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A Protocol for co-Injecting Cells with Pulverized Fibers for Improved Cell Survival and Engraftment

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Abstract

Adipose tissue is crucial for medical applications such as tissue reconstruction, cosmetic procedures, and correcting soft tissue deformities. Significant advances in the use of adipose tissue have been achieved through Coleman's studies in fat grafting, which gained widespread acceptance due to its effectiveness and safety. Despite its benefits, adipose tissue grafting faces several limitations, including high absorption rates due to insufficient support or anchorage, replacement by fibrous tissue, migration from the intended site, and loss of the initial desired morphology post-administration. To counteract these constraints, there is a need for improved grafting techniques that enhance the predictability and consistency of outcomes. Biomaterials are extensively used in tissue engineering to support cell adhesion, proliferation, and growth. Both natural and synthetic materials have shown promise in creating suitable microenvironments for adipose tissue regeneration. PLGA, a synthetic copolymer, is particularly notable for its biocompatibility, biodegradability, and tunable mechanical properties. Here, we describe a protocol using milled electrospun poly(lactic-co-glycolic acid) (PLGA) fibers combined with lipoaspirated tissue to create a fibrous slurry for injection. By pulverizing PLGA fiber mats to create fiber fragments with increased pore size and porosity, we can influence key cellular responses and enhance the success of adipose tissue–grafting procedures. This approach improves anchorage and support for adipocytes, thereby increasing cell viability. This method aims to enhance vascularity, perfusion, and volume retention in adipose tissue grafts, which addresses many of the limitations of current approaches to adipose tissue grafting and holds promise for more consistent and successful outcomes.

Key features

- Adipose tissue for tissue reconstruction.
- Need for improved engraftment and volume retention.
- Pulverized PLGA fiber mats to create a fibrous "slurry" that allows injection.
- PLGA fibers co-injected with lipoaspirated tissue.
- Improved adipose engraftment outcomes (e.g., perfusion, vascularity, and retention of graft volume).

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Keywords: Pulverized PLGA fibers, Adipose tissue engraftment, Volume retention, Increased vascularity, Tissue reconstruction, Soft-tissue augmentation.

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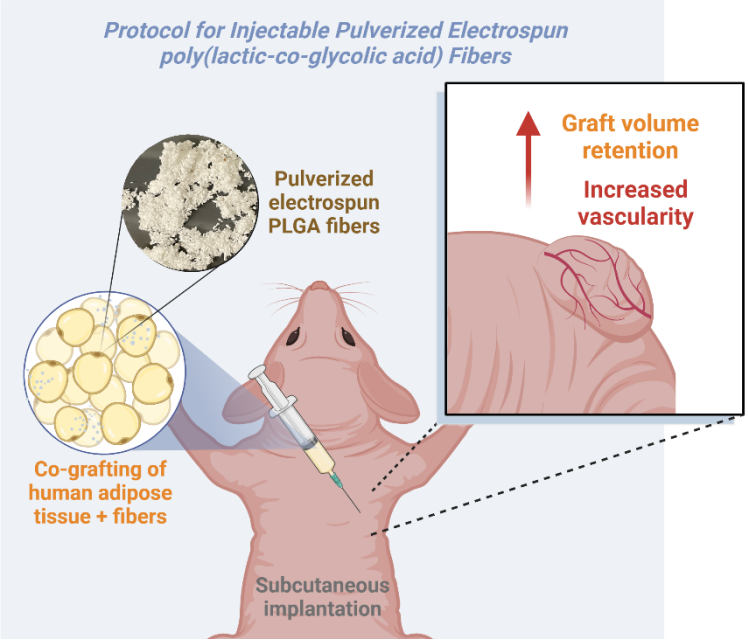
Graphical overview



Overview of the subcutaneous implantation of human adipose tissue and pulverized electrospun poly(lactic-co-glycolic acid) (PLGA) fibers in a mouse model

Background

Adipose tissue plays a crucial role in numerous medical applications, such as tissue reconstruction, cosmetic procedures, and the correction of soft tissue deformities. The first use of autologous adipose tissue in humans was described in 1889 by Van der Meulen [1]. While many subsequent studies have explored this technique to correct various defects, a significant advancement in fat transplantation occurred with the publication of Coleman's studies in fat grafting [2]. The Coleman technique, which utilizes autologous adipose tissue, has gained widespread acceptance due to its effectiveness and safety [3]. This method involves harvesting adipose tissue via liposuction, processing it, and reinjecting it into various tissue depths. Studies have demonstrated its efficacy in yielding viable adipocytes and sustaining optimal cellular function within fat grafts, making it a valuable method for soft-tissue augmentation and tissue repair [4].

