

Cleanroom-Free Microfluidic Chips with Capacitively Coupled 3D Liquid-Metal Electrodes as a Platform Technology for Controlled Electrical Field Stimulation of 2D and 3D Cell Cultures

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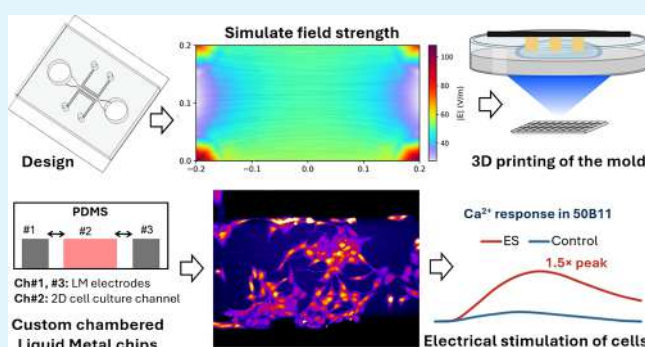
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ABSTRACT: Existing in vitro electrical stimulation platforms are limited by cleanroom-dependent fabrication, direct electrode–electrolyte contact that generates cytotoxic byproducts, and poor compatibility with 3D culture systems. This study reports a modular, cleanroom-free workflow for fabricating polydimethylsiloxane (PDMS) microfluidic chips with capacitively coupled 3D liquid metal (EGaIn) electrodes using digital light processing (DLP)-printed molds. The process achieves sub-day turnaround at electrode resolutions of $\sim 200\ \mu\text{m}$, which extends to $\sim 25\ \mu\text{m}$ via a hybrid DLP/two-photon polymerization approach. A $\sim 150\ \mu\text{m}$ PDMS dielectric barrier isolates the electrodes from the culture medium to eliminate faradaic reactions. COMSOL and ANSYS HFSS simulations show uniform electric fields of 27–108 V/m (three-channel chip) and $(1.72\text{--}2.63) \times 10^3\ \text{V/m}$ (five-channel chip) across the culture chamber. Platform performance was validated across multiple cell types and culture formats. Stimulus-evoked calcium signaling was demonstrated in 50B11 dorsal root ganglion neurons (2D and 3D collagen) and in primary cortical neurons. Daily stimulation ($\sim 4.68 \times 10^3\ \text{V/m}$, 60 kHz) of OCY454 osteocytes in 3D collagen resulted in an 8-fold increase in cell proliferation (compared to 2-fold in controls) and a significant reduction in sclerostin expression. An automated pipeline combining Cellpose segmentation with hierarchical clustering identified three distinct cell subpopulations based on their calcium signaling dynamics. These results establish a customizable, cleanroom-free platform for delivering quantified 3D electrical fields to diverse cell cultures.

KEYWORDS: liquid metal electrodes, microfluidic chip, electrical stimulation, capacitive coupling, 3D cell culture, calcium signaling, DLP printing



1. INTRODUCTION

Precisely controlled exogenous electrical fields modulate a wide range of fundamental cellular functions, including migration, differentiation, proliferation, and calcium signaling, as well as tissue-scale processes, such as wound healing, bone regeneration, and nerve maturation.^{1–3} Externally applied fields are increasingly used in both diagnostic and therapeutic contexts, from deep brain stimulation and spinal cord neuromodulation to capacitively coupled bone fracture-healing devices, underscoring the growing need for well-characterized in vitro platforms to systematically study how electrical fields drive cellular responses.^{4–6} Despite this need, adoption of such platforms for both 2D and 3D cultures remains limited by four persistent engineering challenges: (i) cleanroom-dependent fabrication, (ii) poor electric-field characterization, (iii) limited electrode design flexibility, and (iv) reliance on manual data analysis. The most common approach relies on the manual

placement of metal electrodes directly into cell culture dishes or well plates.⁷ These “direct-coupled” configurations are operationally simple but suffer from a fundamental problem: electrode–electrolyte contact drives faradaic electrochemical reactions that generate reactive oxygen species, pH shifts, and metal-ion leaching, which compromise cell viability during extended stimulation protocols. Manual electrode placement also introduces well-to-well variability in the field geometry, undermining reproducibility.⁸ “Capacitively coupled” setups

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address the toxicity issue by separating electrodes from cells with a dielectric barrier. However, the added impedance requires substantially higher applied voltages to achieve field strengths comparable to direct-coupled systems. Agar–salt bridge configurations avoid direct contact but are ill-suited to long-term culture due to progressive ion diffusion, which alters the media composition.⁷

Cells cultured on planar microelectrodes experience inherently nonuniform fields due to the two-dimensional (planar) geometry of the electrodes, and fabricating such electrodes at high resolution requires cleanroom photolithography, equipment, and expertise that are unavailable to most cell biology laboratories. Microelectrodes integrated into PDMS microfluidic devices offer improved control over both the cellular microenvironment and stimulation conditions, but their fabrication requires precision multilayer alignment of 2D metal features with elastomeric channels, making the process technically demanding and slow.⁹ Critically, nonuniform fields generated by all 2D electrode configurations remain unsuitable for 3D culture applications, where cells distributed throughout a matrix volume must receive equivalent stimulation to enable meaningful biological comparisons.¹⁰ Table 1 summarizes these approaches and the liquid–metal strategies discussed below, contrasting each with the present platform across variables most relevant to in vitro electrical stimulation.

Three-dimensional electrodes represent a more appropriate geometry for volumetric cell culture stimulation. Prior approaches to forming 3D electrodes include metal deposition and electroplating, infusion of conductive pastes (e.g., silver or carbon), and injection of low-melting-point liquid metals (LM).^{17–20} Among these, gallium-based alloys such as eutectic gallium–indium (EGaIn) are particularly attractive: they are room-temperature liquids with high electrical conductivity ($\sigma = 3.4 \times 10^6$ S/m), low viscosity (approximately twice that of water), and they form a surface oxide near-instantaneously upon air exposure that provides mechanical stability within microchannels without requiring solidification. Despite these favorable properties, few devices incorporating LM electrodes have been developed specifically for cell culture. Hallfors et al. demonstrated that directly coupled LM electrodes integrated into a PDMS chip could stimulate 2D-cultured

neurons, but direct coupling introduces the same electrochemical instability risks as conventional metal electrodes.¹⁴ Pavesi et al. used capacitively coupled PDMS–silver flake composite electrodes to show that 3D-cultured cancer cells exhibit reduced metastatic potential under AC fields, but the composite fabrication process is not readily customizable.²¹ As a result, no existing platform simultaneously offers cleanroom-free fabrication, physical electrode–cell separation, compatibility with 3D culture, largely uniform field delivery, and rapid design iteration (Table 1).²²

