# Application of direct current electric fields to cells and tissues *in vitro* and modulation of wound electric field *in vivo*

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**It has long been known that cells can be induced to migrate by the application of small d.c. electric fields (EFs), a phenomenon referred to as galvanotaxis. We recently reported some significant effects of electric signals of physiological strength in guiding cell migration and wound healing. We present here protocols to apply an EF to cells or tissues cultured in an electrotactic chamber. The chamber can be built to allow controlled medium flow to prevent the potential development of chemical gradients generated by the EFs. It can accommodate cells on planar culture or tissues in 3D gels. Mounted on an inverted microscope, this setup allows close and well-controlled observation of cellular responses to electric signals. As similar EFs are widely present during development and wound healing, this experimental system can be used to simulate and study cellular and molecular responses to electric signals in these events.**

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## INTRODUCTION

Endogenous d.c. electric fields (EFs) occur naturally *in vivo*. This was first demonstrated in wounds by Emil DuBois-Reymond[1]. More than 150 years ago, he measured electric currents of approximately 1 μA flowing out of a cut he made in his own finger. Using various modern techniques, including micro-glass electrodes and vibrating probes, we and others have detected a similar electric current flow in wounds in both the skin and cornea of every species we have studied, including human skin[2–6]. In cornea and skin, a laterally oriented, wound-induced EF is generated instantaneously when the epithelium is damaged, and it persists until re-epithelialization restores the electrical resistance barrier function of the epithelium. These EFs are estimated to be at least 40–50 mV $mm^{-1}$ at cornea wounds and 100–150 mV $mm^{-1}$ at skin wounds[2–6]. Growing experimental evidence suggests an important role for such electric signals in directing cell migration in wound healing[6–14].

Endogenous d.c. EFs have been measured during development and regeneration and after damage to non-epithelial tissues[10,11,14–20]. These EFs arise because of spatial and temporal variations in epithelial transport of charged ions such as $Na^+$, $K^+$ and $Cl^-$, and spatial variations in the electrical resistance of epithelial sheets. Disruption of the endogenous electrical gradients during development induces skeletal and neural abnormalities[16,19,21]. It has been shown that the spinal cord responds to damage by generating large and persistent electrical signals, and in turn applied electric stimulation can promote spinal cord repair in human and other mammals[22–25].

On the grounds that there are endogenous EFs, and disruption of these EFs disrupts wound healing and development, research has been conducted into cellular response to EFs for several decades. Among the various signals hypothesized to guide cell migration and division in development and wound healing, electric signals have not been well studied. The biological and medical research community generally is not familiar with the possible roles of EFs as a directional signal in guiding cell migration to heal a wound. The experimental techniques available to study how electric signals control cellular behaviors are therefore only known by a limited number of researchers.

Many *in vitro* experiments show that EFs of strength equivalent to those measured *in vivo* control important cell behaviors such as directional cell migration (galvanotaxis or electrotaxis) and cell division orientation[14,26–34]. Our recent letter to *Nature* provides further experimental evidence that the electric signal as a directional cue probably plays a far more important role in directing cell migration in wound healing of epithelium than previously believed. We also reported in the letter two genes important for EF-induced cellular response[35]. After publication, there has been strong demand that we make our "technological expertise widely available so that others may embark on these investigations"[36].

### Experimental setup

The protocols we use are based on those pioneered and used by a handful of laboratories to apply EFs to cells *in vitro*[27–29,31,32,37–44]. The basic electro-physical theories backing up these protocols include calculating field strength and current, 2D and 3D resistivity and units. These theories have been illustrated in detail by Robinson for both endogenous and applied electrical currents[45], which need to be studied and understood properly to calculate the relevant bioelectrical properties of the system. We have modified and used electrotactic chambers to accommodate cells growing in planar culture or in 3D gels, *en bloc* tissue cultures in 3D and some small embryos, such as those from frog and zebra fish. We have therefore demonstrated that it is also possible to apply EFs to *in vivo* systems. The EF is applied to the cells or tissues cultured in a custom-designed electrotactic chamber (**Box 1** and **Fig. 1**) via agar salt bridges, Steinberg's solution and Ag/AgCl electrodes. The depth of the electrotactic chamber is adjustable to accommodate different thicknesses of the samples while maintaining a reasonably stable voltage and current flow, temperature, calcium level and pH in the chamber. It is possible to apply EFs to cells and tissues for extended periods up to several days while cell behaviors are monitored

## BOX 1 | SYSTEM PREPARATION FOR APPLICATION OF ELECTRIC FIELDS

*For planar cell cultures*

1. To confine the cells to the region of the electrotactic chamber, we use a glass well made from cover slips (**Fig. 1a,b**) that is removed before adding the roof of the chamber (**Fig. 1h**). Prepare 22 × 11–mm$^2$ cover glass strips by cutting 22 × 22–mm$^2$ cover glasses (thickness no.1) in half with a diamond pen. Glue four pieces of cover glass together with 3140 silicone to make the glass well shape as in **Figure 1a,b**. Be sure to align the two cover glasses parallel to each other and perpendicular to the other two. The interior dimensions of the glass well should be 22 × 10 mm$^2$. For larger sample collection purposes, for instance collection of protein samples for western blotting analysis, more cells are required, and therefore longer cover glass strips (50 × 11 mm$^2$) should be used—glue two pieces of 50 × 11 mm$^2$ cover glass with two pieces of 22 × 11, 40 × 11 or 50 × 11–mm$^2$ ones at both ends with the same shape as above. The interior dimension of this glass well should be 50 × 10, 50 × 20 or 50 × 40–mm$^2$, respectively. Allow the glass wells to dry completely overnight, then sterilize by autoclaving before use. See **Figure 1a** for a schematic drawing and **Figure 1b** for a photograph of the actual assembled glass well. The cover glass strips can be stored in a 60 °C oven for up to 1 month.
▲ CRITICAL STEP The glass wells must be sealed tightly to avoid any leakage during cell subculture. The four pieces of cover glass comprising the glass well must be maintained free-standing and intact during the drying period. Pre-cast moldings to support the glass wells during drying are recommended.
2. Prepare the cover glass strips for the side walls of the electrotactic chamber (see **Fig. 1c**). The length of the cover glass strips can be adjusted to suit experimental purposes. For cell migration/division experiments, 22 × 11–mm$^2$ glasses are prepared by cutting 22 × 22–mm$^2$ cover glasses (thickness no. 1) in half with a diamond pen. For larger sample collection purposes, longer cover glass strips (50 × 11 mm$^2$, no. 1) are required to accommodate the 50 × 10, 50 × 20 or 50 × 40–mm$^2$ glass wells prepared in step 1 above. Cover glass strips need to be sterilized in an autoclave and fully dried in a 60 °C oven before use. The cover glass strips can be stored in a 60 °C oven for up to 1 month.
3. This step is optional. For high-magnification experiments with an oil objective lens, prepare glass-based Petri dishes. Drill circular holes of diameter 35 mm in the center of 100-mm Petri dishes. Autoclave a 48 × 64–mm$^2$ cover glass and dry completely in a 60 °C oven. Glue the sterilized cover glass with DC4 in the center of the dish to cover the hole. Sterilize prepared dishes under UV light overnight.
4. Assemble the electrotactic chamber. First glue the sterilized cover glass strips to the Petri dishes (100 mm) with DC4, making sure they line up in parallel to each other with a gap of either 10, 20 or 40 mm in between (see **Fig. 1c,d**). Then build up four strips of 3140 silicone rubber blockers (barriers) at each side of the cover glass strips, leaving a gap of 10 mm at each end of the electrotactic chamber (see **Fig. 1d**). Make sure 3140 silicone runs to the edge of the Petri dish side wall for a watertight seal. The chambers can be stored at room temperature (25 °C) for 1 week.
▲ CRITICAL STEP The gap between two cover glass strips must be no more than 10, 20 or 40 mm to avoid leakage of cell culture medium. Allow a minimum of 24 h for 3140 silicone to solidify completely.
5. This step is optional. Certain cell types require the culture surface to be pre-coated with fibronectin, collagen, laminin or another substratum. Various pre-coated Petri dishes are commercially available, which is convenient for experiments at lower magnification. In this case follow the instruction in steps 1, 2 and 4 above. For higher-magnification experiments using oil objective lenses, surface coating needs to be done on glass-based Petri dishes before the experiments. Use glass-based dishes prepared in step 3 above then follow the instruction in step 4 above. Investigators need to follow the manufacturer's instruction to pre-coat materials of interest.

