Chapter 19

Remote Patterning of Transgene Expression Using Near Infrared-Responsive Plasmonic Hydrogels

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Abstract

The development of noninvasive technologies for remote control of gene expression has received increased attention for their therapeutic potential in clinical scenarios, including cancer, neurological disorders, immunology, tissue engineering, as well as developmental biology research. Near-infrared (NIR) light is a suitable source of energy that can be employed to pattern transgene expression in plasmonic cell constructs. Gold nanoparticles tailored to exhibit a plasmon surface band absorption peaking at NIR wavelengths within the so called tissue optical window (TOW) can be used as fillers in fibrin-based hydrogels. These biocompatible composites can be loaded with cells harboring heat-inducible gene switches. NIR laser irradiation of the resulting plasmonic cell constructs causes the local conversion of NIR photon energy into heat, achieving spatially restricted patterns of transgene expression that faithfully match the illuminated areas of the hydrogels. In combination with cells genetically engineered to harbor gene switches activated by heat and dependent on a small-molecule regulator (SMR), NIR-responsive hydrogels allow reliable and safe control of the spatiotemporal availability of therapeutic biomolecules in target tissues.

Key words Hydrogel, Gene therapy, Gold, Infrared, Nanoparticle, Biomaterial, Scaffold, Transgene, Spatiotemporal, Plasmon

1 Introduction

Heat-shock protein genes encode a small group of proteins (HSPs) found in virtually all living organisms from bacteria to higher vertebrates. HSPs accumulate transiently to elevated levels in cells exposed to certain physical and chemical stressors, heat being the most powerful inducer of these genes [1–4]. Induced expression of *hsp* genes is mediated by heat-shock factors (HSFs) that interact with heat-shock elements located in their promoters [5]. The human HSP70B gene is one of the most highly inducible HSP genes [6–8]. Its promoter has a very low basal activity, which can be induced several thousandfold upon thermal treatment. Interestingly, the magnitude of HSP70B promoter activation is a function of the intensity of the heat treatment in terms of both

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temperature and length of exposure, an attribute that makes this promoter extremely attractive for the deliberate control of transgene expression [9]. The main problems associated with uses of HSP promoters in gene therapy are the insufficiently long periods of transgene expression as well as the possibility of inadvertent expression of transgenes [10–13]. These problems are avoided by gene switches that combine an HSP70B promoter and an SMR-activated transactivator. Gene switches employing transactivators activated by different SMRs successfully provide spatiotemporal control of transgene activity [14, 15].

To administer heat in a focused fashion, technologies based in ultraviolet (UV), short-wavelength visible (vis), or infrared (IR) laser-light irradiation have been developed. However, the main limitation of these approaches arises from the poor penetration of UV, vis, or IR light in biological tissues, which are less prone to absorb or scatter light in the wavelength range of the TOW (i.e., 650–1100 nm). As a consequence, NIR laser light may penetrate at least 10 cm through deep tissue [16, 17]. On account of the phenomenon of localized surface plasmon resonance, gold nanoparticles (GNPs) tailored to strongly absorb NIR light can be used as highly efficient nanotransducers for converting this kind of photon energy into heat. This nanotechnology resource, known as plasmonic photothermia, has been successfully employed for inducing the expression of transgenes driven by the human HSP70B promoter [18, 19].

This chapter describes the detailed procedure for preparing fibrin-based hydrogels that integrate in their structure GNPs with a plasmon surface band absorption peaking at NIR wavelengths within the TOW. The protocol describes the steps for encapsulating cell populations within the NIR-responsive biomaterial to obtain three-dimensional assemblies or injectable cell constructs. The chapter also includes the procedures for in vitro and in vivo NIR irradiation of plasmonic scaffolds populated by genetically modified cells, which enable a tight spatiotemporal control of transgene expression patterns.

2 Materials

Prepare all reagents inside a tissue culture hood, at room temperature. Diligently follow waste disposal regulations when disposing waste materials.