The work presented here prioritizes capability development and rigorous validation of the enabling technology. Our aim is a well-characterized, reproducible platform that other groups can adopt off-the-shelf to design their own electrical-stimulation studies. To address the challenges identified above, we present a general framework for rapidly designing, fabricating, and operating PDMS microfluidic chips that deliver 3D electrical fields to 2D and 3D cell cultures via capacitively coupled, pre-aligned 3D liquid metal electrodes. Specifically, this work contributes: (i) a fabrication workflow that uses only digital light processing (DLP), enabling sub-day design cycles at ~ 200 μ m electrode resolution, extendable to ~ 25 μ m via a hybrid DLP/two-photon polymerization (TPP) approach; (ii) quantitative 3D electric-field characterization by analytical, COMSOL Multiphysics, and ANSYS HFSS methods across three- and five-channel chip geometries; (iii) functional validation, in both 2D monolayer and 3D collagen culture, across three cell types spanning distinct electrical phenotypes: 50B11 dorsal root ganglion (DRG) neurons and primary cortical neurons (both electrically excitable) and OCY454 osteocytes (non-excitable); and (iv) an automated Cellpose-based calcium imaging analysis pipeline that replaces operator-dependent manual region-of-interest selection, enabling unbiased single-cell quantification across hundreds of cells per experiment. We also identify key engineering limitations of the current platform to facilitate informed adoption by the broader research community. Together, (i)–(iv) constitute the development and quantitative validation of the platform itself, while the cell-based experiments described in (iii) function as capability demonstrations that establish the platform's readiness for future, hypothesis-driven biological investigations.

Table 1. Comparison of the Present Platform with Existing In Vitro Electrical-Stimulation Approaches

platform/approach	coupling mechanism	Faradaic byproducts/corrosion	3D-gel compatibility and field uniformity	biocompatibility/reconfigurability
direct-coupled metal electrodes (Pt, Pt/Ir, IrOx) ¹¹	conductive; Faradaic charge injection	yes—ROS, pH shift, gas, possible electrode dissolution at high charge	mostly 2D/surface; point-source-like, non-uniform fields in 3D	biocompatible but rigid; fixed geometry
agar/salt-bridge systems ¹²	conductive via remote electrolyte bridge	generated at remote electrode; kept away from cells by the bridge	chamber-scale, fairly uniform but bulky; not compatible with microfluidic devices.	biocompatible; bulky, low reconfigurability
planar microelectrode arrays (MEA) ¹³	capacitive or Faradaic (electrode-dependent)	material-dependent; reduced for capacitive coatings	primarily 2D, monolayers; surface field decays into 3D	biocompatible; fixed microfabricated layout
prior liquid–metal (EGaIn) electrodes ^{14,15}	conductive/direct contact; Faradaic	yes, at contact; Ga/In ion release if unencapsulated	mostly surface/implant; geometry-dependent field	soft, biocompatible when encapsulated; moderately moldable
capacitive (dielectric-coated) platforms ¹⁶	capacitive through dielectric barrier	none/greatly reduced	typically planar; limited 3D	depends on material; low reconfigurability
this work: EGaIn + thin-PDMS dielectric, DLP multi-channel	capacitive through thin PDMS dielectric	none—no electrode/medium contact; no Ga/In leaching (fully PDMS-encapsulated)	native 3D microfluidic gel; tunable, near-uniform field	soft (EGaIn/PDMS), biocompatible; highly customizable (DLP-printed)

2. RESULTS AND DISCUSSION

Results and their interpretation are presented below, in the order the platform was developed and then applied: fabrication, quantitative field characterization, electrode-geometry optimization, and finally functional demonstration across electrically excitable and non-excitable cell types in 2D and 3D formats. Because the functional experiments were designed to validate platform performance, sample sizes are modest by design, and several outcomes are explicitly flagged as preliminary; they are intended to establish feasibility and to motivate dedicated, adequately powered, hypothesis-driven studies that use this platform.

2.1. LM-Chip Design and Fabrication

Three- and five-channel LM chips were fabricated by replica casting of PDMS using DLP-printed master molds, followed by injection of gallium–indium (EGaIn) into selected microchannels (Figure 1A,B). The central channel was designed for either a 2D monolayer or a 3D culture (cells embedded in extracellular matrix). LM electrodes were generated in channels 1 and 3 for 2D cultures, and in channels 1 and 5 for 3D cultures (Figure 1D). The three-channel chip had a channel height of 200 μm , with 200 μm -wide LM electrode channels flanking a 400 μm -wide cell-culture channel. The five-channel chip had a channel height of 300 μm , with 200 μm -wide LM electrode channels, 400 μm -wide media channels, and a 500 μm -wide central gel channel (Figure 1C,D). Print-substrate flatness was

maintained by gimbaling leveling, accepted only when the corner-to-corner height difference across a flat 12×8 mm, 200 μm -tall reference structure (measured at its four corners) remained below 20 μm , and channel widths were within ± 5 μm of nominal. Bonding integrity was validated by a dye-fill leak check on a cohort of 47 chips across six independent batches; 41 passed (87%), with failures due to PDMS–glass delamination, port leakage, and dye crossover. Chips that passed these tests were used in this work (S1). The near-instantaneous formation of a surface oxide layer upon EGaIn injection provided mechanical stability within the channels. Electrical stimulation (ES) was delivered by applying the function generator output across the pair of LM electrodes via a jumper wire, with the ~ 150 μm PDMS barrier separating electrodes from cells and culture media, making this a minimally invasive, nontoxic configuration well suited to long-term culture protocols.

This capacitively coupled architecture was not the starting point of the design process. Direct coupling of LM electrodes to the culture medium was explored first but proved incompatible with prolonged stimulation: voltages above ~ 1 V caused immediate electrode retraction, whereas repeated sub-threshold stimulation produced toxic precipitates, possibly due to electrochemical degradation of the gallium oxide interface (S9, Figure S5). Capacitively coupled chips incorporating a ~ 150 μm PDMS dielectric barrier were instead used to physically isolate the electrodes from the culture medium; subsequent attempts to further thin this barrier via chemical etching or by incorporating silver nanoparticles were explored but abandoned due to poor reproducibility (S10). This failure-driven design history is reported to help groups adopting this platform avoid retesting directly coupled LM chips for their cell culture projects.