*For application of electric fields to 3D culture*

1. Prepare the glass wells as shown in 'For planar cell cultures' step 1, above.
2. Prepare cover glass strips or slide glass for making the side walls of the electrotactic chamber. Depending on the thickness of the tissue/organ to be studied, either no. 0, 1, 2 or 3 cover glass or ordinary microscope-slide glass can be used. Several cover slips can also be glued together with DC4 to achieve an appropriate height for the thickness of the tissue/organ. As above, use a diamond pen to cut cover glass or slide glass into equal-sized strips, autoclave and dry completely before use.
3. Assemble the chamber as shown in 'For planar cell cultures' step 4, above.

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continuously. Modification of the system will allow high-resolution imaging using cover glass–based dishes as well as lower-magnification imaging for big cell sheet movement in tissue or organ culture with 3D tracking of cell migration and cell division *in vitro* and *ex vivo*.

Migration rates of different types of cells vary significantly. Some fast-moving cells, for instance neutrophils or *Dictyostelium discoideum*, migrate at rates of 20–30 μm min$^{-1}$ (refs. 35,46). Electrotactic experiments with those types of cells are relatively easy, as the temperature, calcium level and pH are normally very stable within the 30–60-min duration of experiments. Some other types of cells migrate very slowly, so it takes longer to record a detectable distance change and to quantify the rate and direction of cell migration. It is therefore crucial to be able to maintain the stability of the temperature, calcium level and pH in the EF chamber. In our hands, embryonic stem cells, progenitor cells and neurons migrate slowly; hence time-lapse recording is normally required for approximately 5–8 h. In this protocol, we provide data from continuous recording of changes in temperature, calcium level and pH in electrotactic chambers of different dimensions, with various types and volumes of media at the reservoirs and subjected to different voltages. These data provide a useful reference for choosing appropriate experimental conditions (see PROCEDURE and ANTICIPATED RESULTS). A temperature-control unit was designed on our imaging system to provide a stable ambient temperature. The depth of the electrotactic chamber should be kept to a minimum to reduce the Joule heating effect (see PROCEDURE). On exposure to the air (low atmospheric $CO_2$, 0.04%), pH changes in the chamber can be observed when high voltage is applied and the electric current is strong. Routine cell culture media use sodium bicarbonate as the primary buffering system to stabilize pH[47]. As most of our experiments with EF application are done outside $CO_2$ incubators, we use the following methods to control pH in the electrotactic chamber: (i) placing an

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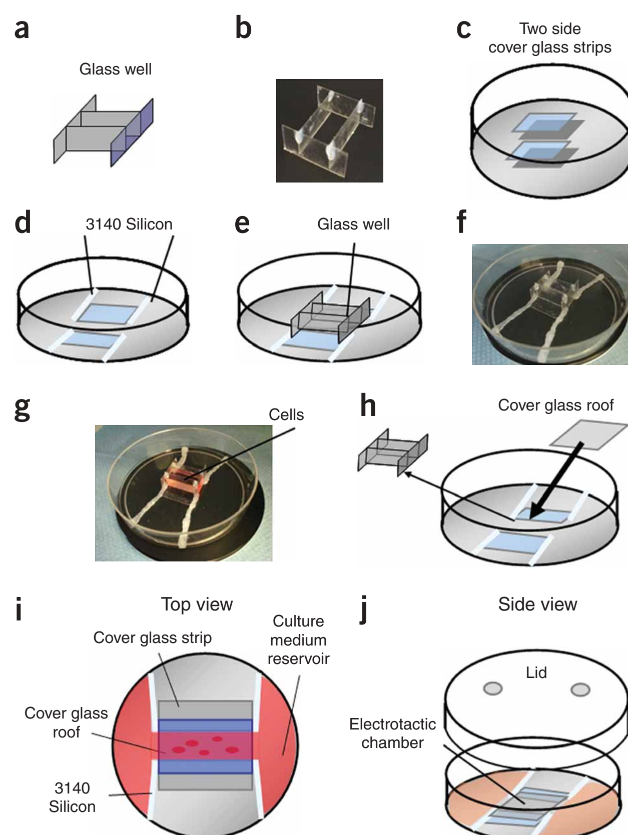


**Figure 1 |** Electrotactic chamber. (**a**,**b**) Glass well. (**c**,**d**) Setting up of the electrotactic chamber base, which consists of two cover glass strips glued in parallel onto a Petri dish 10 mm apart and four 3140 silicone blockers, which build up two isolated reservoirs at the sides of the electrotactic chamber. (**e–g**) Placing the glass well on top of the electrotactic chamber base before and after seeding cells in the well. (**h**) The glass well is removed from the electrotactic chamber before electric field (EF) application, and a cover slip roof is glued onto the cover glass strips, which completes the electrotactic chamber. (**i**) The top view of the electrotactic chamber covered with the cover glass roof. (**j**) The electrotactic chamber covered with a lid, which is ready for an EF experiment.

incubator around the microscope with an additional 95% $O_2$/5% $CO_2$ supply, (ii) using commercially available $CO_2$-independent medium if possible, and (iii) adding HEPES buffer into the medium (see PROCEDURE). Optimal experimental conditions can be achieved by a combination of temperature-control unit with $CO_2$ supply and $CO_2$-independent medium/HEPES-buffered medium.

