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Non-invasive gene electrotransfer in skin

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Short title: ID GET

ABSTRACT

The skin is considered as well suited for gene therapy and vaccination. DNA vaccines elicit both broad humoral and cellular immune responses when injected in the skin. Physical and chemical methods are needed to boost the expression. Gene electrotransfer (GET) is one of the most effective approaches. This step by step protocol describes the procedures to get an efficient GET targeted to the skin by using easy-to-use non-invasive electrodes after intradermal plasmid injection (ID GET). A specific pulse sequence is reported. Expression is observed by *in vivo* fluorescence imaging during more than 2 weeks as the plasmid was coding for tdtomato. The protocol is efficient for the transient expression of clinical proteins.

KEYWORDS

Gene transfer, Electroporation, skin

INTRODUCTION.

The skin is not only a protective barrier that shields the body from external pathogenic agents. This organ is considered as well suited for gene therapy and vaccination, due to its accessibility, large surface area and the presence of numerous immune cells such as dendritic and Langerhans cells that can elicit appropriate immune responses ^{1,2}.

DNA vaccines have been shown to elicit both broad humoral and cellular immune responses when injected in the skin ³. However, due to the barrier properties of the skin, the cellular penetration of DNA and resulting applications of skin DNA immunization are limited. Several chemical, biological and physical methods have been implemented to enhance skin DNA delivery (see for review ⁴). The highly efficient viral gene transfer presents several side effects such as generation of novel infectious agents due to the risk of generating replication competent virus, immunogenicity of the vector and mutational insertion of viral DNA. Safe non-viral gene transfer approaches should be used where gene electrotransfer (GET) appears as the most promising ¹.

The structural morphology of mouse skin displays successive layers: stratum corneum (cornified dead cells), epidermis (epithelium), dermis (connective tissue) and hypodermis (adipose tissue). The epidermis is a stratified, squamous epithelium layer. The epidermis composed mainly of keratinocytes contains dendritic cells and Langerhans cells, involved in the immune system. The hypodermis fat also has immune-modulatory functions, and macrophages within adipose tissue should be considered as antigen-presenting cells ⁵. Gene transfer can transfect adipocytes within the hypodermis, but most transfected cell types were in the dermis and epidermis ⁶. Therefore, skin appeared to be a good target for immunization with an easy access ^{7,8}.

Skin gene electrotransfer (GET) is obtained by a local intradermal injection of a small volume of a plasmid solution followed by electrical voltage pulses delivery between electrodes in contact with the skin (ID GET). GET results from an electrophoretic driven accumulation of the plasmid (pDNA) towards the surface of the target cells, that are electropermeabilized ⁹. The electric parameters that control the pDNA electrophoretic drift

in the tissue and the level of target cell membrane permeabilization should be selected to preserve the cell viability¹⁰.

A complex pulse sequence (so called HV LV) where a short high field pulse (to trigger membrane permeabilization) is followed by a long low field pulse (to electrophoretically push the plasmid towards the permeabilized surface) is considered as the most effective to obtain a high level of expression¹¹. This sequence has to be optimized for each cell system as the cell viability must be preserved. The total production of the transgene is controlled by the tissue volume that is affected. This volume is under the dependence of the electrode design. For dermal GET, the field distribution must focus its high value only in the thin layer of the skin. This is obtained by non-invasive electrodes in contact with the skin as the stratum corneum is permeabilized within microseconds, and its contribution to the field distribution in the skin tissue becomes negligible¹². Several electrode models have been described. The skin can be pinched between plated electrodes and the field is across the skin and the tissue underneath, where expression is detected¹³. An array of small rod-shaped electrodes (multi-electrode array (MEA)), in contact with the skin brought a pattern of localized expression. Transgene-expressing cells were observed exclusively in the epidermal layer of the skin due the very localized field distribution¹⁴⁻¹⁶. The surface intradermal EP (SEP) device where the electrodes are minimally invasive brings expression deeper in the skin⁶.