2.2. Scaling to High-Resolution Chips Using DLP and Two-Photon Polymerization

The same fabrication route also scales to substantially smaller features. Multiscale master molds were printed by combining DLP printing with two-photon polymerization (TPP), with the same resin formulation serving both the DLP- and the TPP-defined regions, and were replica-cast into PDMS chips carrying LM electrode pairs ~ 25 μm wide flanking a central media channel ~ 25 μm wide (Figure 2A,B). Two variants were successfully fabricated: a 25 μm central media channel and a 10 μm central media channel, each flanked by TPP-defined LM electrode microchannels (S2, Figure S2 and Table S2). EGaIn was injected into these microchannels at room temperature and remained stably confined within the TPP-defined geometry. Since TPP is a point-by-point scanning-based process, making master molds is low-throughput and slow. Therefore, this is reported as a demonstration of fabrication capability, while all following results use DLP molds to fabricate three- or five-channel LM chips.

2.3. Characterization of Electrical Fields

Directly measuring the electric field within the ROI is infeasible due to the small dimensions of these chips. Moreover, the field probe becomes a conductor that could locally short the surrounding material and perturb the field it is meant to sample. This is most obvious in 3D cultures with collagen (1.25×10^{-3} S/m), whose high impedance relative to the media (1.5 S/m) concentrates the field in the culture channel, and the

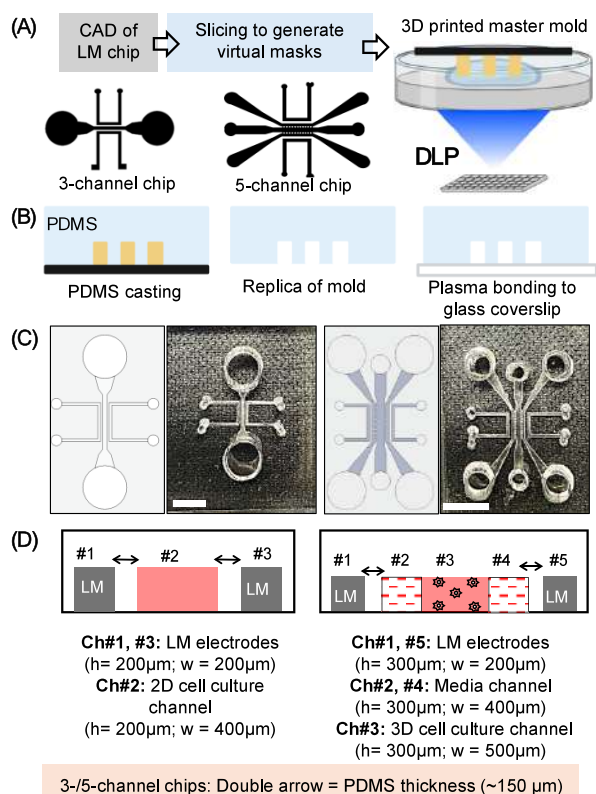


Figure 1. (A) Process workflow to print master molds (DLP: digital light processing). (B) Cross-sectional schematics showing the PDMS replica casting process. (C) CAD (top view) and photographs of three-channel chip (left) and five-channel chip (right); scale bar 5 mm. (D) Schematic showing chip dimensions for both LM chips.

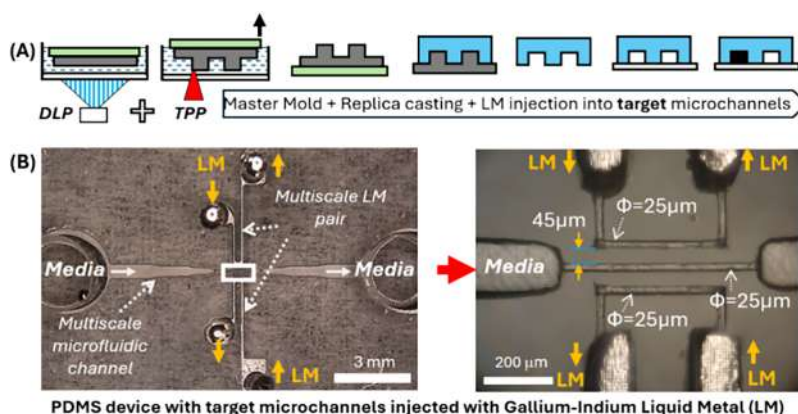


Figure 2. (A) Schematics showing the sequential use of DLP and TPP to generate PDMS chips with microscale channels. (B) Photograph of the 3-channel LM chip; the zoomed image shows a pair of LM electrodes ($\Phi \sim 25 \mu\text{m}$) flanking the central media channel ($\sim 25 \mu\text{m}$ wide).

presence of the probe would negate the impedance contrast (S4). Hence, the 3D electric-field $|E|$ distribution within the cell-culture ROI was characterized by using three complementary modeling methods: an analytical circuit model, ANSYS HFSS (transient solver), and COMSOL Multiphysics (AC/DC module, electric currents, and time-dependent). Full details for each model and the material properties are provided in S3, S5, and S6 (Table S3 and Figure S3). The analytical model, treating each material layer as a parallel RC element in a series capacitive stack, estimated peak field strengths of $\sim 21 \text{ V/m}$ and $\sim 1.06 \times 10^3 \text{ V/m}$ in the three- and five-channel chips, respectively, under 10 V, 10 Hz square-wave excitation (Figure 3B). Applying the same model to the high-resolution TPP chip (Figure 2), in which the PDMS barrier separating the electrode channels from the media channel is reduced from $\sim 150 \mu\text{m}$ to $45 \mu\text{m}$, gave $\sim 71 \text{ V/m}$ under identical excitation (Figure S6).