**Application of EFs to 3D cultures, tissue blocks or embryos**

Application of EFs to 3D cultures, tissue blocks or embryos is technically demanding, especially when there is a need to experiment for a long period of time. A deeper electrotactic chamber is required if thicker tissues are to be studied. This inevitably increases heat generation in the chamber owing to the Joule effect. We have used several configurations for 3D tissue culture to minimize the temperature rise as a result of the increase in the depth of the chamber. Depending on the thickness of the tissue to be studied, the depth of the side panels of the electrotactic chamber can be adjusted (see PROCEDURE). Another common problem of 3D culture is *en bloc* movement of the tissue/organ in the chamber. The tissue/organ tends to move or slide away from the original position if not immobilized, which makes the tracking of cell behavior in the block difficult and unreliable. We have developed techniques to stabilize tissue blocks with either Matrigel or fibrinogen/aprotinin/thrombin gel (see PROCEDURE).

Examples of EF application to 3D cultures include the aortic ring angiogenesis model, cornea scratch wound model, skin wound healing model and chicken embryo development model. EF-controlled angiogenesis can be studied using aortic rings harvested from rat or mouse and cultured in a 3D electrotactic chamber. Newly regenerated blood vessel–like structures could be detected from day 1 after embedding the aortic rings into 3D gels, and these are detectable most obviously on day 3. EF application could produce a polarized regeneration of vessel-like structures toward the anode (see ANTICIPATED RESULTS). The cornea scratch wound model is used to study the EF-modulated cell migration and division in 3D; it can be incorporated into the 3D electrotactic chamber system for *ex vivo* study[33,35] or performed *in vivo* utilizing the wound-induced endogenous EF and pharmacological approaches (see ANTICIPATED RESULTS). The skin wound healing model can be used both *ex vivo* in a 3D electrotactic chamber and *in vivo*, and is very useful to study the cross-reaction between two or three different types of cells, for instance fibroblasts and keratinocytes. Chicken embryo development and angiogenesis can also be monitored in 3D electrotactic chambers under EF application (data not shown).

This electrotactic chamber system can be easily set up in conjunction with a confocal microscope, which is useful for a variety of purposes, including pH-reporter dye use and membrane recruitment of signaling molecules. A vibrating microprobe system is currently used in experiments involving pharmacological manipulation of endogenous EFs[6].

Application of EFs can generate chemical gradients in the culture chamber. Although the interaction of chemical cues and electrical cues is highly likely *in vivo*, it is critical to exclude or minimize this interaction in electrotactic experiment. This can be achieved using a ‘cross-flow’ electrotactic chamber so that a continuous medium flow can be maintained to disrupt possible chemical gradient build-up[28] (see **Box 2**). In the absence of media cross-flow, the pH/ion/chemical gradient build-up depends on the EF strength and length of application. Under low-voltage or short-term EF treatment, the influence of these gradients is minor; therefore setting the cross-flow is not always necessary, as discussed in details in ref. 35 (see Supplementary Figure 8 from ref. 35). This is especially helpful when the preparations will not tolerate cross-flow—for example, extremely delicate settings or preparations that must not be moved at all owing to long shutter times in confocal microscopy. The cross-flow system is recommended for (i) high-voltage EF application, (ii) long-term EF application, (iii) experiments that require absolutely no interference with pH/ion/chemical gradients and (iv) comparison experiments of different effects between pH/ion/chemical gradients and EF treatment.

**Pharmacological manipulation of endogenous EFs *in vivo***

To test the effect of electric signals *in vivo*, we developed an alternative approach to that achieved by using electrodes, by modulating endogenous wound EFs pharmacologically in a cornea wound healing model *in vivo*. Wounding the tissue instantly

## BOX 2 | SYSTEM SETTING OF CROSS-FLOW SYSTEM ● TIMING Approximately 1 h

This requires similar preparation as described in Step 2A in the PROCEDURE. The only difference between the standard chamber and a cross-flow chamber is that instead of two 22 × 11–$mm^2$ strips glued to the Petri dish, four 9 × 11–$mm^2$ strips are glued onto the dish as indicated in **Figure 2c**. Add two extra 3140 blockers at each side of the gap in the middle of the strips. Secure silicone tubes in place to generate a closed circulation system (see **Fig. 2c**). Secure the silicone tubes in two timer-controlled pumps. Connect one tube to a fresh medium source and the other tube to a waste beaker.

Investigators need to evaluate the cross-flow speed and volume to suit the individual experiment plan. The flow system can be set either at a constant flow or at an interval controlled by the timer attached to the pumps. Make sure the fresh medium source does not run empty.

▲ CRITICAL Make sure all the tubes and containers for fresh medium are sterilized before the experiment. Sterile techniques are required in all procedures.

See **Table 1** for further guidance.

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generates an endogenous electric current that can be measured directly[4,6,7,22,48]. Pharmacological or chemical agents that modulate ion transportation are applied to a wound to either enhance or decrease ion transportation, and thus to enhance and decrease endogenous wound EFs accordingly[6,28,33,35]. Adams and Levin have contributed in-depth studies of using pharmacology to dissect endogenous bioelectric signals[49]. The effects of such modification on wound healing can be studied *in vivo*. Our protocols for applying drugs to modulate EFs are also provided (see **Box 3**). Cornea wound healing is used as a model system in this protocol.

The whole protocol can be completed in 2 d.