2.1 Fibrin-Based Plasmonic Hydrogels

1. Lab scale.
2. Ultrasonic bath.
3. Refrigerated benchtop centrifuge.
4. Sterile metallic bent spatula.

5. Sterile tweezers.
6. Disposable biopsy punches.
7. Conical centrifuge tubes, 15 and 50 mL.
8. Hemocytometer.
9. Eppendorf tubes, 1.5 mL.
10. Syringes.
11. Sterile syringe filter with a 0.2 μm polyethersulfone (PES) membrane.
12. Sterile multiwall plates.
13. Double-distilled water.
14. Penicillin-streptomycin (P/S) solution 10,000 U/mL.
15. Fetal bovine serum heated for 30 min at 56 °C with mixing to inactivate complement (FBSi).
16. Dulbecco's phosphate-buffered saline (DPBS).
17. Dulbecco's modified Eagle's medium incorporating L-glutamine, high glucose (4.5 g/L) and phenol red (DMEM).
18. Trypsin-EDTA solution: 0.25 % Trypsin/0.91 mM EDTA with phenol red. Prewarm to 37 °C before use.
19. GNP stock solution. Weigh lyophilized nanomaterial in a glass vial and add sterile double-distilled water to a final concentration of 1 mg/mL. In order to break particle agglomerates and facilitate nanomaterial dispersion in water, the vial is kept immersed in ice water for 30 min in an ultrasonic bath operating at 40 kHz and a power of 80 W. Sterilize GNP solution by ultraviolet germicidal irradiation for 12 h. Store GNP stock solution at 4 °C.
20. P/S-DMEM solution: Add penicillin-streptomycin solution to DMEM at a final concentration of 1,000 U/mL. Store at 4 °C.
21. Cell culture media: Add 0.1 volumes of FBSi to P/S--DMEM. Store at 4 °C.
22. Fibrinogen suspension: Weigh bovine fibrinogen (Sigma) in a 50 mL centrifuge tube and add P/S-DMEM to a final concentration of 20 mg/mL of clottable protein. Gently stir the solution with a vortex for 20 s and incubate at 37 °C in a water bath for 10 min. Repeat this step twice and then centrifuge at 3,000 × *g* for 5 min in a benchtop centrifuge refrigerated at 4 °C. Check that fibrinogen is completely solubilized and there are no traces of foam or insolubilized fibrinogen. Otherwise, gently stir the solution with vortex for 20 s, incubate for an additional 10 min at 37 °C, and then repeat centrifugation step at 4 °C. Filter solution using a sterile syringe filter with a 0.2 μm PES membrane.

23. Fibrinogen conjugate suspension: Labeled fibrinogen from human plasma is commercially available in four fluorescent colors (Alexa Fluor -488, -546, -594, -647 or Oregon-488; Molecular Probes). Reconstitute 5 mg of lyophilized product in 3.33 mL of 0.1 M sodium bicarbonate (pH 8.3) to obtain a 1.5 mg/mL stock solution. Complete solubilization may take 1 h or more with occasional gentle mixing. Divide the solution into aliquots and freeze at −20 °C protected from light.
24. Thrombin solution: Add double-distilled water to lyophilized thrombin from bovine plasma (Sigma) to a final concentration of 500 U/mL. Store at −20 °C. Prepare the working solution by diluting stock solution at 40 U/mL in chilled P/S--DMEM. Filter solution using a sterile syringe filter with a 0.2 μm PES membrane.

2.2 Animal Experimentation

1. Table-top anesthesia system: Isoflurane vaporizer with O_2 H-Tank regulator, fluosorber charcoal canister, oxygen hose, and rodent nose cone (Harvard Apparatus).
2. Bead sterilizer.
3. Heat lamp for use during small animal surgery (75 W infrared bulb).
4. Warming blanket.
5. Electric clipper for small animals.
6. Surgical instruments.
7. 7 mm wound clips, wound clips applier, and wound clip remover.
8. Sterile gloves.
9. Sterile syringes.
10. 20 and 27 G needles.
11. High-density foam.
12. Tegaderm™ absorbent clear acrylic dressing (3 M).
13. Aluminum foil.
14. Ophthalmic ointment.
15. Topical depilatory cream.
16. Povidone-iodine 10 % solution.
17. Sterile gauze pads.
18. Sterile surgical drapes.

