetrate the cell walls. Do not allow this incubation to exceed 10 minutes in total.
9. Add 14.0 mL of warm media to the flask to stop the trypsinization.
10. Transfer the cell suspension to a 50.0 mL conical centrifuge tube.
11. Centrifuge the tube at 400g for 4 minutes.
12. Aspirate and carefully discard the media supernatant, taking special care not to aspirate any cells from the pellet.
13. Re-suspend the cell pellet in 10.0 mL of warm media, mixing it thoroughly until the solution is homogenous using an appropriate serological pipette.

14. Use a cell counter to determine the concentration of cells / mL (*see Equipment Setup)*. Since the cells are suspended in 10 mL of media, multiply the number of cells per mL by 10 to get the total number of cells suspended in the centrifuge tube.
15. *For isolation:* Take the necessary number of cells from the 10 mL suspension (as dictated by the experiment) and transfer them to a new 50.0 mL conical centrifuge tube, diluting as necessary to reach the final concentration required for the experiment. Keep the cell suspension on ice throughout the experiment. Use the following equation to determine the appropriate volume to remove:

$$\frac{Number\ of\ cells\ required\ for\ experiment}{Concentration\ of\ cells\ (cells\ /\ mL)} = X\ mL$$

16. *For splitting:* Add 27.0 mL of warm media to a new cell culture flask. Calculate the volume to take from the 10 mL cell suspension so that you collect 5×10^6 cells. Add this volume to the new culture flask and incubate it in the cell incubator (5.0% CO_2, 37 °C). Use the following equation to determine the appropriate volume to remove:

$$\frac{5 \cdot 10^6\ cells}{Concentration\ of\ cells\ (cells\ /\ mL)} = X\ mL$$

XENOGRAFT IMPLANTATION

MATERIALS

Reagents

- Matrigel® (356234, Corning Life Sciences, Teterboro, NJ, US)
- Isoflurane (Isoflurane Liquid Inhalation 99.9% Glass Bottle, 1182097, Henry Schein, Melville, NY, USA) **!CAUTION** Isoflurane can cause severe skin burns and eye damage and may cause drowsiness, dizziness, and respiratory irritation if inhaled. Isoflurane is suspected to cause damage to unborn children.
- SW1222 cell line (12022910, Sigma-Aldrich, St. Louis, MO, USA)

Equipment

- Insulin syringes for xenografting (188450, McKesson Corporation, Irving, TX, USA *or equivalent*)
- Ethanol wipes (22-363-750, ThermoFisher Scientific, Waltham, MA, USA) **!CAUTION** Ethanol is highly flammable.
- Tumor measuring device (Peira TM900 device, Ketelaarstaat 8, Belgium *or equivalent*)
- Gauze (Dynarex Sterile Gauze Pads, 3353, Dynarex, Orangeburg, NY, USA *or equivalent)*
- Complete animal anesthesia system (V-9 Wall Mount Laboratory Animal Anesthesia System with Accessory Kit, 901810, VetEquip, Livermore, CA, USA *or equivalent*)

PROCEDURE

!CRITICAL The following protocol is for implanting xenografts in nude athymic mice. Protocols may need to be altered if you are using a different type of mouse.

1. Isolate enough cells so that you will have 5×10^6 cells per mouse in a 50.0 mL conical centrifuge tube. **!CRITICAL** It is usually a good idea to round up and isolate 15-20% more cells than necessary to ensure you will have more than enough for the whole procedure.
2. Dilute the cells to a concentration of 66.67×10^6 cells/mL with cell media. If your cell suspension is not concentrated enough, spin the cells down (400g, 4 min) and discard the supernatant. Then dissolve the cells in media to make a final concentration to 66.67×10^6 cells/mL.
3. Place the cell suspension on ice.
4. Make a 1:1 solution of cell suspension:Matrigel®. **!CRITICAL** As you are making and handling the Matrigel® solution, keep everything on ice. Once the Matrigel® starts to warm, it will thicken, which is ideal for when it is in a body to help with the implantation of tumor cells but not ideal for preparing the solutions to use.
5. Aliquot 1.0-1.5 mL of the cell suspension:Matrigel® mixture into several microcentrifuge tubes. Keep the microcentrifuge tubes on ice.

Transfer the microcentrifuge tubes on ice to the animal facility and begin xenografting.

!CAUTION When using isoflurane for animal protocols, perform all procedures in a well-ventilated hood. Isoflurane is toxic to humans. Refer to your animal facility's guidelines on isoflurane usage.

a. Anesthetize the first cage of mice with isoflurane in the induction chamber (or whatever anesthetization method is approved in your laboratory's IACUC-approved animal protocol).
b. Before drawing the first dose from the microcentrifuge tube, mix the cell suspension:Matrigel® solution by drawing it into the syringe a few times. Visually make sure the suspension is homogenous before you start drawing doses.
c. Draw 150 µL of cell suspension:Matrigel® mixture into the insulin syringes. Make enough syringes for the whole cage (we typically have five mice in one cage).
d. Place one mouse in the prone position with its snout in the anesthesia cone to ensure the mouse stays anesthetized as you work.
e. Wipe the right shoulder (or the tumor desired location) with ethanol wipes thoroughly.
f. Insert the insulin needle (containing the cells) just under the skin at a 15° angle, bevel up, on the right shoulder (or desired location). Gently pull the needle up *slightly* to ensure you are just under the skin. When you pull up here, you should see a very thin layer on top of the needle – if its thick, you may need to re-do the insertion.
g. Slowly inject the cell suspension under the skin (~150 µL in 5 seconds). A small ball will form as you inject the cells. Once all the solution has been added, hold the needle in place for around 10 seconds. This allows the Matrigel® to start to thicken and will prevent leaking of the cells.
h. Remove the needle slowly and twist as you pull it out to prevent leaking of the cells.
i. If you still see leaking of the cell suspension solution out of the injection site, hold a piece of gauze over the site gently for a few seconds as the Matrigel® continues to thicken. Wipe the area with an ethanol wipe.
j. Place the mouse back in its cage.
k. Repeat steps a-j with the rest of the mice.
l. Observe the mice for 20 minutes as they regain consciousness. Make sure that there are no significant behavior changes in the mice and that there is no leakage from the injection site.

6. After xenografting, measure the tumors every 2 days using the tumor measurement device until tumors have reached an ideal size (~100 mm^3).

Results

The SW1222 tumors typically reach the ideal size for experiments (~100 mm^3) after approximately 14 days.