## MATERIALS

### REAGENTS

- Primary cultured cells or cryopreserved cells (or cell lines) derived from human, mouse or other species of interest
- Cell culture medium
- Heat-inactivated serum (Sigma, cat. no. F4135)
- PBS, without calcium or magnesium (Sigma, cat. no. P4417)
- Trypsin–EDTA (GIBCO, cat. no. 25300054)
- Soybean trypsin inhibitor (GIBCO, cat. no. 17075029)
- $CO_2$-independent medium (GIBCO, cat. no. 18045) (see REAGENT SETUP)
- DMEM with 25 mM HEPES (GIBCO, cat. no. 12430-054)
- HEPES buffer (GIBCO, cat. no. 15630-049)
- L-GIn (GIBCO, cat. no. 25030149)
- Penicillin/streptomycin (GIBCO, cat. no. 15140148)
- Steinberg's solution (see REAGENT SETUP)
- Agar (Sigma, cat. no. A7002)
- Coating material (fibronectin, laminin, collagen, etc. if required) or pre-coated Petri dish
- Matrigel basement membrane matrix (BD Biosciences, cat. no. 354234)
- Fibrinogen/aprotinin/thrombin (Sigma, cat. nos. F4883, A6279, T4393) for 3D gel (see REGENT SETUP)
- Hypnom and diazepam
- Balanced salt solution (BSS), artificial tears (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, with pH adjusted to 7.4)
- Ophthalmologic fluorescent dye strips
- 3D gel (see REAGENT SETUP)
- Aminophylline (10 mM), prostaglandin (0.1 mM), ascorbic acid (1 mM), $AgNO_3$ (1 mM), ouabain (10 mM), furosemide (1 mM)

### EQUIPMENT

- Direct current power supply (CONSORT E332, voltage range 3–125 V, current range 2–200 mA, precision required 1 V per 1 mA) and cables

## BOX 3 | PHARMACOLOGICAL MODULATION OF ENDOGENOUS WOUND ELECTRIC FIELDS ● TIMING Approximately 2 d

1. Anesthetize the experimental animals with anesthetic(s) of choice. We have used intramuscular hypnom (0.3 ml $kg^{-1}$) and intraperitoneal diazepam (0.5 ml $kg^{-1}$).

! CAUTION Experiments involving live animals must conform to national and institutional regulations.

2. Under the dissecting microscope, mark the corneas with a shallow incision using a trephine. The size of the circular wound depends on the purpose of the experiments and the size of the trephine can be adjusted accordingly (normally 2–3 mm for mice, and 3–5 mm for rats).

▲ CRITICAL STEP Make sure the trephine incision is made to an appropriate depth to leave a clearly visible mark after washing with tear solution and to keep the stroma intact. Make sure the circular incision by the trephine is made evenly across the whole area.

3. Wash with sterilized artificial tear balanced salt solution (BSS).
4. Under the dissecting microscope, gently scrape off the cornea epithelium within the marked circular area using ophthalmologic surgical scalpels.

▲ CRITICAL STEP Make sure to use equal force with the scalpel across the whole area to scrape off the whole thickness of the epithelium, avoiding damage the stroma underneath.

5. The circular wound can be tested and visualized by fluorescent dye staining (see **Fig. 3a**).
6. The positive-effect drugs aminophylline (10 mM), prostaglandin $E_2$ (0.1 mM), ascorbic acid (1 mM) and $AgNO_3$ (1 mM) or the negative-effect drugs ouabain (2 mM) and furosemide (1 mM) can be used to enhance or reduce the endogenous wound electric fields (EFs), respectively. Investigators need to decide which drugs are suitable for their individual experiments. Please refer to Adams and Levin's recent book chapter for full details[49]. Apply three drops of drug topically onto the wounded cornea every 2 h, for up to 36 h. The endogenous wound EFs can be modulated pharmacologically and maintained until the wound healing process is complete (**Fig. 7b,c**).

▲ CRITICAL STEP Make sure to establish appropriate controls by applying sterile artificial tear BSS on identical wounds.

## BOX 4 | PREPARATION OF CULTURE MEDIUM

It is inevitable that the pH of the medium will change during electrotaxis if $CO_2$-dependent media are used, especially during a long experiment. To minimize this, two types of media are available [$CO_2$-independent medium as above or the special medium of choice with 10–25 mM (final concentration) HEPES buffer added]. $CO_2$-independent medium is recommended for cells that do not have a special requirement for culture medium. Cells that need a special culture medium require the addition of HEPES buffer to maintain the pH during experiments, and the final concentration of HEPES for each type of cell needs to be tested by investigators before experiments. For the majority of the cells we studied, $CO_2$-independent medium gave the best stability of pH, usually up to 4–5 h without changing the medium in the system. If the time-lapse experiment is longer than 4–5 h, it is important to exchange the medium in the electrotactic chamber no later than 4–5 h after the experiment starts in order to stabilize the pH. Make sure to warm up the fresh medium before exchanging the old medium in the system. For cell types that require a special culture medium, for example keratinocytes, which require keratinocyte growth medium, or stem cells, which require special knockout medium with serum replacement, add 10–25 mM HEPES into the final medium immediately before the experiment.

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- Custom-designed resistance box (optional, to fine-tune the voltage; suggested resistance values are between 4 and 28 kΩ)
- Voltage meter
- Standard glass slides
- No. 0, no. 1, no. 2 cover glass, size 22 × 22 $mm^2$, 22 × 40 $mm^2$ and 48 × 64 $mm^2$
- Silver wire electrode (Advent, cat. no. AG549109)
- Platinum wire, diameter 0.38 mm (World Precision Instruments, cat. no. AGT1510)
- Silicone tubing for cross-flow experiment, diameter 2 mm
- Tissue culture Petri dishes with 10-mm diameter
- Glass tubes (inner diameter approximately 7 mm)
- Electrical insulating compound silicone grease (DC4; Dow Corning)
- Silicone rubber compound (Dow Corning, cat. no. 3140)
- Glass-cutting diamond pen or glass saw
- Gas burner
- Pumps with timer control
- Time-lapse imaging system, ideally with functions of *X*/*Y*/*Z* multiple position recording and multiple wavelength recording, as well as a $CO_2$-supplied temperature control chamber incorporated onto the microscope; we are currently using the MetaMorph imaging system (Molecular Devices, UK)
- Hoffman modulation contrast/Nomarski DIC microscope
- 3–5 mm Trephine (a circle-cutting surgical instrument)
- Ophthalmologic surgical scalpels
- Dissecting microscope

### REAGENT SETUP

**Steinberg's solution** 60 mM NaCl, 0.7 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.3 mM $CaNO_3 \cdot 4H_2O$, 1.4 mM Tris base, pH 7.4. For convenience, it is recommended to keep 10× concentrated stock solutions of each individual reagent and to prepare from the stock solutions. Steinberg's solution can be prepared and sterilized through autoclaving in advance and kept at 4 °C for up to a month.

**$CO_2$-independent medium** 10–15% heat inactivated serum, 1% L-GIn, 1% penicillin/streptomycin and 1% non-essential amino acid.