2.3 Patterning of Transgene Expression in Fibrin-Based Plasmonic Hydrogels

1. Rapamycin (Invivogen): Dissolve rapamycin solid in *N*-dimethylacetamide (DMA) to prepare a stock solution of 3 mg/mL and store at −20 °C. For in vitro assays use rapamycin at a final concentration of 10 nM. For in vivo assays, dilute stock in a mixture of 50 % N,N-dimethylacetamide, 45 %

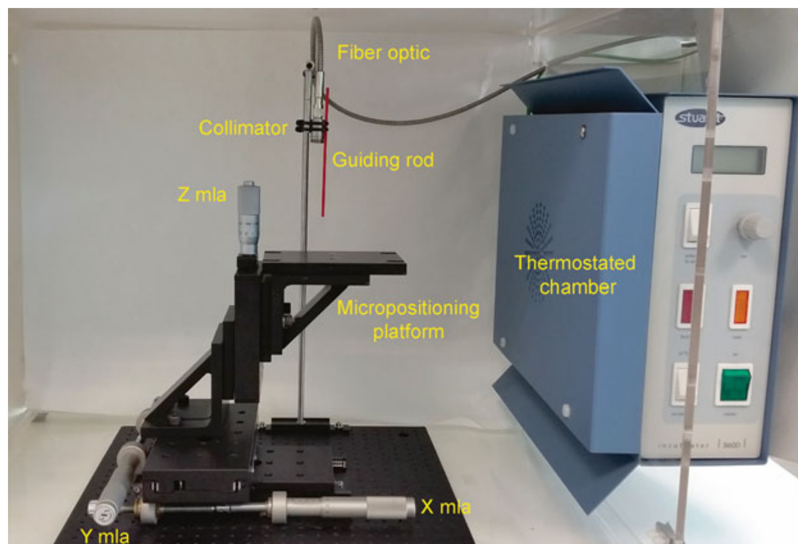

Fig. 1 Experimental setup for NIR irradiation. *mla* micro-linear actuator

polyethylene glycol (average molecular weight of 400 Da), and 5 % polyoxyethylene sorbitan monooleate.

2. NIR irradiation setup (Fig. 1) consisting of a laser diode emitting at a wavelength of 808 nm coupled to a fiber optic of 400 μm of diameter (MXL-III(FC) model, Changchun New Industries Optoelectronics Technology Co., Ltd) connected to a fixed focus collimator (Thorlabs). The collimator is mounted on a micropositioning platform to ensure that the laser beam illuminates the sample orthogonally. For in vitro irradiation assays, the fiber-optic and the positioning system are placed inside a thermostatically controlled cabinet to establish the environmental temperature at 37 °C. For in vivo irradiation assays, the micropositioning platform is covered with a thermal blanket to maintain the corporal temperature of the anesthetized animal. Control measures of NIR laser hazard include laser warning signs, laser safety barriers, and safety laser goggles (all from Thorlabs).

3 Methods

To illustrate the methodology, the procedures detailed below use cells, derived from C3H/10T1/2 cell line (ATCC CCL-226), which stably harbor a firefly luciferase gene under the control of a heat-activated and rapamycin-dependent gene switch (Fig. 2). In rapamycin-treated cells, levels of transgenic luciferase expression increase as function of the intensity of an activating heat treatment.

