Supplementary material

Handling of data and specimens

Appropriate data transfer and use agreements (DTUA) and material transfer agreements (MTA) will be obtained. Clinicodemographic data (Table 4) will be de-identified and shared via secure email with the coordinating center. Facial photographs will be shared via secure email and stored in a password protected file on a password protected institutional computer. Biological specimens will be labeled with their content, the date/time of collection, and a unique patient identifier code that does not include any of the 18 Health Insurance Portability and Accountability Act (HIPAA) identifiers. Special care will be taken to ensure that harvested nerves avoid contact with any kind of marking pen ink. Published results will exclude identifying protected health information.

1st Stage Surgical Specimens: Three 2 milliliter (ml) Eppendorf tubes will be used for sample collection, designated for the distal donor facial nerve branch, proximal sural nerve graft, and distal sural nerve graft, respectively. These specimens will be initially preserved in regular formalin, with a subsequent transfer to ethanol exactly 24 hours later. They will then be transported to the coordinating center via FedEx courier services. These samples will be stored and shipped at room temperature.

2nd Stage Surgical Specimens: The only harvested sample (distal tip of the CFNG) will be divided into two Eppendorf tubes.

- The first tube will undergo the same process as the 1st stage samples (initial preservation in formalin with a switch to ethanol at exactly 24 hours, stored at room temperature).
- The second tube will contain a facial nerve fixative solution for electron microscopy composed of 2.5% Glutaraldehyde/4% Paraformaldehyde/0.1 molar (M) Cacodylate buffer pH

7.4. Please refer to “Instructions for facial nerve fixative preparation” below for more information on the exact preparation process. This specimen will need to be refrigerated at -4°F if it is going to be stored for more than 48 hours.

Upon transfer, the coordinating center will be responsible for embedding, processing, and storing the samples received from the corresponding author’s institution. The biopsies will be placed in 3% glutaraldehyde, postfixed in 2% buffered sodium tetroxide, and embedded in epoxy resin. The plastic molds will be cut into 1-µm sections using the microtome, stained with toluidine blue, and imaged at 10X and 40X per established by Terzis et al. [11]. Axon counts will be manually counted by a blinded investigator.

Microscopy: We will utilize immunohistochemistry (IHC) and antibody method for the first stage of surgery that includes 3 samples fixated in formalin and embedded in paraffin. For the second stage of surgery, we will have two identical samples from graft, one will be fixated in formalin and the other will be fixated in glutaraldehyde, embedded in epoxy plastic and then stained with heavy metal to allow direct histomorphometric analysis. The formalin fixated samples will be analyzed via IHC and antibody method with light microscopy (3 from first stage and 1 from second stage). The glutaraldehyde fixated sample will be analyzed via electron microscopy (1 sample from the second stage). This will allow a good comparison of axonal count between baseline (first stage) and post-regeneration (second stage), as well as accuracy and reliability of light microscopy and electron microscopy.

Instructions for facial nerve fixative preparation:

1. Sodium cacodylate buffer (0.2M) at pH 7.4

Reagents

- 43.0 g sodium cacodylate trihydrate (Electron Microscopy Science, Hatfield, PA 19440Cat # 21140)
- 1000ml distilled H2O

Instructions

- Fill a 1L graduated cylinder with 800 mls distilled water.
- Add 43.0 g sodium cacodylate trihydrate and stir until completely dissolved.
- Adjust pH with 1 N HCL to pH 7.4. Fill the cylinder to 1000mls with distilled water.
- Vacuum filter with Millipore Express Plus 0.22 µm Vacuum Driven Disposable Bottle top filter.
- Store in 4°C refrigerator.

2. 25% Glutaraldehyde Solution (Electron Microscopy Sciences, Hatfield, PA 19449, Cat # 16220)

3. 20% Paraformaldehyde Solution (Electron Microscopy Sciences, Hatfield, PA 19449, Cat # 15713)

4. In a 100 ml graduated cylinder:

- Add 10 mls of 25% Glutaraldehyde solution
- Add 20 mls of 20% Paraformaldehyde solution
- Add 50 mls of 0.2M Cacodylate buffer pH 7.4
- Add 20 mls of distilled water

Note: The Glutaraldehyde and Paraformaldehyde solutions are made in water. By using 50 mls of 0.2M buffer along with the rest of the reagents in water, the final buffer concentration is 0.1M.