Full-wave time-domain simulations in ANSYS HFSS, performed for the three-channel chips using the same dimensions and material parameters as above and excited with the experimental driving waveform (10 V peak, bipolar square wave, $< 25 \text{ ns}$ rise time), yielded peak field magnitudes of 40–60 V/m through the ROI, with a relatively uniform distribution across the central region and higher values ($\sim 100 \text{ V/m}$) localized near channel boundaries. For the five-channel chip, HFSS returned values of $2.60 \times 10^3 \text{ V/m}$, with only modest spatial variation away from the boundary edges (Figure 3C). The five-channel map looks asymmetric, but this reflects the plotting scale rather than the field itself: across the ROI, the magnitude spans only about (2597 to 2635) V/m, a variation of roughly 1.4%, so the field is effectively uniform. HFSS auto-scales its color bar to this narrow range, stretching sub-percent fluctuations from mesh discretization and interpolation across the full color spectrum and producing a visually striking but physically negligible pattern. Equivalent COMSOL simulations yielded 27–108 V/m for the three-channel chip and largely uniform fields of $(1.72\text{--}1.79) \times 10^3 \text{ V/m}$ for the five-channel chip (Figure 3D), with comparable fringing-related deviations near channel edges. The electrical field distribution within the five-channel gel channel was simulated in COMSOL, with $|E|$ extracted on six XY planes at successive Z positions spanning the full 300 μm depth from the bottom to the top PDMS interface (Figure S3). Across the four interior planes ($Z = 50, 100, 200,$ and $250 \mu\text{m}$), the

simulated bulk field was uniform at $(1.7\text{--}1.73) \times 10^3 \text{ V/m}$, varying by only 1–2% over the full XY extent. Edge enhancement to $\sim 1.78 \times 10^3 \text{ V/m}$ was confined to a sub-30 μm layer at the gel–PDMS interfaces.

The analytical model underestimates field magnitudes compared with finite-element methods because it models the device as an idealized 1D capacitive stack with spatially averaged impedances, omitting 3D fringing fields, nonuniform current redistribution, finite electrode dimensions, and interface-dependent effects. Discrepancies between HFSS and COMSOL arise from differences in meshing algorithms and solver formulations. Importantly, the COMSOL and HFSS values bracket the range of field strengths experienced by cells: for the three-channel chip, cells experienced (27–108) V/m, and for the five-channel gel, $(1.72\text{--}2.63) \times 10^3 \text{ V/m}$. Chip-to-chip reproducibility of these estimates is set by the geometry of the capacitive divider. Two-terminal resistances of all EGaIn lines fell below the 1Ω resolution of the measurement, negligible against the series impedance of the PDMS and gel, so the field is governed by the PDMS barrier thickness. That thickness measured $152 \pm 5 \mu\text{m}$ across 15 chips from three fabrication batches ($\text{CV} = 3.3\%$); because $|E|$ scales inversely with barrier thickness, this bounds chip-to-chip field variation at $\sim 3\%$, far below the spread between solver formulations (S4). The analytical model is best suited to order-of-magnitude estimates and parametric scaling, whereas HFSS and COMSOL provide spatially resolved field magnitudes for quantitative experimental design. A summary of calculated electrical-field values and stimulation conditions is provided in S7 (Tables S4 and S5).

2.4. Design Optimization of the Electrode Configuration

Because the electrode layout determines the shape of the field within the culture region, three electrode geometries were fabricated and compared to select the configuration used in the experiments that follow: symmetric partial-coverage opposing electrodes, a V-shaped converging configuration, and an asymmetric same-side configuration (Figure 4A). COMSOL simulations predicted distinct field distributions for each geometry (Figure 4B). The symmetric opposing configuration (1.5 mm of electrode coverage along the 3.2 mm channel) produced a plateau of near-constant $|E|$ ($\sim 62 \text{ V/m}$) spanning the electrode-covered region, falling to roughly half the peak value at the electrode edge and decaying steadily beyond it.