**Preparation of culture medium (See Box 4).**

**3D gel** Fibrinogen (Sigma, cat. no. F4883) 2 mg $ml^{-1}$, aprotinin (Sigma, cat. no. A6279) 250 STV, medium of choice, thrombin (Sigma, cat. no. T4393) 0.625 μ $ml^{-1}$. For example, 225 μl fibrinogen and 3 μl aprotinin are required to make up 300 μl final volume. Mix these well with 67.3 μl medium of choice, and then add 4.67 μl thrombin into the mixture immediately before the experiment.

**Agar gel** Dissolve 2% (wt/vol) agar powder into Steinberg's solution and autoclave the mixture. The agar gel can be kept in a sterilized condition at room temperature (25 °C) for up to a month.

### EQUIPMENT SETUP

For full details of how to prepare an electrostatic chamber for use when applying EFs to planar cell cultures and 3D culture see **Box 1**.

For preparation of glass tubes for agar bridges see **Box 5**.

## PROCEDURE

### Preparation of cells ● TIMING Approximately 1 week

**1|** Adapt cells to $CO_2$–independent medium. For best growth performance, some cell types may require either direct (option A) or sequential adaptation (option B) to $CO_2$-independent medium. The number of cells should be predetermined before the adaptation experiment. Cells should be in mid-logarithmic growth phase with high (greater than 90%) viability in either case.

**(A) For direct adaptation**

(i) Inoculate stock cultures at normal seeding densities and incubate using a closed cap in a humidified (37 °C) incubator with 0% $CO_2$.

(ii) Monitor growth daily and subculture cells at 80–90% confluence.

▲ CRITICAL STEP If the cell cultures fail to maintain acceptable growth and viability over three to five passages during direct adaptation, the sequential adaptation method should be used.

**(B) For sequential adaptation**

(i) Initially inoculate cells into a 50:50 ratio (vol/vol) of $CO_2$-independent medium and the current medium.

## BOX 5 | PREPARATION OF GLASS TUBES FOR AGAR BRIDGES

Cut 10-cm lengths of glass tube with a diamond pen or glass saw. Heat the glass tubes in a Bunsen flame at about 4 cm away from both ends and bend the tubes to around 90° to produce Π-shaped bridges (as shown in **Fig. 2a,b**). Autoclave the glass bridges and dry in a 60 °C oven. The glass tubes can be stored for up to 1 month and can be washed and reused.

▲ CRITICAL Wear protective glasses and gloves to prevent injury. Fire-polish both ends of the glass tubes in a Bunsen flame to round-off sharp edges and allow maximum contact with the electrotactic chamber.

▲ CRITICAL Heat-resistant gloves must be worn to avoid burning.

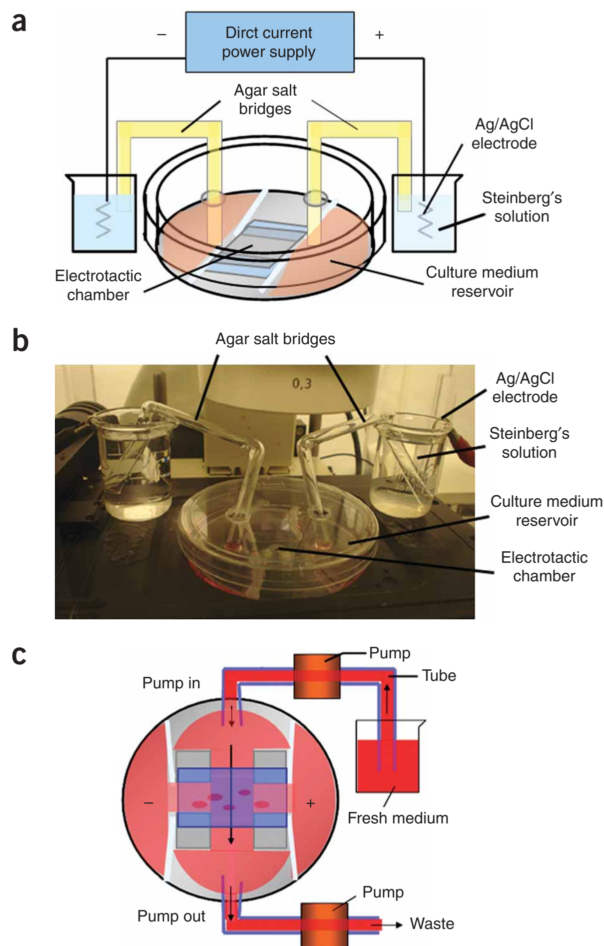


**Figure 2 |** Electrotactic chamber assembly on the microscope stage. (**a**) Schematic drawing of the electric field (EF) application. (**b**) A photograph of the actual assembling of the EF application system. Electric currents from the power supply are passed through the chamber via Ag/AgCl electrodes, Steinburg's solution and agar salt bridges. (**c**) Schematic drawing of cross-flow system setting. For clarity, the agar bridges at the ends of the chamber are not shown. Two silicone tubes are placed at the two reservoirs at the sides of the electrotactic chamber and connected to a pump to keep continuous flow of fresh medium perpendicular to the long axis (EF vector) of the electrotactic chamber.

(ii) Subculture the flasks at 80–90% confluence and inoculate with a 75:25 ratio (vol/vol) of $CO_2$-independent medium and the current medium.

(iii) Subculture subsequently using 100% $CO_2$-independent medium and maintain as described in option A above.

### System preparation for application of EFs ● TIMING Approximately 2 d

**2|** When preparing the system for experiments using planar cells, follow option A. When using tissues, follow option B.

**(A) For planar cell cultures**

(i) Load the sterilized glass well on top of the electrotactic chamber in the dish prepared in steps 4 and 5 of **Box 1**. Make sure the glass well matches the exact position of the electrotactic chamber on the dish (see **Fig. 1e** for the schematic drawing and **Fig. 1f** for the actual assembly).

(ii) Trypsinize off the cells to be studied using trypsin–EDTA and soybean trypsin inhibitor and subculture into the glass well above the electrotactic chamber (see **Fig. 1g**). When you are using $CO_2$-independent medium for EF experiments, cells should already have been directly or sequentially adapted (see Step 1). The number of cells to seed into the electrotactic chambers should also have been pre-determined. Transfer the chambers to a 37 °C incubator with a 5% $CO_2$ supply. Wait 4–12 h for cells to attach to the surface of the chamber.

▲ CRITICAL STEP To avoid drying up of the medium during subculture, the incubator used to maintain the electrotactic chambers should be well humidified. The volume of the culture medium inside the glass well should be at least 1 ml for a 22 × 10–mm$^2$ chamber and 3 ml for a 50 × 10–mm$^2$ chamber.

