ON CHIP SPATIOTEMPORAL ELECTRIC FIELD SHAPING TO LOCALLY ELECTROPORATE CELL MARKERS INTO MOUSE EMBRYONIC TISSUES

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ABSTRACT

We have developed a microsystem and the associated protocols for the electroporation of genetic markers into mouse embryos. Noticeably, using 3D insulating guides and adjusting the electric pulse sequence we could target discrete regions of these delicate organisms and thus follow tissue movements by imaging fluorescent proteins.

KEYWORDS

Transgenesis, Development, Morphogenesis, Embryo culture, Cell viability.

INTRODUCTION

During embryonic development, interactions between cell groups enable the growth, migration, and specification of various tissues. The dynamics of these phenomena can be unraveled by manipulating gene expression spatiotemporally, for instance thanks to electroporation of DNA constructs [1]. However, 5.5 to 7.5 days-old mouse embryos are very small (~ 150 − 500 µm long) and necessitate new dedicated tools. Sharp metallic electrodes generate harmful chemical species (e.g. gas, protons) [3] and cannot be approached close to the outer epithelium in order to localize transgenesis [2]. Thus, an "electrodeless" strategy [4] has been set up where the electric field is generated by remote platinum pads and focused by dielectric microtunnels [5] (Fig. 1a). Moreover, we systematically investigated cell viability and pore generation so as to establish reliable protocols. The device we here propose is easy-to-use and allows a precise, safe, and reproducible targeting of desired regions in early post-implantation mouse embryos.

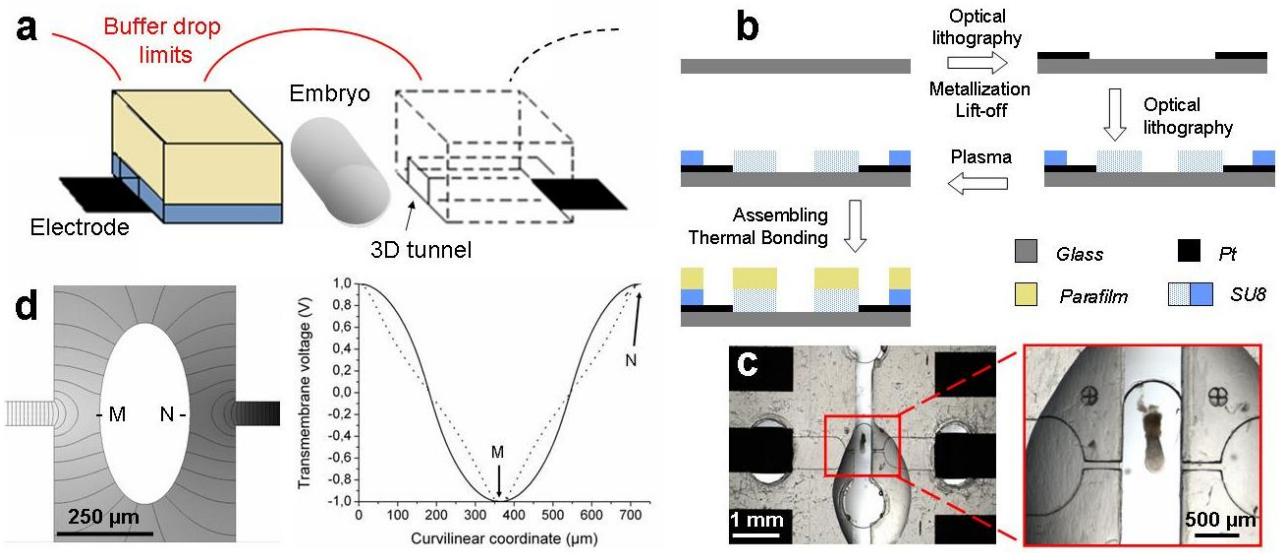

Figure 1. (a) Schematic representation of the device. (b) Corresponding fabrication including platinum electrodes deposition on a glass wafer, dielectric guides patterning in SU8-2050, plasma hydrophilization, and Parafilm covering the assembly. (c) Optical micrographs of the chip welcoming a E6.5 embryo. (d) 2D FEM computation of the electric potential in the transgenesis chamber and of the voltage drop across the membrane of the embryo outer cells. The normalized results for 3D tunnels [···] are compared with the ones for bare electrodes having the same geometry [—].

EXPERIMENTAL

We realized the microsystem on a glass substrate, according to a straightforward four steps process (Fig. 1b): (i) the electrodes were obtained by patterning nLOF2070 (AZ Electronic Materials) by optical lithography, evaporating a 20 nm thick of Ti and a 50 nm thick of Pt film, and lifting it in acetone. The resulting features consisted in pairs of aligned stripes, 2 mm large and 30 mm long, separated by 5 mm gap; (ii) the 90 µm high and 60 µm large dielectric guides, as well as the electroporation chambers and the electrode reservoirs were photolithographed in SU8-2050 (MicroChem), a resist allowing high aspect ratio structures; (iii) the polymer surface was rendered hydrophilic using a 300 mTorr air plasma treatment for 50 secondes in a plasma cleaner reactor (Harrick); (iv) the tunnels were closed by thermal bonding at 60 °C of a 127 µm thick Parafilm (Pechiney) ceiling in which appropriate access holes had been punched (Fig. 1c).