The use of adipose tissue has led to numerous advancements in the use of autologous fat grafts not only in aesthetic treatments but also in various medical specialties such as therapies for breast cancer [2,5]. The regenerative potential of adipose tissue is attributed to the presence of stem cells, which makes it an ideal filler due to its availability, low donor-site morbidity, cost-effectiveness, and biocompatibility [6]. Adipose-derived stromal cells can secrete various growth factors, such as VEGF, HGF, and TGF-β, which play a crucial role in tissue remodeling. These growth factors influence the differentiation of stem cells and promote angiogenesis, thereby enhancing the efficacy of adipose tissue in tissue engineering approaches [7]. Moreover, after transplantation, ischemic adipocytes attract macrophages, initiating revascularization through neoangiogenesis, suggesting thus that the foreign body response is associated with increased vascularization [6,8].

However, existing autologous adipose engraftment methods have several limitations that impact the success and consistency of clinical outcomes. In clinical practice, a major issue with adipose tissue autotransplantation is the absorption rate over time, which ranges from 25% to 70% of the total implanted volume [2,6,9]. In 1987, the American Society of Plastic and Reconstructive Surgeons reported that only 30% of the injected autologous fat was

expected to survive for one year [9]. Additionally, it was observed that the transplanted tissue often became filled with connective tissue, indicating the death of the adipose tissue followed by its replacement with fibrous tissue or newly formed metaplastic fat [9]. In contrast, Peer proposed the cellular survival theory, which suggests that the final volume after an adipose tissue transplant depends on the number of living adipocytes at the time of transplantation [9,10]. These challenges underscore the need for better grafting techniques to improve the predictability and consistency of adipose grafting results.

Biomaterials are extensively utilized in tissue engineering for their capability to support cell adhesion, proliferation, and growth during the development of new tissues. For instance, porous scaffolds and hydrogels, both natural and synthetic, have shown promise in improving adipose tissue grafting procedures. These materials have been found to enhance cell viability, promote vascularization, and support the growth of adipocytes derived from progenitor cells. Collagen-based scaffolds, hyaluronan-heparin-collagen hydrogels, fibrinogen hydrogels, and decellularized extracellular-matrix scaffolds are among the natural materials that have demonstrated potential in creating a suitable microenvironment for adipose tissue regeneration [11–13]. On the other hand, synthetic materials such as poly(N-isopropylacrylamide) (PNIPAm), polyglycolic acid (PGA), polymeric nanocomposites, and poly(lactic-co-glycolic acid) (PLGA) have shown promise in tissue regeneration, contributing to adipose tissue grafting [14,15].

In this context, we propose a protocol to improve grafting outcomes using milled/pulverized electrospun PLGA fibers combined with lipoaspirated tissue to create a fibrous slurry for injection into recipient tissue. PLGA is a copolymer widely used in tissue engineering due to its excellent biocompatibility, biodegradability, and mechanical properties. PLGA degrades into lactic acid and glycolic acid, which are naturally metabolized by the body, minimizing its toxicity [16]. Additionally, the mechanical properties (e.g., degradation rate) can be adjustable by altering the ratio of lactic acid to glycolic acid monomers. Higher lactic acid content results in slower degradation, while higher glycolic acid content leads to faster degradation. The ability to control the degradation rate by adjusting the ratio is a unique feature of PLGA that sets it apart from other commonly used biodegradable polymers, such as PGA or polylactic acid (PLA) [17,18]. While PLGA can be easily electrospun into microscale fibers for scaffolding in cell culture, these fibers must be processed into small pieces to make them suitable for injection. Therefore, we pulverize the electrospun fiber mats using a mini mill to create small fibrous clusters with increased pore size and porosity, which are critical for influencing key cellular responses.