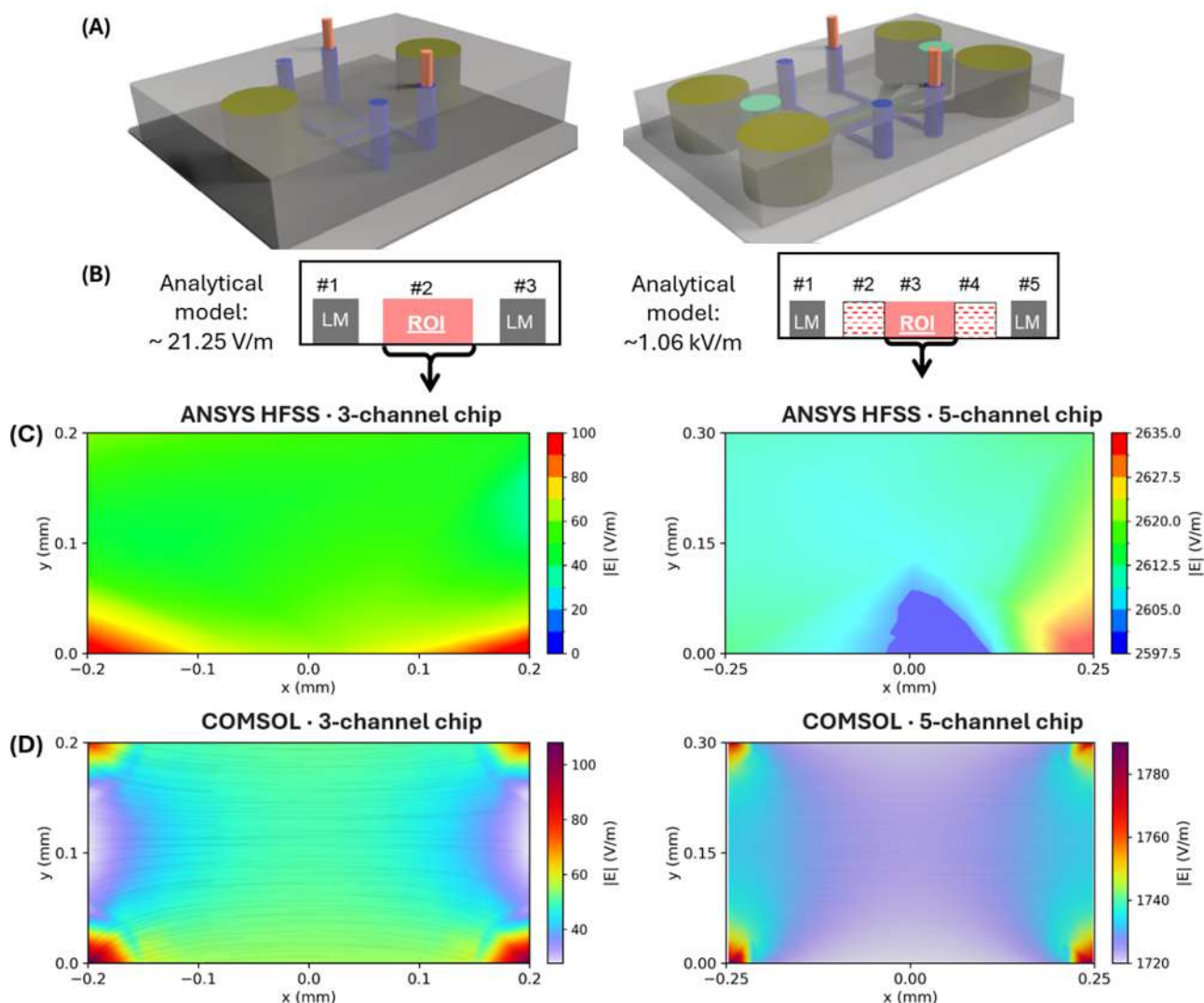


Figure 3. (A) CAD design of LM chips. (B) Schematic cross-section showing the ROI within the chips, and the electrical field strength calculated from the analytical model: ~ 21.25 V/m and $\sim 1.06 \times 10^3$ V/m for the three- and five-channel chips, respectively. Electric field distribution obtained using (C) ANSYS HFSS and (D) COMSOL.

The V-shaped design reached a comparable peak (~ 58 V/m) but confined it to a narrow convergence zone (~ 200 to 300 μm), with no plateau. The asymmetric same-side configuration (400 to 500 μm electrode gap) produced the highest magnitudes (~ 310 V/m at the center) but varied continuously along the entire channel, falling to ~ 45 V/m at 2 mm.

Because a plateau of constant $|E|$ is what allows all positions within the ROI to receive equivalent exposure, uniformity rather than peak magnitude was the selection criterion. Only the symmetric opposing configuration produced such a plateau (coefficient of variation ~ 2 – 3% across the central ROI; S8, Figure S4 and Table S6); the asymmetric configuration, despite its higher magnitude, imposed a continuous gradient. Because the plateau tracks the electrode-covered length and decays beyond the electrode edge, electrode coverage was extended to the full length of the culture channel, extending the uniform region across the entire ROI. This maximum-coverage symmetric configuration was used for all subsequent experiments.

The V-shaped and same-side geometries remain available for localized or directional stimulation instead, and this interchangeability follows directly from the fabrication route. Because DLP-printed molds make electrode layout a free design variable, new geometry costs only a new print, with no change to casting, bonding, or EGaIn injection. This is the practical distinction from photolithographic microelectrode arrays, where the layout is fixed at fabrication, and each variant requires a new mask. The same design and fabrication workflow extends to high-resolution (~ 25 μm) LM chips (DLP-TPP) without changing the resin, casting, or injection steps. Thus, new chips for custom applications that require user-defined (localized or gradient) electrical fields across the ROI can be produced by changing only the mold (Figures S4, S8 and Table S6).

2.5. Electrical Stimulation of 50B11 DRG Neurons in 2D Culture

As an initial functional validation of the platform, we first evaluated the effect of stimulation duration on the viability of

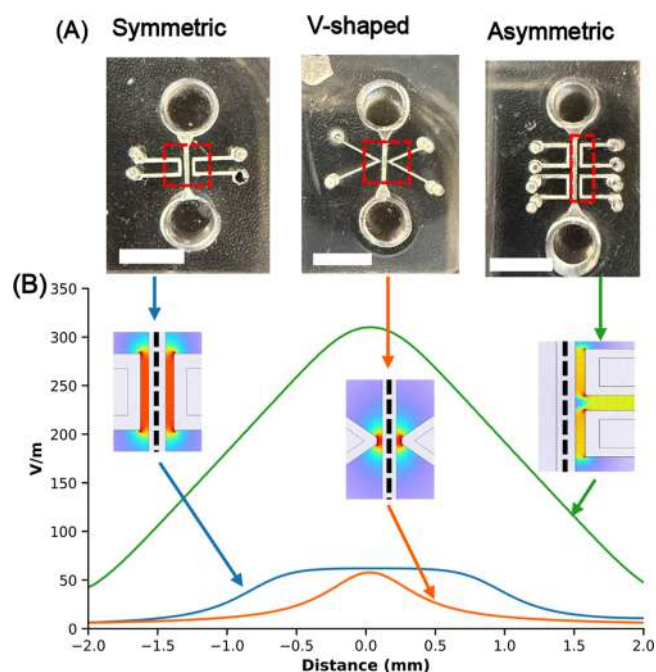


Figure 4. (A) Digital masks and simulated field strengths for three LM electrode designs; scale bar 5 mm. (B) Electric field magnitude (V/m) as a function of distance along the length of the cell culture channel (dashed line).

50B11 cells cultured in 2D in the central channel of the three-channel LM chip (Figure 5A,B). Following stimulation with a 10 V square wave at 10 Hz for 5, 15, or 60 min, cells were assessed using a live/dead assay (Calcein AM/EthD-1). The chip was mounted on an imaging stage with electrical interconnects to the LM electrodes (Figure 5C), and phase-contrast imaging confirmed the integrity of the PDMS barrier between the cell/media region and the LM electrodes (Figure 5D). Viabilities were $98 \pm 2.8\%$, $96 \pm 4.2\%$, and $93 \pm 2.9\%$ for 5, 15, and 60 min stimulation, respectively ($n = 5$ chips per condition, one field of view per chip; Figure 5E). A one-way ANOVA with Tukey post-hoc test revealed no statistically significant difference among the three durations ($p = 0.21$), indicating that the capacitively coupled protocol did not induce acute cytotoxicity even after one hour of continuous stimulation. Baseline viability in unstimulated chips cultured for the same period was $98 \pm 1.6\%$ ($n = 5$ chips), confirming that the chip itself does not reduce cell viability relative to standard culture conditions. A 5 min stimulation duration was used in all subsequent experiments. Because each daily session lasted only 5 min, cumulative exposure over the full multi-day protocol (2 days; 10 min total) remained below the 60 min of continuous stimulation shown here to be noncytotoxic (Figure 5E).