We introduce a different approach with contact electrodes where the field is focused along the skin and where a large skin volume can be positively electropermeabilized¹⁷. This approach was used for electroimmunization in anti-angiogenesis developments¹⁸. We optimize the pulse protocol for this set of contact electrodes for ID GET by using a more sophisticated pulse generator. A reporter gene coding for a fluorescent protein allowed a quantified follow-up by using animal imaging. Histological investigation of the pulsed skin shows no damage in the tissue.

MATERIALS

Sterile phosphate-buffered saline (PBS) pH 7.2. (Gibco, Thermofischer)

Plasmids adjusted at 1 mg/ml in sterile PBS (see Troubleshooting 1).

Prepare 1 ml per animal plus 10 % to compensate the loss when filling the syringes.

Hair removal lotion (Veet, France)

Ethanol 70 %.

Sterile syringes with needle 29 G × 1/2 in (Terumo)

B-10 electropulsator (Betatech, St Orens, France)

Contact electrodes ¹⁷.

Gel for electrodes (Comepa, St Denis, France)

Pico oscilloscope 2000 serie, (Picoscope St Neots, UK)

Current clamp EN3 (Chauvin Arnoux, Paris, France)

Fluorescence Macroscope (Leica, Wetzlar, Germany) with a cooled CCD camera (Roper, USA)

MICE:

Animal studies are conducted in accordance with the principles and procedures outlined by the European convention for the protection of vertebrate animals used for experimentation.

Female C57Bl/6 mice 6 to 9 weeks old were obtained from Janvier laboratory (Le Genest St. Isle, Saint Berthevin, France) (see troubleshooting 1)

PLASMIDS:

pCMV-tdTomato Vector is a 5.4 kb plasmid DNA coding for tdTomato fluorescent protein (from Clontech, Mountain View, CA), amplified in *Escherichia coli* DH5 α and purified with the Maxiprep DNA Purification System (Qiagen, Germany) according to the manufacturer's protocol (see troubleshooting 2).

EXPERIMENTAL PROCEDURE**Intra-dermal DNA injection:**

Hair on the back is removed with a hair removal lotion (Veet, France) 2 days before each electro-transfection. Skin inflammation may result from using razor rather than cream.

Animals are kept under isoflurane/air anesthesia during the whole procedure. The comfort of the lab animal is very important and it should not be stressed. Gas anesthesia is therefore better than chemical injection approaches. Anesthetize the mouse in a closed box with 4 % isoflurane under a high flow rate 400–500 ml/min to make it deeply asleep quickly. This is obtained within about 1 min. The closed box can be opened (under a hood if possible). You can then manipulate the mouse and transfer it on the electropulsation stage (see troubleshooting 8) with an inhalation mask where a lower flow (200-250 ml/min) of isoflurane at a lower dose (2%) is continuously delivered during the GET experiment (18). Clean the naked skin with ethanol 70 %.

Mice are injected in two sites with 25 μ g of plasmid in 25 μ l PBS by intra-dermal (ID) route using a 300 μ l syringe with a 29G needle (Terumo, France) (Fig.1). The injection should take no less than 5 seconds to avoid hemolytic transfection (see troubleshooting 3).

A mark with a permanent ink can clearly indicate the area of the skin where the injection was performed.

Electric pulses delivery

Animals are kept under gas anesthesia.

We used non-invasive wire contact electrodes designed to focus the field in the tissue layer close under the skin between the wires (Fig.3). Treated skin surface is 0.5 cm².

Apply conducting gel (Comepa, St Denis, France) on the edge of the electrodes to ensure good electrical contact with the skin surface. The gel should be present only on the electrodes and should not build a short-circuiting bridge between them (see troubleshooting 4). Hair removal prevents short circuits along the hairs.

Place the electrodes so that the bleb on the skin (see Troubleshooting 3) is between their two contacts with the skin.

Hold the contact electrodes firmly against the skin as a muscle contraction is present during the pulse delivery.

Following ID injection of plasmid DNA, an electrical field pulse train is applied on each injection site with 10 mm long x 1mm diameter contact wire electrodes. The distance between the electrodes is 4 mm.