(iii) Immediately before the experiment, gently wash the glass well with fresh medium several times (either $CO_2$-independent medium or other special medium of choice). Pipette away most of the culture medium from the glass well, then take the glass well from the electrotactic chamber (see **Fig. 1h**). The glass well can be washed and autoclaved for reuse. Glue a no. 2 cover glass roof with DC4 electric compound onto the two cover glass strips to cover the area of the subcultured cells (see **Fig. 1h**). This forms the electrotactic chamber. Add sufficient medium to both reservoirs of the electrotactic chamber, normally 5 ml per reservoir in most of our studies (see **Fig. 1i**).

▲ CRITICAL STEP When gluing the cover glass 'roof' onto the two side cover glass strips, push the cover slip roof down to minimize the depth of the chamber formed. This is important to maintain a stable voltage across the chamber as well as to reduce the Joule heating effect. Take extra care to avoid air bubbles entering the chamber. In general, the more

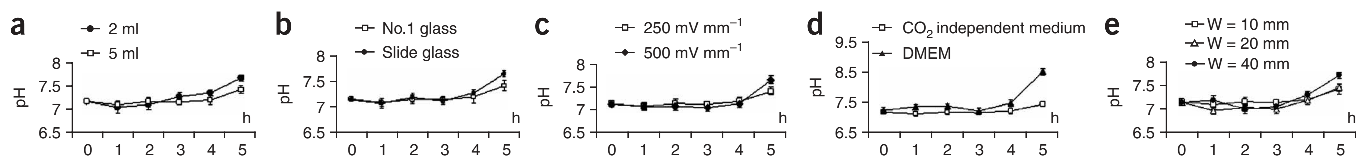


**Figure 3 |** Stable pH is maintained in various conditions for approximately 4 h in an electric field (EF) and much longer in $CO_2$-independent medium. The effect on the pH value of following factors was studied. (**a**) The volume of medium (2 and 5 ml) added to the EF chamber reservoirs; (**b**) chamber depths equivalent to no. 1 cover glass or slide glass; (**c**) different voltages; (**d**) $CO_2$-independent medium and DMEM with 25 mM HEPES; (**e**) different EF chamber widths (10, 20 and 40 mm). Compared with DMEM with 25 mM HEPES, $CO_2$-independent medium yields better capacity to stabilize pH even after 4 h of EF application (**d**). Unless specifically stated, experiments are performed at 37 °C using 5 ml $CO_2$-independent medium in each of the EF chamber reservoirs; the depth of the EF chambers is that of no. 1 cover glass, and the width is 10 mm; EF = 250 mV mm$^{-1}$.

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culture medium added into EF chamber reservoirs, the better the effect in terms of maintaining a stable pH, temperature and calcium level.

(iv) Prepare a lid to cover the dish. Drill two holes on the Petri dish lid corresponding to the ends of the electrotactic chamber. Cover the dish with the lid. The cells are now ready for EF application. (see **Fig. 1j**).

▲ CRITICAL STEP When drilling the holes in the dish lid, make sure the diameter of the holes is not much larger than the diameter of the glass bridges. This is to minimize evaporation of the medium during the experiment.

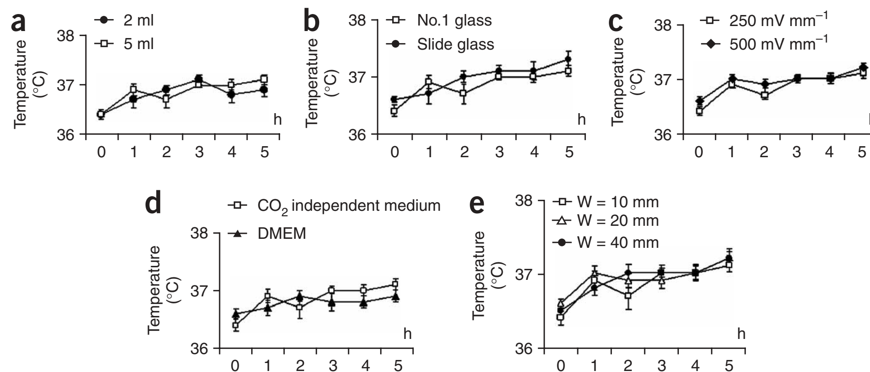


**Figure 4 |** Stable temperature is maintained in various conditions during electric field (EF) treatment. The effect on temperature of the following factors was studied: (**a**) the volume of medium (2 and 5 ml) added to the EF chamber reservoirs; (**b**) chamber depths equivalent to no. 1 cover glass or slide glass; (**c**) different voltages; (**d**) $CO_2$-independent medium and DMEM with 25 mM HEPES; (**e**) different EF chamber widths (10, 20 and 40 mm). Temperatures at time 0 were slightly lower than during the rest of the time course because the incubator doors were kept open for setting up the chambers, electrodes and wiring.

**(B) For 3D cultures**

(i) Prepare Matrigel or fibrinogen/aprotinin/thrombin gel to immobilize the tissue/organ in position. Matrigel has to be transferred to 4 °C the day before the experiment. Pre-cool pipette tips on ice, as well as the dish/chamber to be used. Prepare the samples of tissue/organ to be studied on ice. Transfer the samples to the electrotactic chamber prepared in the "System preparation for application of EFs to 3D culture" part of **Box 1**. Pipette out 100–200 µl Matrigel to cover the samples on the chamber. Depending on the purpose of the study, a smaller volume of Matrigel can be used to cover only the base of the tissue, leaving the top part exposed to the culture medium. Alternatively, fibrinogen, aprotinin and culture medium (either $CO_2$-independent medium or medium of choice) can be mixed before preparing samples, followed by addition of thrombin immediately before immobilization of the samples into the chamber. Investigators need to evaluate which method is more suitable for the tissue block to be investigated.

▲ CRITICAL STEP The whole procedure must be done on ice if Matrigel is used to fix the samples as Matrigel can solidify very quickly at room temperature (25 °C).

(ii) Transfer the chambers to 37 °C to solidify the gel. This takes approximately 30 min for Matrigel and 5 min for fibrinogen/aprotinin/thrombin gel. Add $CO_2$-independent medium or special medium with HEPES supplement to cover the samples. Cover the chamber with cover glass roof (as in **Fig. 1**). The chamber is now ready for EF application.