We present a detailed protocol for using milled electrospun PLGA fibers co-injected with adipose tissue, aimed at enhancing vascularity, perfusion, and graft volume retention. This approach offers the potential to improve the consistency and success of adipose grafting techniques, addressing the limitations of current methods.

Materials and reagents

Reagents

1. Polylactide-co-glycolide acid (PLGA) 82:18 lactide:glycolide (Corbion Purac, catalog number: PLG8218)
2. Hexafluoroisopropanol (Oakwood Chemical, catalog number: 3409)
3. 0.9% sodium chloride solution (saline) (Henry Shein, catalog number: 1047098)
4. Ethanol, 200 Proof (100%) (Decon Labs, catalog number: 2701)
5. Betadine (Henry Schein, catalog number: 67618–150-09)
6. Isopropyl alcohol (Fisher Scientific, catalog number: A416P-4)
7. Isoflurane (Primal Health Care, catalog number: NDC 66794–017-10)
8. Artificial tear ointment (Henry Schein, catalog number: 1338333)
9. Nairing cream (Nair, catalog number: 339823)

Solutions

1. 70% ethanol solution (see Recipes)

Recipes

1. **70% ethanol solution**
   Mix 35 mL of 100% ethanol with 15 mL of sterile water, to create 50 mL of 70% ethanol solution.

Laboratory supplies

1. 0.5 mm sieve (IKA, model: 2939000)
2. 0.25 mm sieve (IKA, model: 2938900)
3. 2 mL Eppendorf Tubes (VWR, catalog number: 87003-298)
4. 50 mL Falcon tubes (VWR, catalog number: 89039-660)
5. Alcohol swabs (Henry Shein, catalog number: HS1007)
6. Cotton tip applicator (McKesson, Q-Tip, catalog number: 785468)
7. Liquid glue (3M Vetbond, catalog number: B07Q39FL9M)
8. 6–0, C-22 black braided silk sutures (Henry Schein, catalog number: 101–2636)
9. Surgical tape (3M, catalog number: 1527–0)
10. 18-gauge needle (Central Infusion Alliance, catalog number: BD 305196)
11. 3 mL syringe (Fisherbrand, catalog number: 14–955-457)
12. Parafilm (Bemis, catalog number: PM999)

Equipment

1. Electrospinner (Glassman High Voltage, model: PS/FJS0R02.4)
2. Micro-miller (IKA Mini-Mill, model: 2836001)
3. Cold centrifuge (VWR, Eppendorf, model: 5430 R, catalog number: 76458-526)
4. Weigh scale (VWR, model: VWR-6001E, catalog number: 10204-996)
5. Analytical balance (VWR, model: VWR-310AC/CAL, catalog number: 89422-676)
6. Slide warmer with temperature control (4MD Medical, catalog number: CASCXH-2001)
7. Somnosuite low-flow anesthesia system (Kent Scientific, catalog number: 13-005-111)
8. Induction chamber (Kent Scientific, catalog number: Somno-0705)
9. Warming pad (Kent Scientific, catalog number: RT-0501)
10. Electric razor with vacuum (Remmington, catalog number: VPG-6530)
11. Tenotomy scissors (Fine Science Tools, catalog number: 14066–11)
12. Ridge-less forceps (Fine Science Tools, catalog number: 11016-17)
13. Germinator 500 (Braintree Scientific, catalog number: GER5287120V)