Next, 2D-cultured 50B11 cells were loaded with the Ca^{2+} indicator, Fluo-4, and subjected to ES (10 V, 10 Hz). In stimulated cells, the fluorescence signal increased at the onset of stimulation, peaked at ~ 150 s, and then either decreased or

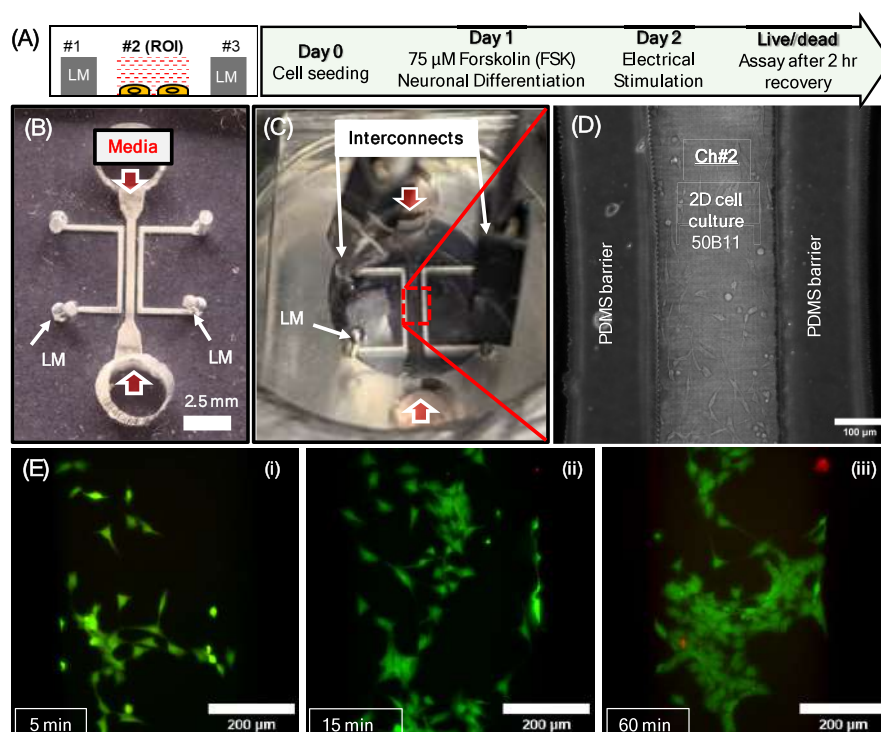


Figure 5. (A) Schematic of the 3-channel LM chip and the experimental timeline. (B) LM chip setup; scale bar 2.5 mm. (C) Setup mounted on the imaging stage, with electrical interconnects connected to the LM electrodes. (D) Phase-contrast image showing a top view of the ROI, with PDMS barriers separating the cell/media region from the LM electrodes; scale bar 100 μm . (E) (i–iii) Representative fluorescence images of cell viability after ES for different durations (green: live; red: dead); $n = 5$ chips per condition; scale bar 200 μm .

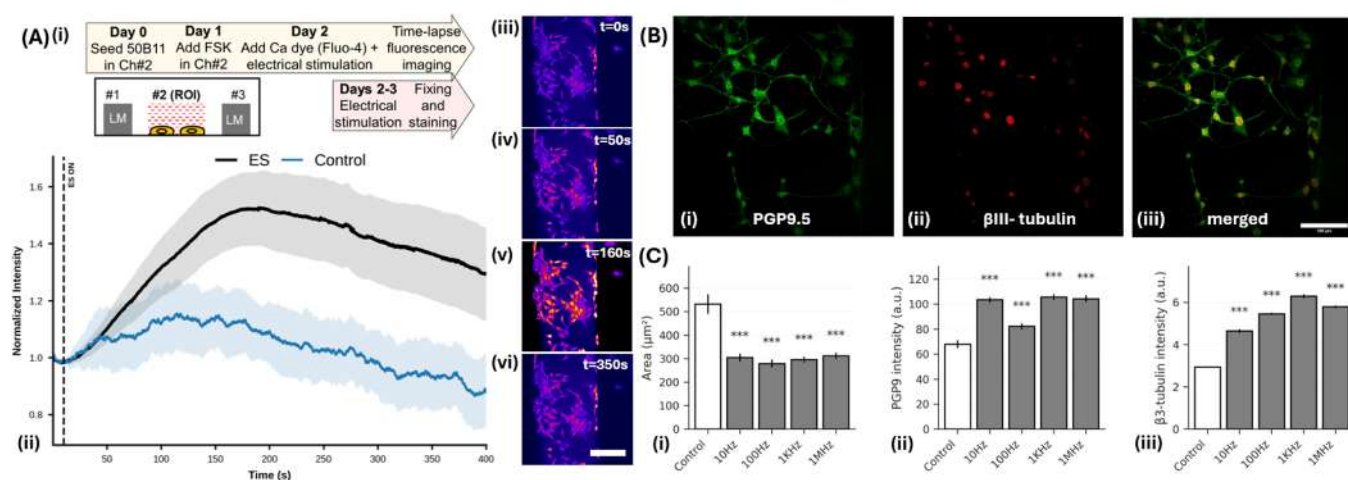


Figure 6. (A) Calcium signaling dynamics in 50B11 DRG neurons with and without ES, initiated at $t = 10$ s, (i) experimental timeline; (ii) mean normalized fluorescence intensity of the 20 most active cells across 5 chips stimulated (ES, black) and control (blue) conditions, with shaded bands indicating $\pm$ SD; and (iii–vi) representative time-lapse fluorescence snapshots with ES at $t = 0, 50, 160$, and 350 s. Scale bar $200 \mu\text{m}$. (B) Immunofluorescence characterization of neuronal markers with ES (5 min/day on days 2 and 3) in FSK-differentiated 50B11 neurons: (i) PGP9.5 (green), (ii) β III-tubulin (red), and (iii) merged; scale bar $100 \mu\text{m}$. (C) Effect of stimulation frequency on cell area and neuronal marker expression. Data presented as mean $\pm$ SEM; *** $p < 0.001$ vs control.

plateaued (Figure 6A (ii)); a subset of cells within the ROI showed no detectable change in fluorescence. Traces represent mean normalized fluorescence intensity of the 20 most active cells recorded from separate chips under stimulated (black) and control (blue) conditions, with shaded bands indicating $\pm$ SD ($n = 5$ chips per condition). Representative time-lapse snapshots illustrate calcium signaling dynamics at indicated time points (Figure 6A (iii–vi)). Overall, stimulation significantly increased both the magnitude and frequency of calcium signals compared with unstimulated controls ($p < 0.001$).