### Application of EFs ● TIMING Approximately 1 h

**3|** Heat up the agar gel either in a microwave or on a hot-plate until boiling.

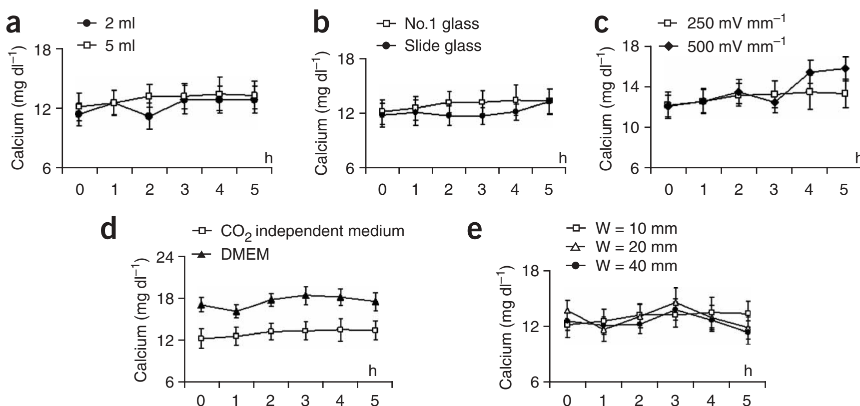


**Figure 5 |** Stable calcium level is maintained in various conditions during electric field (EF) treatment. The effect on calcium level in the medium of the following factors was studied: (**a**) the volume of medium (2 and 5 ml) added to the EF chamber reservoirs; (**b**) chamber depths equivalent to no. 1 cover glass or slide glass; (**c**) different voltages; (**d**) $CO_2$-independent medium and DMEM with 25 mM HEPES; (**e**) different EF chamber widths (10, 20 and 40 mm). Calcium level is stabilized during the whole time course of EF treatment in all conditions studied.

**4|** When the agar gel cools to approximately 60 °C, fill the autoclaved glass tubes with a sterile plastic Pasteur pipette. The agar gel will solidify as it cools. The bridges can be kept in sterilized PBS solution temporarily until the start of the experiment.

▲ CRITICAL STEP When filling the glass tubes with agar gel, be careful to prevent air bubbles entering the tubes.

**? TROUBLESHOOTING**

**5|** If required, turn on the temperature-controlled chamber and adjust temperature setting before the experiment starts. Set up parameters required for the time-lapse recording on the imaging system.

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**6|** Transfer the dish containing cells/samples to be studied to the temperature-controlled chamber of the imaging system and switch on the $CO_2$ when needed. Fill the reservoirs at both side of the electrotactic chamber with plenty of $CO_2$-independent medium or special medium with HEPES (normally 5 ml per reservoir). Cover the dish with the pre-prepared lid with holes. Fill two beakers with Steinberg's solution. Connect the beakers of Steinberg's solution to the medium reservoirs of the electrotactic chamber with the pre-made agar glass bridges through the holes on the lid. Connect the beaker of Steinberg's solution with the d.c. power supply via an Ag/AgCl electrode and electric cables (see **Fig. 2a,b**).
▲ CRITICAL STEP It is essential to establish appropriate controls for EF application by running experiments in the absence of applied EFs.

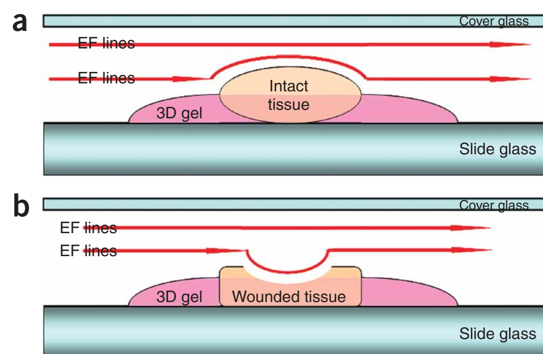


**Figure 6 |** Schematic drawings illustrate the patterns of current flow resulting from applied fields in complex 3D tissues. (**a**) When intact tissues are embedded in the 3D chamber and an electric field (EF) is applied, the EF lines flow in parallel above the surface of the tissue; (**b**) when wounded tissues are treated with EF, the EF lines will run into the wound site, and the EF lines within the wound will run in parallel with the surface of the wound.

**7|** Switch on and adjust the voltage across the electrotactic chamber using a voltage meter.
▲ CRITICAL STEP It is convenient to have thin platinum wires at the end of the voltage meter cables dipped separately into the two reservoirs at the ends of the chamber, so that the voltage setting can be monitored and changed as required during the experiment.

**8|** To use cross flow system (optional), two tubes are connected to the reservoirs at the sides of the electrotactic chamber through times controlled pumps, to provide a continuous flow of fresh medium running perpendicularly to the long axis of the electrotactic chamber (see **Fig. 2c**).

**9|** Start time-lapse recording on the imaging system. If multiple position recording is required, make sure the cables connecting the beakers and the power supply are free to move with the stage.

**10|** Check pH changes every 2–3 h if longer recording is required. When possible, change the medium at the reservoirs every 4–5 h during the experiment.
**? TROUBLESHOOTING**

## ● TIMING

Preparation of reagents: Steinberg's solution, approximately 1 h; culture medium, approximately 30 min; 3D gel, approximately 1 h; agar gel, approximately 30 min
Adaptation of cells to $CO_2$ independent medium: approximately 1 week (optional)
Preparation for 2D EF application (**Box 1**): approximately 2 d
Preparation for 3D EF application (**Box 1**): approximately 2 d
Preparation of agar glass bridges: approximately 1 h
Setting up for EF application: approximately 1 h
System setting of cross-flow system (**Box 2**): approximately 1 h
Pharmacological modulation of endogenous wound EF (**Box 3**): approximately 2 d

## ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

**TABLE 1 |** Troubleshooting table.

| Problem | Possible reason | Solution |
|---|---|---|
| Cell culture medium leaking out of the glass well during subculture | The glass wells were not sealed tightly | Change to a tightly sealed glass well |
| | The width of the glass well is less than the width of the electrotactic chamber | Reduce the electrotactic chamber width by pushing the cover slips further in with forceps |
| Medium leaking into the cover slip roof area | Too much medium added | Build a thin DC4 blocker at both ends of the cover slip roof |
| No current running across the electrotactic chamber | Air bubbles in the agar glass bridges | Change to new agar bridges without air bubbles |

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**TABLE 1 | Troubleshooting table (continued).**

| Problem | Possible reason | Solution |
|---|---|---|
| Unstable voltage across the electrotactic chamber | Ag/AgCl electrodes are too old | Change to new electrodes |
| | Small bubbles in the agar bridges | Change to new agar bridges without bubbles |
| Temperature of the chamber increasing too quickly | Too little medium in the system | Add sufficient medium to the system |
| | Too much DC4 used between the cover slip roof and strips | Reduce the volume of DC4 or push the roof down firmly |
| pH of the medium changes too quickly | Too little medium in the system | Add sufficient medium to the system |
| Rapid medium loss from the electrotactic chamber | Medium evaporation because of the heating of the temperature-controlled chamber | Moisturize the temperature-controlled chamber with plenty of water |
| Medium contamination during experiment | Lack of sterile technique | Make sure sterile technique is used in every step of the procedure |
| Cells not migrating | Contamination or old medium | Change to fresh medium |
| Image shifting | Chamber is not secured on the stage | Secure the chamber on the stage |