Procedure

A. Pulverized electrospun PLGA fiber fabrication

For fiber fabrication, electrospinning was used to create ultra-fine fibers from poly(lactic-co-glycolic acid) (PLGA) polymer. The process begins with the preparation of a solution where PLGA is dissolved in an organic solvent (hexafluoroisopropanol), resulting in a homogeneous solution. This solution is then loaded into a syringe fitted with a needle, and a high-intensity electric field is applied between the needle tip and a grounded collector plate at a constant flow rate. The electric field induces the formation of a liquid droplet at the needle's tip, which elongates into a jet and is ejected toward the collector. As the jet travels, the solvent evaporates, and the polymer solidifies into very thin fibers, forming a non-woven mat. This process is driven by the interaction between electrostatic forces and the surface tension of the polymer solution, and it depends on factors such as the solution's viscosity, the strength of the electric field, and the distance between the needle and the collector. Pulverization of PLGA is then performed by breaking down the electrospun fiber mat into fine fragments. This

is achieved through mechanical grinding, where the mat is subjected to shear and impact forces, reducing it to smaller fragments. Milled and un-milled fibers were obtained from Nanofibers Solutions (Columbus, OH) as outlined below:

1. Dissolve PLGA, at a ratio of 82:18 lactide to glycolide, in hexafluoroisopropanol at 6% w/w (Figure 1).

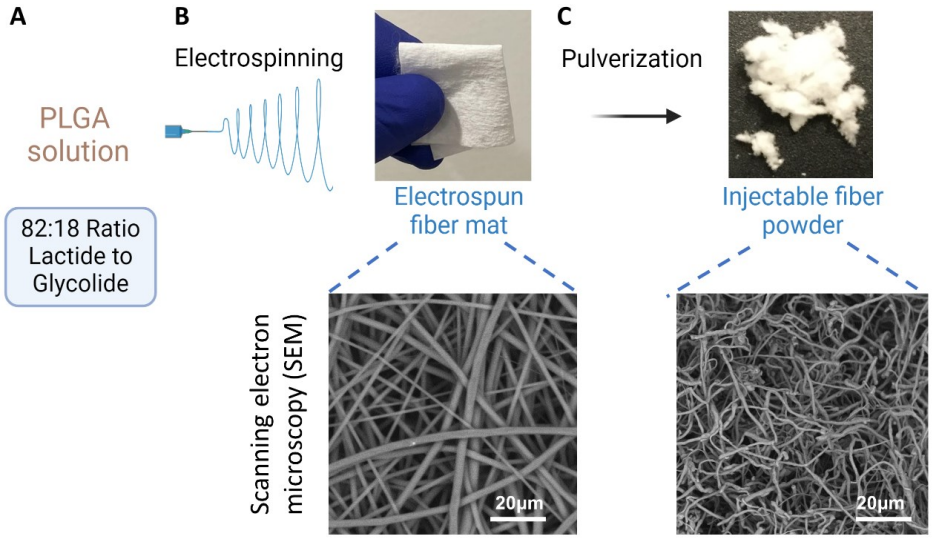


Figure 1. Pulverized electrospun poly(lactic-co-glycolic acid) (PLGA) fibers. A. PLGA at a lactide to glycolide ratio of 82:18. B. Electrospinning to get an electrospun fiber mat. C. The resulting PLGA injectable fiber fragments. Scanning electron microscopy (SEM) micrographs of the PLGA before and after the milling process.

2. Mix at room temperature for at least 24 h.
3. Electrospin the polymer solution at +12 kV/-3 kV at a flow rate of 5 mL/h until reaching a 0.1–0.3 mm thick nanofiber sheet at room temperature and humidity with a tip-to-collector distance of 20 cm using an 18-gauge needle.
4. Evaporate the solvent from the nanofiber sheet for ~16 h at room temperature.
5. Cut the electrospun mat into 1 cm wide strips and feed into the mini mill. This will grind the fibers into smaller fragments.
6. Perform milling with the 60-mesh sieve for the first pass. Then, re-run the fibers that passed through that sieve through the 40-mesh sieve mill. Then, run the fibers that passed through that sieve through the 20-mesh sieve to achieve the final desired particle size.

B. Co-injectable nanofiber and adipose tissue preparation

Adipose tissue was sourced from Cosmetic & Plastic Surgery of Columbus via standard minimally invasive procedures such as single port cannula liposuction; no further characterization of the tissue was done (See General Note 1).

Note: Preparation should be done in a sterile environment for best results.