This effect is mechanistically consistent with field-induced polarization of the neuronal membrane, which biases cells toward depolarization and increases the open probability of activating voltage-dependent Ca^{2+} channels (VDCCs), the principal route of activity-dependent Ca^{2+} entry in these neurons.^{23,24} Using the spherical cell approximation ($\Delta V_m \approx 1.5 \cdot E \cdot R \cdot \cos \theta$) with a representative soma radius $R \approx 7.5 \mu\text{m}$ and the COMSOL-estimated field of $27\text{--}108 \text{ V/m}$, the transmembrane polarization at the cell poles ($\cos \theta = 1$) is predicted to be approximately 0.3 mV at the lower field bound and up to approximately 1.2 mV at the peak field. These values are subthreshold relative to a typical action potential but sufficient to modulate channel-gating probability and bias network-level excitability.^{25–27} The enhanced Ca^{2+} transients observed at 10 Hz are consistent with rhythmic VDCC activation, producing modest cumulative shifts in membrane potential that promote network activity.^{23,24}

To assess the influence of stimulation frequency on neuronal marker expression, 2D-cultured 50B11 cells were treated with $75 \mu\text{M}$ forskolin (FSK) to induce differentiation and stimulated daily for 5 min, 10 V , square wave at 10 Hz , 100 Hz , 1 kHz , or 1 MHz ($n = 5$ chips per condition). Stimulated cultures showed significantly higher expression of both PGP9.5 and β III-tubulin compared to nonstimulated controls at all tested frequencies (Figure 6B,C; $p < 0.001$ vs control). PGP9.5 expression was comparable across 10 Hz , 1 kHz , and 1 MHz (~ 1.5 -fold increase), with a modest reduction at 100 Hz (~ 1.2 -fold). β III-tubulin showed a more

progressive, frequency-dependent response, peaking at 1 kHz (~ 2 -fold increase) and declining toward 10 Hz (~ 1.7 -fold increase). Cell area decreased under all stimulation conditions relative to controls ($p < 0.001$). Total neurite length, quantified from the β III-tubulin images, shows no change between stimulated and control conditions. As a platform-capability finding, this appears to be the first report of ES-induced upregulation of neuronal markers in the 50B11 cell line, illustrating the kind of new observation the platform is intended to enable.

PGP9.5 and β III-tubulin were selected as differentiation markers because the 50B11 line has a short differentiation window (maximum $\sim 72 \text{ h}$): β III-tubulin is a well-established early marker of neuronal differentiation and axonogenesis, and PGP9.5 is a standard marker of peripheral DRG neurons. Late-stage markers, such as NeuN (minimally expressed within this window) or MAP2 (dendritic marker less appropriate for pseudounipolar DRG neurons),^{28,29} were not chosen for this study. The divergence in frequency-response relationships between the two markers (PGP9.5, β III-tubulin) suggests they are differentially regulated and exhibit distinct frequency sensitivities, a profile broadly consistent with the literature. DC stimulation of neural stem cells has been shown to enhance β III-tubulin and NeuN expression, and application of an electrical field to forebrain neural precursor cells shows a 1.5-fold increase in β III-tubulin-positive neurons.^{28,30–35} This trend does not follow the low-frequency preference of directly coupled systems, where stimulation in the $1\text{--}20 \text{ Hz}$ range aligns with endogenous firing and calcium-channel kinetics.^{36–38} In a capacitively coupled, square-wave configuration, frequency does not set the field amplitude delivered to the cells, which is fixed by the voltage step at each transition; it instead sets the number of field transients the cells experience per unit time, so that each frequency over a fixed stimulation window delivers many effective transients which can account for the upregulation observed across the entire 10 Hz – 1 MHz band rather than a single-frequency optimum. The small, nonmonotonic differences between frequencies are not interpreted further pending

direct measurement of the delivered field at each condition. These results highlight that stimulation parameters optimized for direct-coupled systems cannot be assumed to translate to capacitively coupled configurations.

In contrast to marker expression and cell area, total neurite length was unaffected by stimulation. This most likely reflects the stimulation regimen (5 min/day on two days): a cumulative exposure sufficient to acutely modulate intracellular calcium and upregulate early neuronal markers, but too brief to drive measurable neurite elongation. Two factors likely contribute: FSK differentiation may have already maximized outgrowth across all conditions, masking any incremental effect (a ceiling effect), and neurite remodeling is inherently slower than calcium or protein-expression changes, so longer stimulation may be needed before a frequency-dependent difference emerges.

2.6. Electrical Stimulation of 50B11 DRG Neurons in 3D Culture

To validate platform performance in a physiologically more relevant 3D format, 50B11 cells encapsulated in type I collagen (3 mg/mL) in the central channel of a five-channel LM chip were treated with FSK via side channels (channels 2 and 4) and subjected to ES (10 V, 10 Hz). Traces represent the mean normalized fluorescence intensity of the nine most active cells and show a gradual, sustained increase in Ca^{2+} fluorescence following stimulation onset, peaking at ~ 270 s, substantially later than the ~ 150 s peak observed in 2D culture (Figure 7A). Representative time-lapse snapshots illustrate the progression of calcium signaling over the imaging period (Figure 7B). This temporal lag likely reflects two compounding factors: slower diffusion of calcium ions through the tortuous collagen network, and reduced direct cell-to-cell connectivity at equivalent time points in 3D vs 2D. Notably, the electrical field strength within the collagen gel is substantially higher than that experienced by cells in 2D media because the higher impedance of the collagen matrix (conductivity $\sim 1.25 \times 10^{-3}$ S/m) relative to culture media (1.5 S/m) concentrates the voltage drop across the gel.³⁹ This elevated field

possibly compensates for the resistive losses imposed by the collagen matrix and generates sufficient transmembrane potential to activate VDCCs and trigger Ca^{2+} entry.^{40,41} Because VDCC activation is threshold-dependent, pronounced field heterogeneity could in principle drive position-dependent responses. HFSS modeling indicates the field varies by less than 1.5% across the gel, so cells throughout the gel experience near-identical stimulation. In this work, calcium transients were acquired from an ROI at the center of the inter-electrode gap, within this uniform region. A position-resolved comparison of calcium signaling as a function of ROI location relative to electrodes could provide a direct test of field-to-response coupling and is planned for future work. The delayed but robust Ca^{2+} response confirms that neurons embedded in collagen remain fully electrically excitable and that the five-channel chip architecture is well suited to 3D neural stimulation applications.