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## ANTICIPATED RESULTS

Our modified EF experimental system has been proved to be stable and useful in studying cellular responses to d.c. electric signals[34,35,44,46]. Investigators can test different types of cells with various molecular and genetic manipulations and quantify any difference statistically using the data recorded from the imaging system. Investigators can also further their studies with molecular and genetic approaches by collecting protein/DNA/RNA samples from the cells in the electrotactic chambers or staining the cells of interest *in situ*. Single-cell migration/division during wound healing and nerve regeneration can be tracked in 3D using the modified 3D model system[35]. Continuous monitoring of the temperature, calcium level and pH changes during EF application revealed that stable pH could be maintained in various experimental settings up to 4 h in an EF. This suggests that culture medium needs to be exchanged every 4 h to maintain a stable pH during EF treatment when higher voltage is used (**Fig. 3**). More culture medium in the EF chamber reservoir helped to stabilize pH (**Fig. 3a**); minimizing EF chamber depth also contributed to stable pH (**Fig. 3b**). $CO_2$-independent medium stabilized pH much better than DMEM with 25 mM HEPES (**Fig. 3d**). Narrow EF chamber width maintained stable pH compared with wider chambers (**Fig. 3e**). Stable temperature and calcium level are maintained in various settings up to 5 h after EF treatment (**Figs. 4** and **5**). Comparison experiments were performed in exactly the same way as the measurement of pH. Please note that the temperatures at time 0 were lower than the rest of the time course because at this time point the doors of the incubator around the chamber were kept open to allow setup of the electrodes, wiring etc. (**Fig. 4**).

Schematic drawings are provided to help investigators to understand the

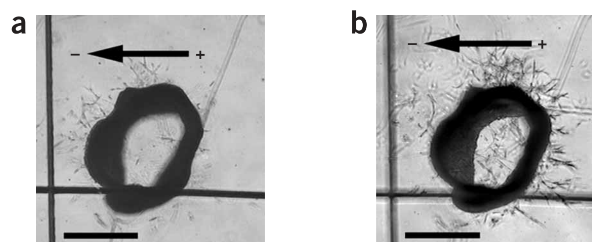


**Figure 7 |** Directional angiogenesis was controlled by a small applied electric field (EF) in the 3D electrotactic chamber. The vessel-like structures from rat aortic ring grew toward the anode. (**a**) Day 0, immediately after embedding the aorta ring; (**b**) day 3, after 200 mV mm$^{-1}$ EF treatment. Bar = 500 μm.

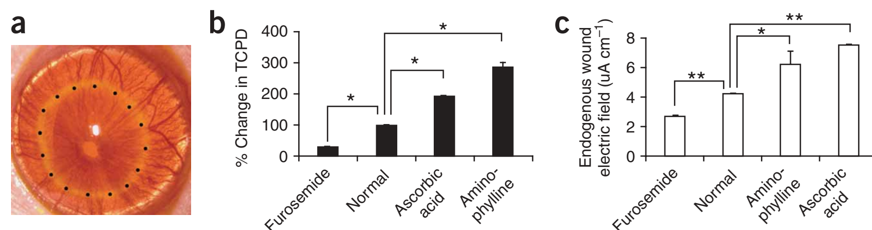


**Figure 8 |** Pharmacological modulation of endogenous wound electric field (EF). (**a**) A circular wound made on a rat cornea. The wounded area was visualized by fluorescent dye staining; the wound edge is indicated with black dots. (**b**) Positive-effect drugs (ascorbic acid, aminophylline) and a negative-effect drug (furosemide) significantly enhanced or reduced the transcorneal potential difference (TCPD). (**c**) Wound-induced endogenous EF was detected immediately after cornea wounding, and this endogenous EF is enhanced by aminophylline and ascorbic acid or reduced by furosemide. $*P < 0.05$; $**P < 0.01$. Student's *t*-test. Data were confirmed by a minimum of six independent experiments.

pattern of current flow resulting from applied fields in complex 3D tissues (**Fig. 6**). In brief, when intact tissues are embedded in the 3D chamber and EF is applied, the EF lines will flow in parallel above the surface of the tissue (**Fig. 6a**). When wounded tissues are treated with EF, the EF lines will run into the wound site (**Fig. 6b**). The 3D chamber has been proven to work well with a directional angiogenesis assay in EF treatment. Directional angiogenesis was induced by a small applied EF in the 3D electrotactic chamber with newly regenerated blood vessels oriented toward the anode (**Fig. 7**).

Transcorneal potential difference can be modulated pharmacologically using drugs with different mechanisms *in vivo* (see **Fig. 8**), which is the mechanism of wound-generated endogenous EF. Wound-induced EF can be measured directly after wounding the cornea *ex vivo*; application of pharmacological or chemical agents could enhance (ascorbic acid, aminophylline) or reduce (furosemide) the endogenous EF respectively (see **Fig. 8c**).

Endogenous EF can be modulated not only pharmacologically, using various drugs, but also by applying EFs directly onto the wound in the 3D chamber or *in vivo* (**Fig. 9**). Applying EF in the default direction of wound healing (**Fig. 9**, brown arrow) will enhance the wound-induced endogenous EF (**Fig. 9**, red arrow) and therefore increase the wound healing rate. In contrast, reversing the EF direction (**Fig. 9**, blue arrow) against the default wound healing direction will suppress the endogenous wound EF (**Fig. 9**, red arrow) and therefore decrease or even abolish the normal wound healing behavior.

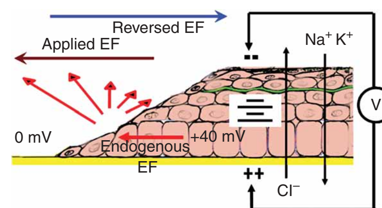


**Figure 9 |** Schematic drawing illustrating the cross-action between endogenous electric field (EF) and applied EF. Endogenous EF can be modulated by applying EFs directly onto the wound in the 3D chamber or *in vivo*. Applying EF in the default direction of wound healing (brown arrow) will enhance the wound-induced endogenous EF (red arrow) and therefore increase the wound healing rate. In contrast, reversing the EF direction (blue arrow) against the default wound healing direction will suppress the endogenous wound EF (red arrow) and therefore decrease or even abolish the normal wound healing behavior.

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