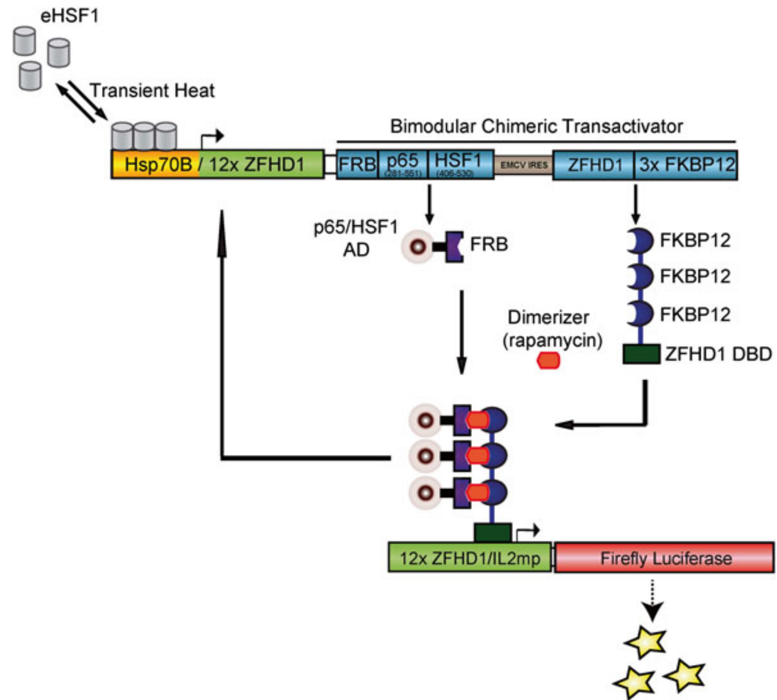

Fig. 2 Outline of the heat-activated gene switch that employs a dimerizer-regulated transactivator to control firefly luciferase expression. A bicistronic gene encoding the two component proteins of a transactivator regulated by a dimerizer (rapamycin) is expressed under the control of promoter cassette HSP70B/12xZFHD12 that responds to activated endogenous heat-shock factor 1 (eHSF1), the transcription factor that mediates heat-induced expression of HSP genes, as well as the dimerizer-activated transactivator. Transactivator-responsive promoter 12xZFHD12/IL2mp drives the expression of the linked transgene. *AD* activation domain. *DBD* DNA-binding domain. *See* ref. 15 for a detailed description of the gene-switch components

Note that if heating conditions are too harsh, the level of induced luciferase activity may decrease substantially due to cell damage caused by thermal stress [20].

3.1 Fibrin-Based Plasmonic Hydrogels Encapsulating Genetically Modified Cells

3.1.1 Preparation of Cellular Component of Fibrin-Based Plasmonic Hydrogels

Carry out all procedures under aseptic conditions in a tissue culture hood.

1. Remove culture medium and eliminate residual serum by rinsing cell monolayers with 5 mL of DPBS per 25 cm^2 of surface area of cell culture. Let the DPBS sit on cells for at least 30 s to remove as much extracellular proteins as possible.
2. Aspirate DPBS and add 1 mL of trypsin-EDTA solution per 25 cm^2 of surface area of cell culture (*see* **Note 1**).
3. Incubate at the tissue culture incubator (37 °C/5 % CO_2/100 % humidity) for ~5 min (*see* **Note 2**).

4. Neutralize the trypsin by adding 10 mL of culture medium per mL of trypsin-EDTA solution and distribute cell suspension in centrifuge tubes of appropriate size.
5. Centrifuge cells at $200 \times g$ for 5 min in a benchtop centrifuge.
6. Following centrifugation, aspirate the media and resuspend the cell pellet in 0.25 mL of P/S-DMEM per 25 cm^2 of initial surface area of cell culture.
7. Count cells using a hemocytometer.
8. Dilute cells in P/S-DMEM to a final concentration of 5×10^6 cells/mL and keep cell suspension on ice.

3.1.2 Preparation of the Polymerizable Mixture for Fabricating Fibrin-Based Plasmonic Cell Constructs

Carry out all procedures under aseptic conditions in a tissue culture hood. All samples must be kept on ice unless otherwise specified.

1. To break up agglomerates of GNPs formed during storage of the sample, immerse the vial containing the GNP stock solution for 10 min in an ultrasonic bath filled with ice water that operates at 40 kHz and a power of 80 W.
2. Add 0.01–0.1 volumes of sonicated GNP stock solution to 0.5 volumes of fibrinogen suspension and pipette briefly to ensure uniform dispersion of the nanomaterial (*see* **Note 3**).
3. According to the volume used of GNP stock solution, add pre-chilled P/S-DMEM to make up to 0.7 volumes of plasmonic hydrogel mix. Pipette briefly to homogenize the solution (*see* **Note 4**).
4. Add 0.2 volumes of cell suspension. Pipette gently to distribute the cells among the other components of the plasmonic hydrogel mix.
5. Add 0.1 volumes of chilled thrombin working solution to the plasmonic hydrogel mix that includes the cellular component. Pipette gently to homogenize the sample. The solution is ready for the fabrication of three-dimensional plasmonic assemblies described in Subheading 3.1.3, or for subcutaneous polymerization of plasmonic cell constructs as indicated in Subheading 3.2.2 (*see* **Note 5**).

3.1.3 Fabrication of Three-Dimensional Plasmonic Assemblies

1. Add 1 volume of FBSi to the well selected as mold for the gelation of the plasmonic hydrogel-incorporating cells (*see* **Note 6**).
2. Incubate the well-plate at the tissue culture incubator for 5 min. Aspirate the FBSi.
3. Dispense 1 volume of the mixture obtained in Subheading 3.1.2, **step 5**, into the well pretreated with FBSi. Leave for 5 min at room temperature. Monitor visually the sol-gel transition and incubate at the tissue culture incubator for 1 h to allow consolidation of the plasmonic cell construct.