A Square wave unipolar pulse train is delivered by a voltage pulse generator (ELECTRO cell B10 Betatech, St Orens, France) (see troubleshooting 5).

The proper delivery of the pulses from the pulse generator is monitored on-line on the touch screen. A current-clamp with a wide bandwidth (Chauvin Arnoux, Paris, France) connected to a digitizer (Picoscope, St Neots, UK) is used to register the delivered current profiles. The monitoring of both the voltage and the delivered current allows a perfect control of the electrical operating conditions and the reproducibility of the procedures. All data are stored on-line on a PC laptop to keep an accurate follow-up of the experiments (see troubleshooting 6).

A mixed (HV-MV) train of electric pulses is delivered. High Voltage (HV) pulse consists in 100 μ s square waved pulses of 400 V followed by Medium Voltage (MV) pulse that is 20 ms square waved pulse of 100 V. The intermediate time delay was 50 ms. Pulses in the train are applied at the frequency of 1 Hz. 4 successive pulses are present in the train. All these settings are selected from the pulse generator (Fig.2).

Muscle contractions are observed along each pulse of the train (4 along the procedure) (see troubleshooting 7).

Remove the mask for isoflurane from the mouse nose and observe the animal until it has fully recovered from anesthesia (see troubleshooting 8). Anesthesia lasts only 2-3 min.

Clean the electrodes with ethanol 70%.

Pulses are delivered at only one injection site. The second is used as a control of the lack of expression when the plasmid is only injected.

Expression

Shave and depilate the mice to avoid the auto-fluorescence of the hair.

Maintained the mice under isofluorane anesthesia during the imaging procedure.

Expression of fluorescent proteins is detected in vivo by fluorescence imaging (Macrofluor microscope (Leica, Wetzlar, Germany) equipped with a cooled CCD camera (Roper Coolsnap HQ, Photometrics, Tucson, AZ) using the 0.57 magnification).

The exposure time is set at 1 s with no binning.

Color imaging is obtained by use of CRI Micro*Color 2 Liquid Crystal Technology. The fluorescence excitation is obtained with an EL6000 light source (Leica, Wetzlar, Germany) and the ET mCH/TR (λ_{ex} = 560/40 nm, λ_{em} =630/75 nm) filter sets (Chroma technology, Rockingham, USA). This procedure allows analysis of vector expression on the same animal during several weeks.

For images acquisition and quantification, the MetaVue5.2 software (Universal, Downingtown, PA, USA) is used. Images are processed for contrast and brightness. High-resolution images of 1392×1040 pixels are captured directly on a Dell PC.

Imaging is observed during 20 days after the treatment. Expression (fluorescence emission in the red) is detected after 2 days and peaks at 8 days (see troubleshooting 9).

A quantitative analysis is obtained from the digitized images. We use the NIH Image J software. Processing is obtained on the red channel of the RGB image. Expression (fluorescence emission) is observed only in the tissue present between the electrodes. The profile reflects the distribution of the injected solution where a bleb is obtained and confirms the expected field distribution (Fig.4) (see troubleshooting 10).

No fluorescence is observed at the injection site where no pulses are delivered

Histology

At day 0 and day 7 post-treatment, the mice were sacrificed,

The region of the skin exposed to the treatment was excised with a scalpel. At D0, samples were collected 3h after GET or PBS injection.

Samples were fixed 24H in 10% neutral buffered formalin and then stored in 70% ethanol

After deshydration, skin was embedded in paraffin.

5 µm thick sections were cut in the direction perpendicular to the skin layers.

After rehydration, staining with hematoxylin and eosin was performed.

The samples were mounted in Eukkit medium (Sigma).