Electric potential profiles were computed with the finite element method (FEM) software Comsol Multiphysics

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3.5, using the Conductive Media DC application mode. More precisely, we solved the Laplace equation, i.e. $\Delta V = 0$, in two dimensions over the domain associated with the buffer presence [5]. Both SU8 walls and embryo surface – the latter being modeled as an ellipsoid – were considered insulating (Fig. 1d). Conversely voltages at both electrode tips were fixed.

Freshly dissected mouse embryos were immersed in a 15 µL drop of the electroporation solution that had been deposited on the device so as to cover the electrodes. According to the biomolecule to inject, the 150 mM NaCl 20 mM Hepes buffer, pH 7.0, was supplemented with either 2 mg/mL of FITC-dextran (FD4, Sigma) or 1µg/µL of pCAG-nls-mCherry Electrotransfection was carried out using a TSS20 Ovodyne electroporator (Intracell) directly connected to the microsystem. A train of three square pulses, between 10 and 50 ms long – spaced by 1 s, was selected and the voltage was tuned to yield a 150 to 350 $V.cm^{-1}$ electric field within the drop. After electroporation completion, the embryos were rinsed in the dissection solution (DMEM-GlutaMAX, 4.5 g/L glucose – Invitrogen – supplemented with 25 mM Hepes and 10 % inactivated fetal calf serum). Subsequently, groups of 3 embryos were placed into 200 µL of culture medium (DMEM-GlutaMAX, 4.5 g/L glucose, 100 µg/ml streptomycin, 100 U/ml penicillin, supplemented with 75 % inactivated rat serum) and incubated in 8-wells chambers slides (NUNC) under standard cell culture conditions (37 °C, 5% CO2) for up to 24 h. To differentiate necrotic cells from normal ones, embryos were incubated in 25 ng/ml Propidium Iodide (SIGMA) in dissection medium for 20 min at 37°C.

Embryo positioning during electroporation experiments as well as immediate fluorescence visualization were achieved on a M165FC stereomicroscope (Leica) equipped with GFP and Cy3 filters and connected to a Cool Snap HQ camera (Photometrics). This apparatus was selected because it enables easy embryo manipulation and because it minimizes UV exposure, hence preventing adverse effects related to phototoxicity. However, for detailed investigations, embryos were fixed 20 min in 4% Paraformaldehyde at room temperature, rinsed in PBS containing 0.02 % Tween-20 and incubated 20 minutes at room temperature in 10µg/ml Hoechst 33342 (Invitrogen) in PBS/0.02% Tween-20. Embryos were imaged in PBS/0.02% Tween-20 using a LSM 710 Laser Scanning Microscope (Zeiss).

RESULTS

To decrease the number of cells touched by the electric field, the 2D guides of the first prototype [2] had to be closed by a ceiling [5]. Yet, we found difficult to fill with buffer the resulting 3D structures because they were built in hydrophobic NOA81 [5]. Therefore, we changed technology: the present tunnels are made of SU8 walls which hydrophilicity is systematically improved by air plasma treatment prior to Parafilm lid mounting (Fig. 1a-c). Incidentally, when compared with the previously used 'microfluidic sticker' process [6], fabrication is here greatly simplified.

The guides shape has been devised to facilitate a fine tuning of the transmembrane voltage drop that drives electroporation and to exploit the full working range of the electroporator. Moreover, FEM simulations point that microtunnels yield an even better localization than metallic microelectrodes. Indeed, the curve displaying the transmembrane voltage at the embryo surface exhibits more pronounced variations for the 'electrodeless' device than for a system realized with two bare electrodes of the same size (see the computed profiles around M and N on Fig. 1d). The latter device would result in a larger electroporated area because of the lateral branching of electric field lines all along the metal stripes; on the contrary with tunnels a confinement is ensured up to their openings [5].