1. Transport the tissue on ice and place it in appropriate containers (See General Note 2).
2. Centrifuge at 195× *g* for 1 min at 4 °C (Figure 2).
   Note: This step will separate the tissue from oils and anesthetics that can permeate the tissue during liposuction surgery.

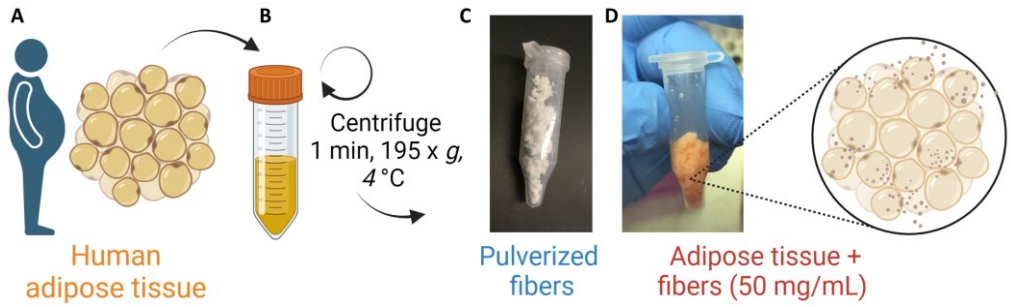


Figure 2. Co-injectable nanofiber and adipose tissue. A. Adipose tissue collected from patients using standard liposuction procedures. B. The tissue is centrifuged to separate fat oils and anesthesia from viable cells. C. Pulverized fibers (50 mg) are combined with (D) adipose tissue (1 mL) to form an injectable solution (50 mg/mL).

3. Discard excess oils and debris.
4. Place 50 mg of fibers into a 2 mL tube.
5. Add 1 mL of tissue to the fiber-containing tube (See General Note 3).
6. Mix tissue and fibers into a 3 mL syringe and cover the end with parafilm for surgical use.

C. Fat engraftment surgery preparation

1. Sterilize all surgical tools, surgical trays, and materials before surgery.
   Note: Between same-day animal surgeries, clean instruments with 70% ethanol solution and sterilize them using the germinator prior to the next surgery.
2. Set and maintain the surgical warming pads at 37 °C during surgery.
3. Place mouse cages onto a slide warmer set to 37 °C.
   Note: Each mouse should be placed in individual cages to prevent agitation of the wound site.
4. Weigh each mouse and record baseline weights.
   Note: Baseline weight will be compared with post-surgery values to evaluate mouse recovery in the form of post-implantation weight loss.
5. Completely remove hair from the incision area 24 h prior to surgery. Shave hair with the razor, apply Nairing cream directly to the skin with a cotton tip applicator, and leave it for 10 s. Remove the cream entirely with warm water and cotton balls.
   Note: It is crucial to remove the Nairing cream thoroughly to prevent serious skin burns.

D. In vivo fat engraftment surgery

2. Place the mouse into the induction chamber (see General Note 4).
3. Set the Somnosuite low-flow anesthesia system at 5% isoflurane in room air at ~500 mL/min and induce flow into the induction chamber to anesthetize the mouse (see General Note 5).
4. At full anesthetic depth, move the mouse to the nose cone on the warming pad.
   Note: Confirm the anesthetic depth of the mouse by testing the toe-pinch reflex.
5. Quickly set the anesthesia system at 1.5%–2% isoflurane at ~250 mL/min and induce flow into the nose cone for the duration of the procedure.
6. Apply artificial tear ointment to the mouse's eyes to maintain moisture.
7. Apply betadine followed by isopropyl to the incision area using cotton tip applicators (repeat this step three times).
8. Create a 1-inch incision horizontally on the upper and lower dorsal area using ridge-less forceps to gently pull the skin taut, allowing for an easier cut (Figure 3).