Observation was performed with a scanner (Pannoramic 250, 3DHISTECH) (Fig.5)

TROUBLESHOOTINGS

1. The thickness of the skin is variable between animals according to their age and sexe; this affect the field distribution and as a result the topology of expression. Avoid fat (male) animals¹⁹.
2. tdTomato plasmid codes for a protein with an emission in the red (wavelength longer than 630 nm). Its expression is easily detected as its spectral range is in skin optical window, where light scattering and absorption are limited and do not alter the detection of the emission²⁰. Td Tomato fluorescence is photostable; its absorption coefficient is high as well as its quantum yield²¹.
3. This step needs practice. ID injection in mice skin has been described for RNA (22). A similar procedure was adapted for pDNA injection in female mice with the appropriated needle. One should be careful in the speed of injection, that must be proceeded in a 5-7 s duration. The formation of the bleb and its observation are very important as they tell us when the pDNA is present and is a guide for positioning the parallel electrodes (Fig.3). The proper ID delivery of the pDNA is shown by a whitening of the skin where the needle is present. No leak of the solution should be observed. A delay between the 25 μ l injection and the removal of the needle should be observed to obtain a decrease in the pressure induced by the injected volume in the skin.
4. The conducting gel is needed to obtain that the electrical contact with the skin is present to make sure that the current will flow in the tissue¹⁸. It should be present all along the electrodes to get the expected field distribution in the tissue. Be careful to clean any trace of gel present on the skin between the two electrodes and linking them. This will build a short circuit and the current will be only along the skin surface and would not penetrate in the tissue. No muscle contraction will be observed and the current will be high.

5. Other pulse generators can be used. The IGEA “cliniporator” and the BTX “Agile pulse” with the “Pulse Agile” technology can deliver the pulse sequence similar to that was used with the B10 electropulsator (2 0.05 ms HV rather than a single 0.1 ms with the BTX generator).

6- This is achievable with the 3 pulse generators mentioned in Trouble shooting 5 with either a direct display or by transfer with an USB key of a CSV file.

7. The proper pulse delivery is shown both by the current transient and the muscle contraction.

8. As the body temperature is observed to decrease along anesthesia, one should either place a heating lamp above the animal or keep the mouse on a heating blanket to ensure successful recovery.

9. A more sustained expression would be obtained by using minicircle plasmid vectors^{23, 24}.

10. This confirms that the injection step is critical for a high level in expression.

CONCLUSION

This protocol is effective with different reporter gene coding plasmids (GFP, minicircles, PgP rich and free constructs). A large surface of the skin is showing a rather homogeneous expression. The long-lived expression suggests that the thin layer of subdermal striated muscle (panniculus carnosus) is affected, a contribution of the MV pulse²⁵. This confirms the Comsol simulation¹⁷ where the field affects all skin layers as with minimally invasive electroporation device⁶. The treatment is very fast as the train of pulses is delivered in 3 s. No deleterious effect is observed. Present investigations confirm its potentiality for transient effects of expression of clinically relevant plasmids coding for IL12.

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Author Contributions: LP designed, performed the biological experiments and contributed in writing the paper; SC designed and performed the biological experiments; EB performed the animal imaging and contributed in writing the paper; MPR contributed in writing the paper; JT and MG designed the main concepts of the experimental work, checked the results and wrote the manuscript.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist

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Figure 1: Visualization of the injection point. Mice were injected i.d. in two sites with 25 μ l of a 1mg/ml DNA plasmid solution. A bleb on the skin is present at each injection site

Fig.2 Settings the parameters on the B10 touch-keyboard

By using of the touch screen (inside the red circle), the proper pulse parameters (durations T, voltages U, interval between the pulses T_i, delay in the train P, number of pulses R) are set (as shown on the right to obtain the HVMV pulse displayed on the lower part of the figure).

Fig.3 Contact electrodes covered with the conducting gel are brought in firm contact with the “shaved” skin of the mouse overlapping the bleb due to the plasmid injection

Fig.4 Imaging of dt Tomato expression after skin GET 8 days after the treatment

No skin damage is observed by the direct light observation. The fluorescent image in the red wavelength is the expression of the gene (size 1 cm x 1 cm). Image analysis gives quantitative results in pseudo-colour.

Fig.5 Histological images

Representative eosin/hematoxylin images, at days 0 and 7, of control skin only injected with PBS (left) and skin electroporated with HVMV pulses in presence of plasmid (right).

Scale bars : 1mm for 2X images and 100 μ m for 20X images.