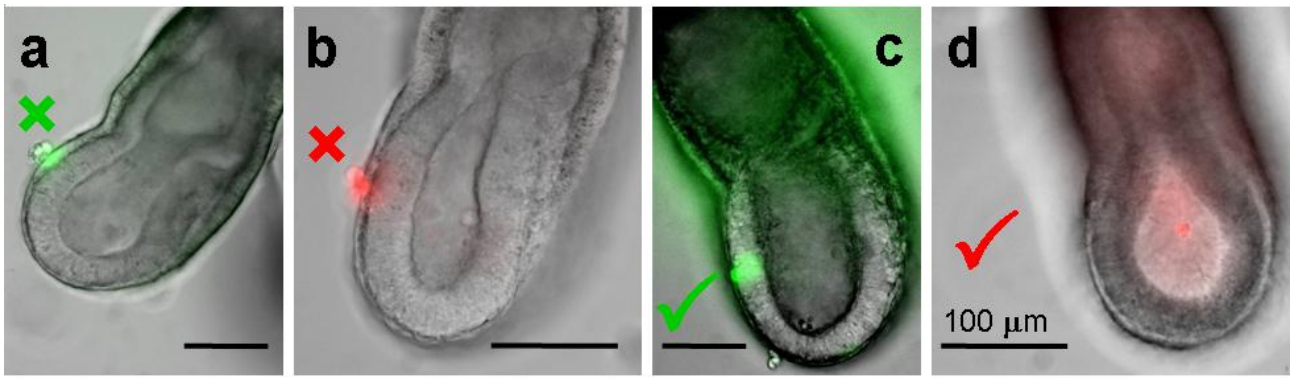

Figure 2. Poration and cell viability tests performed on E6.5 embryos. Bright field and fluorescence images were superposed for two different settings, either (a-b) 6 pulses at 25 V for 100 ms, spaced by 1 s, or (c-d) 3 pulses at 15 V for 50 ms, also 1 s apart. Pore formation is indicated by the penetration of the 4 kDa fluorescein labeled dextran (a and c) whereas dead cells are revealed by staining with Propidium Iodide (b and d).

It is first interesting to note that no bubble was found inside the tunnels during electroporation, all the electrochemical reactions taking place in the dedicated reservoirs. Thus, reproducibility was optimal, as expected.

We could then develop protocols to introduce genetic markers into mouse embryos at 5.5 and 6.5 days of development (E5.5 and E6.5 respectively). Employing two membrane impermeant fluorophores, we started studying the influence onto cell viability and pore generation of the various electrical parameters (i.e. the number of pulses, their intensity and length). Applied during electroporation FITC-dextran, a large polymer, reports on pores formation; used

for subsequent staining propidium iodide, an intercalator, indicates cell death (Fig. 2). We observed that pulses should not exceed 20 V - 50 ms to avoid necrosis (Fig. 2a-b) and should remain larger than 10 V – 20 ms to stay efficient (Fig. 2c-d). Furthermore, E6.5 embryos were found more fragile than E5.5 ones. For the former the best conditions are 3 pulses at 15V – 30 ms, which results in more than 50 % poration and less than 50 % mortality. For the latter, with 3 pulses at 15V – 50 ms no cell death was observed although incorporation of the fluorescent marker was 100 % successful.

In a second series of experiments we performed lineage tracing in E5.5 *Hex-GFP* transgenic embryos that specifically express the green fluorescent protein in their distal visceral endoderm (DVE) cells [7]. Electroporation of a plasmid coding for a nuclear mCherry red fluorescent protein was achieved with one microtunnel facing the DVE (Fig. 3a). It resulted in the overlapping of GFP and mCherry signals after 24 hours culture (Fig. 3b-c). Additionally, in agreement with the literature, DVE cells tagged on the distal side of embryo (Dist.), moved towards the anterior side (Ant.) [7].

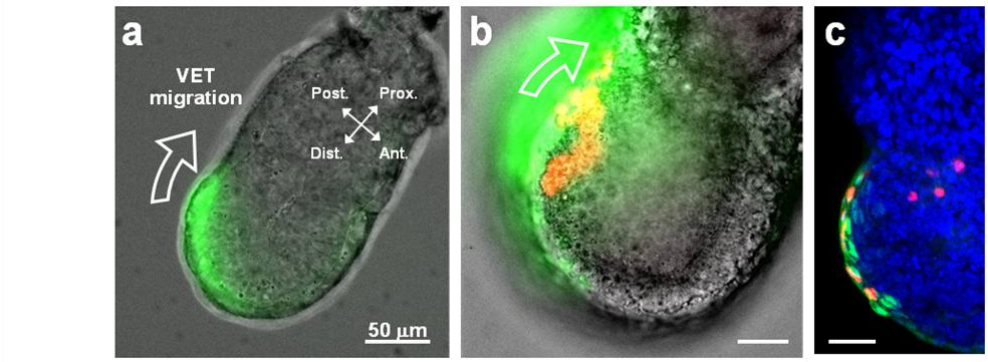

Figure 3. Targeting of DVE cells in a E5.5 *Hex-GFP* transgenic embryo. Bright field and fluorescence images were superposed (a) 3 hours and (b) 24 hours after electroporation with a plasmid coding for mCherry dissolved at 1 µg/µL. The dielectric guide was positioned as shown in blue and 3 pulses at 15 V for 50 ms were applied, 1 s apart. The second observation was performed 24 hours later, the DVE cells having migrated according to the white arrows. Colocalization was confirmed by confocal imaging (c), the blue color reporting for nuclear DNA staining with Hoechst.

CONCLUSIONS

Although localized electroporation of early stage mouse embryos is extremely difficult we succeeded in gently introducing a transgene in a given tissue. More specifically, we were able to efficiently target DVE cells and to follow their movement after 24 h of in vitro culture. The present 'electrodeless' strategy results in localized modification of gene expression while keeping the various species generated by the electric pulse (e.g. gas, protons) far away. Our microchip will help deciphering the dynamics of developmental events by performing lineage tracing studies or loss and gain of function experiments involving morphogens [8].

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