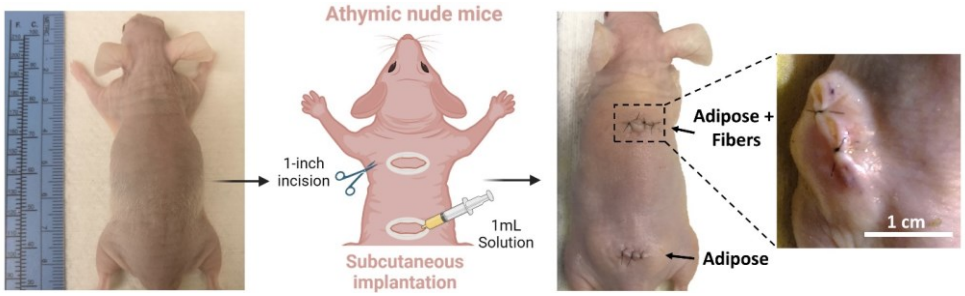


Figure 3. Fat engraftment surgery. Prepare the back of the athymic nude mice for injection. Create a 1-inch incision horizontally on the upper and lower dorsal area for the injection of adipose + fibers and their internal control with only adipose tissue, respectively.

9. Form a cavity under the mouse's skin by detaching the surrounding fascia using tenotomy scissors.
10. Push approximately 100–120 µL of warm saline into each side of the incision.
11. Connect a 3 mL syringe loaded with adipose tissue and fibers to a 4 mm cannula.
    Note: Inject adipose tissue alone as a control, as opposed to the adipose tissue–fiber combination treatment.
12. Push 1 mL from the syringe load into the cavity (see General Note 6).
    Note: Larger syringes (>3 mL) may be required to push the adipose tissue and fibers through the 4 mm cannula, as this can facilitate the displacement of larger volumes while generating less pressure, minimizing the risk of clogging. The syringe may have to be pumped back and forth to ensure that any remaining contents are injected, and it may require readjusting the cannula and extension of the syringe to push in all of the load.
13. Close the cavity by suturing the skin with 6–0 braided sutures along the incision in a single interrupted suture pattern.
14. Clean the skin of the mouse using an alcohol pad.
15. Seal the incision line using liquid glue/bandage.
16. Remove the mouse from the nose cone, weigh the mouse, and record post-surgery weight.
17. Place the mouse into a new, clean cage and allow the mouse to recover.
18. Monitor the status of the mouse until fully awake (1–5 min).
19. Keep mice on the slide warmer at 37 °C throughout the duration of the study.
20. Place mash (i.e., wetted food) and hydrogel in Petri dishes into each mouse cage to facilitate fluid/food intake.

Data analysis

Scanning electron microscopy (SEM) is widely used to characterize the physical properties of electrospun fiber scaffolds before and after milling, evaluating the effects on porosity, pore size, and fiber diameter, which impact cellular responses.

To assess the biocompatibility of the milled fibers with adipose tissue, in vitro cultures of adipocytes with the fibers can be used to run viability assays (e.g., live/dead kit) and fluorescence microscopy for quantifying live cells. Similarly, in vivo biocompatibility can be evaluated through subcutaneous implantation in immunocompetent mice, followed by histological analyses (e.g., hematoxylin and eosin staining) to detect cytoarchitecture differences and immunohistochemistry to analyze immune cell infiltration and expression of inflammatory mediators, as previously described [19].

For evaluating volume retention after adipose tissue implantation, width and length measurements are commonly used to assess graft volume [20–22] (Figure 4). Additional methods include water displacement, magnetic resonance imaging (MRI), computed tomography (CT), and ultrasound. Immunostaining is employed to verify graft vascularity using endothelial markers (e.g., CD31, VEGF, bFGF, lectin) [23,24]. Laser speckle imaging (LSI) can

be used to evaluate graft perfusion, while flow cytometry, MRI, ultrasound, and micro-CT provide further insights into vascularization by measuring endothelial marker expression at the single-cell level (see General Note 7). When using this model, sample sizes should be estimated based on a power analysis. It is recommended to include at least four biological and technical replicates per group in each experiment. Additionally, to minimize bias, blinding of the animals or experimental groups is advised.