4. Add 0.9 volumes of cell culture medium and 0.1 volumes of FBSi to the top of the hydrogel. Incubate at the tissue culture incubator for 1 h to balance the serum content of the hydrogel.
5. Use a bent spatula for unmolding the plasmonic cell construct and transfer the assembly to a larger well containing 4 hydrogel volumes of cell culture medium.
6. Incubate in the tissue culture incubator for 24 h.

3.2 *In Vivo* Implantation of Fibrin-Based Plasmonic Cell Constructs

3.2.1 Surgical Preparation of the Animal

1. Administer preemptive analgesics according to the institutional procedure for Animal Care and Use.
2. Anesthetize the animal with isoflurane. Administer the anesthetic at 5 % for induction and at 2 % for maintenance in O_2 flowing at 1 L/min. Protect animal eyes from desiccation using an ophthalmic ointment.
3. Remove hair from implantation site using an electric clipper.
4. Treat the skin in the region of implantation with depilatory cream for 5 min.
5. Remove loose hair and debris from the animal and clean the surgical area with water and gauzes.
6. Place the animal in the surgical area.
7. Scrub surgical site with povidone-iodine solution.
8. Rinse with 70 % ethanol.
9. Repeat soap scrub and rinse process three times.
10. Drape the animal with sterile, impermeable covering to isolate the disinfected area.

3.2.2 Subcutaneous Polymerization of Fibrin-Based Plasmonic Cell Constructs

1. Pre-chill a 1 mL syringe on ice.
2. Load the syringe with 1 volume of the mixture obtained in Subheading 3.1.2, **step 5**.
3. Attach a 20-gauge needle to the syringe.
4. Insert the needle, bevel up and slightly angled, in the animal skin to access to the subcutaneous space.
5. Lift the skin slightly with the needle and inject slowly the contents of the syringe into the subcutaneous space.
6. Remove carefully the needle from the area of implantation (*see* **Note 7**).
7. Keep the anesthetized animal stationary for 5 min under the infrared lamp to complete the polymerization of the hydrogel.

3.2.3 Subcutaneous Implantation of Three-Dimensional Plasmonic Assemblies

1. Use a scalpel blade to make the smallest possible incision.
2. Use sterile tweezers to open a subcutaneous pocket of sufficient size to fit the hydrogel assembly obtained in Subheading 3.1.3.
3. Introduce the plasmonic assembly in the subcutaneous pocket using sterile forceps.
4. Close the skin incision with 7 mm clips stapled with a wound clip applier (*see* **Note 8**).

3.3 Patterning Transgene Expression in Fibrin-Based Plasmonic Cell Constructs by NIR Irradiation

The coherence and low divergence angle of a NIR laser, aided by focusing from the lens of an eye, can concentrate the radiation into a spot on the retina leading to irreversible damage. When operating with the NIR laser diode, refer to institutional laser safety procedures and always wear certified laser safety glasses (ANSI Z136 and CE).

3.3.1 Patterning Transgene Expression In Vitro

1. To immobilize the hydrogel, with the help of a bent spatula transfer the three-dimensional plasmonic assembly obtained in Subheading 3.1.3 to a well-plate of similar size to that used for gelation. Add 2 volumes of cell culture medium containing rapamycin at a final concentration of 10 nM.
2. Incubate the well-plate in the tissue culture incubator.
3. Place the well-plate containing the three-dimensional hydrogel assembly on the micropositioning platform placed inside the thermostated chamber set at 37 °C.
4. Use the Z micro-linear actuator to position the collimator 1 mm above the top of the lid of the well-plate.
5. Use the X-Y micro-linear actuators to align concentrically the hydrogel-well with the collimator. This position is established as the center of coordinates of the system (*see* **Note 9**).
6. Use the scale graduation of the micro-linear actuators to set the coordinates for the first irradiation spot of the projected pattern.
7. Select continuous-wave (CW) or pulsed mode of NIR irradiation in the driver unit. For the pulsed regime of irradiation, select duty cycle and pulse repetition rate. Switch on the laser driver unit and adjust to the desired output power.
8. After irradiation at the selected illumination spot, turn off the laser driver unit and use the micro-linear actuators to position the collimator at the next irradiation spot.
9. Repeat **steps 5** and **6** to complete the desired pattern of transgene expression.
10. Incubate the well-plate in the tissue culture incubator for the required period of time and measure transgene activity by bioluminescence assay using a coupled charged device (CCD) camera and D-luciferin substrate.