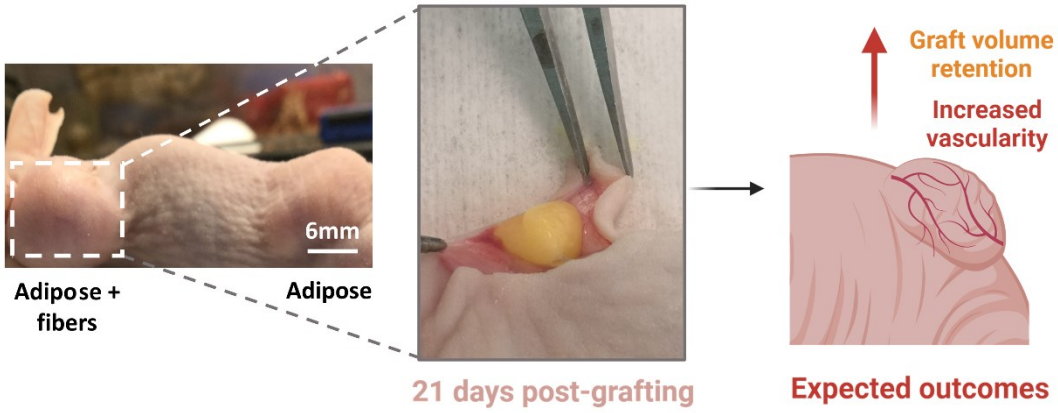


Figure 4. Fat engraftment outcomes. Monitor the volume retention regularly between the day of engraftment and the final day post-engraftment. Remove the graft and evaluate volume retention, displacement, vascularization, and perfusion.

Validation of protocol

This protocol has been used and validated in the following research article:

- Das, et al. [19]. Injectable pulverized electrospun poly(lactic-co-glycolic acid) fibers improve human adipose tissue engraftment and volume retention. Journal of Biomedical Materials Research Part A (Figures 1 and 3).

General notes and troubleshooting

General notes

General note 1: Engraftment outcomes can be affected by the source of adipose tissue, with decreased cell proliferation being linked to factors such as increasing donor age, higher body mass index, diabetes mellitus, and other comorbidities [25].
General note 2: To ensure maximum cell viability, keep tissue samples on ice unless otherwise specified.
General note 3: While this study focused on the application of PLGA fibers, the protocol can be adapted to substitute or incorporate other materials, allowing for modified properties.
General note 4: Consider mouse characteristics such as age, sex, and housing status (single vs. group) as these factors may influence outcomes. This procedure is most commonly performed on mice aged 6–9 weeks. It is important to evaluate the immunoreactivity profile using immunocompetent mice. In this study, we used wildtype C57/BL6 (Jackson Laboratories, stock #000664) mice to test the immunoreactivity of PLGA fibers via subcutaneous implantation. Once the immunoreactivity was evaluated, male athymic nude mice (Jackson Laboratories, strain #002019) were used due to their immunodeficient nature, which allows for the study of human tissue engraftment without immune response interference [26]. Evaluating the immune response is crucial since it could lead to cell death, inflammation, and calcification of the engrafted fat tissue, complicating the study of fat engraftment.
General note 5: While the duration of the procedure and recovery period may vary, we recommend anticipating

approximately 21 min from the initial induction of the mouse with isoflurane (step D2) until the completion of the procedure (step D16). Post-procedure, the mouse should be awake and alert within 3–5 min of the postoperative recovery period (step D18).

General note 6: In this study, human adipose tissue was engrafted into a mouse model. It is important to note that outcomes may vary as different adipose tissue sources or engraftment models are used, as the results are specific to human adipose tissue engraftment in mouse models.

General note 7: One limitation of this study is the evaluation period of adipose tissue engraftments. In this study, evaluations were conducted over 21 days, but a longer period can fully assess the host's response.

Acknowledgments

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Competing interests

Jed Johnson is a co-founder of Nanofiber Solutions, LLC. (Columbus, OH).

Ethical considerations

All animal experiments were performed in accordance with protocols approved by the Laboratory Animal Care and Use Committee at The Ohio State University (IACUC # 2016A00000074-R1 and IACUC # 2009A0006-R3).

All studies involving human adipose tissue were completed in accordance with a protocol that was approved by the Institutional Review Board (IRB) no. 180822–2. Informed consent was obtained by all human subjects prior to study.

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