3.3.2 Patterning Transgene Expression In Vivo

The use of a mask for referencing NIR-irradiation spots on the body of the animal is highly recommended to accurately induce patterns of transgene expression in vivo.

1. Use a sheet of paper to draw the desired irradiation pattern based on circles of 2.5 mm of diameter.
2. Place the template on the top of a sheet of high-density foam.
3. Place a piece of Tegaderm™ transparent film dressing on the template.
4. Use a biopsy punch of 2.5 mm of diameter to trim the Tegaderm™ membrane at points that shape the projected pattern.
5. One hour before irradiation, inject rapamycin intraperitoneally at a dose of 1 mg/kg in a final volume of 50 μL using a 27 G needle.
6. To proceed with NIR irradiation, anesthetize the animal with isoflurane and cover its head with aluminum foil to protect eyes from laser light scattering.
7. Place the irradiation mask at the implantation site.
8. Place the animal on the micropositioning platform covered with the thermal blanket.
9. Operate on Z micro-linear actuator to position the collimator ~5 cm above the region of implantation.
10. Use X-Y micro-linear actuators to align the guiding rod with the first irradiation spot masked in the Tegaderm™ membrane (*see* **Note 10**).
11. Use X micro-linear actuator to position the collimator at the known distance between the center of the collimator lens and the guiding rod, thereby focusing the laser beam at the center of targeted irradiation spot.
12. Select CW or pulsed mode of NIR irradiation in the driver unit. For the pulsed regime of irradiation, select duty cycle and pulse repetition rate. Switch on the laser driver unit and adjust to the desired output power.
13. After irradiation, turn off the laser driver unit and use the micro-linear actuators and the guiding rod to position the collimator at the next irradiation spot.
14. Repeat **steps 6–9** to complete the masked irradiation pattern.
15. Remove irradiation mask and transfer the animal to the recovery cage.
16. After the desired period of time, measure induced transgene activity by bioluminescence assay using a CCD camera and D-luciferin substrate.

4 Notes

1. Trypsin-EDTA solution is suitable for most but not for all adherent cells. Use cell dissociation solution and cell culture medium recommended for subculturing and harvesting the cells of choice.
2. Examine cells under an inverted microscope. Fully trypsinized cells should appear rounded up and no longer attached to the surface of the culture plasticware. Otherwise, increase the time of incubation in the tissue culture incubator for an additional 5 min.
3. Upon NIR irradiation, GNPs embedded in fibrin-based plasmonic hydrogels promote high induction of transgenic activity with negligible cell damage. Optimal GNP concentration in plasmonic cell constructs must be determined empirically by performing procedure 3.3.1 followed by quantification of induced transgene activity and cell viability in the plasmonic cell construct.
4. To monitor dynamic degradation of plasmonic cell constructs, add 0.02 volumes of a human fibrinogen conjugate solution that must be substracted to the amount of P/S-DMEM to complete 0.7 volumes of plasmonic hydrogel mix. Hydrogel degradation over time can be estimated by fluorometric quantification techniques or fluorescence imaging.
5. After thrombin addition, slow fibrin polymerization may occur in chilled samples. Work fast and minimize handling time between adding the thrombin solution and dispensing/injecting the plasmonic hydrogel mix containing cells.
6. Multiwell-plates of 6-, 12-, 24-, and 48-wells are convenient formats to generate three-dimensional assemblies. If a smaller size is desired, glass, polystyrene, or Permanox® plastic slides with a removable silicone chamber for cell culture (Nunc) can be used as mold. Alternatively, biopsy punches of different diameters can be used to obtain small samples from gelated three-dimensional assemblies of higher size.
7. Needle should be removed gradually from the injection area to minimize leaking of the polymerizable solution. At the time of withdrawing the needle tip, wait for 20 s to allow clogging of the punctured skin by fibrin polymerization.
8. After the skin incision has healed, surgical clips must be withdrawn using the wound clip remover.
9. To facilitate the positioning of the collimator, a circle with same diameter as the collimator housing and concentric to the center of the target well can be plotted on the lid of the multiwell-plate. Using X, Y, and Z micro-linear actuators, the collimator can be positioned according to the marked reference.

10. To position the collimator for in vivo irradiation, a plastic rod of 0.5 mm of diameter can be attached to one side of the collimator. The known distance between the center of the collimator lens and the guiding rod will be used to align the collimator over the masked irradiation spot.

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