2.7. Electrical Stimulation of Primary Cortical Neurons in 2D Culture

To further validate the platform's versatility across neuronal subtypes and to illustrate compatibility with pharmacological co-treatment, primary cortical neurons (PCNs) were cultured in three-channel LM chips and subjected to ES stimulation (10 V, 10 Hz). Given the limited yield of primary cortical neurons per dissection, the pharmacological conditions were run as single chips ($n = 1$ per drug) and are described qualitatively. Time-lapse fluorescence imaging of the ES-only condition showed a rise in calcium signaling that peaked approximately 90 s after stimulation onset (Figure 8B,C), consistent with the temporal profile observed in 50B11 cells. Unstimulated control chips exhibited moderate spontaneous fluctuations with a gradual baseline drift. Two pretreatment conditions were run alongside the ES-only and unstimulated controls, each on its own exposure schedule. In one chip, calcitriol (1 μM), the bioactive form of vitamin D_3 , which modulates L-type voltage-gated calcium channels in both genomic and non-genomic pathways,^{42–44} was introduced on day 2 and maintained for 48 h prior to stimulation on day 4. In a separate chip,

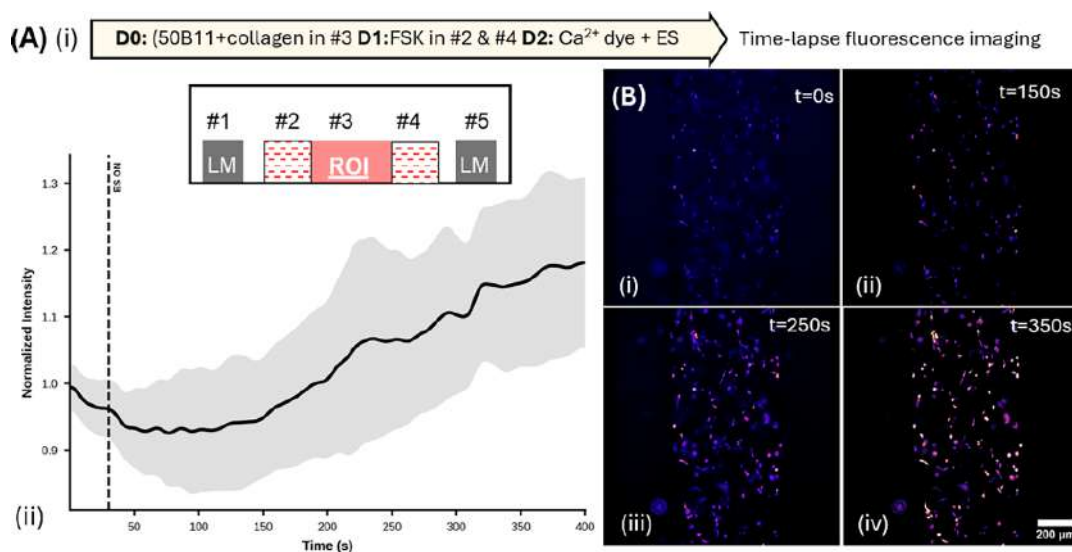


Figure 7. (A) (i) Schematic of the experimental timeline for electrical stimulation of 3D-cultured DRG neurons. (A) (ii) Mean normalized fluorescence intensity of the 9 most active cells under across 3 chips stimulated (ES, black) with shaded bands indicating $\pm$ SD (B) (i–iv) representative time-lapse fluorescence snapshots with ES at $t = 0, 150, 250,$ and 350 s of 3D-cultured 50B11 neurons in the five-channel LM chip; scale bar $200 \mu\text{m}$.

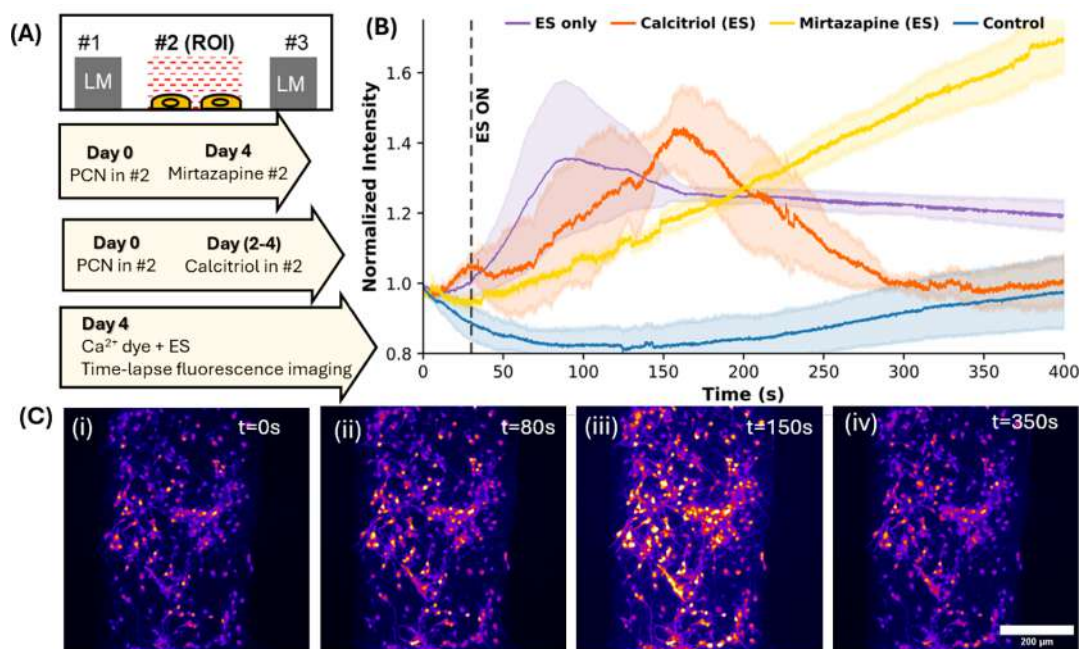


Figure 8. (A) Schematic of experimental design. (B) Mean normalized Ca^{2+} fluorescence intensity traces ($\pm$ SD) showing calcium signaling dynamics in PCNs under different stimulation conditions: ES-only and control $n = 3$ chips; calcitriol and mirtazapine $n = 1$ chip each. (C) Representative snapshots during time-lapse fluorescence microscopy of PCNs under ES only; scale bar 200 μm .

mirtazapine (10 μM), a clinically used antagonist at α_2 -adrenergic and 5-HT₂/5-HT₃ receptors,^{45–47} was applied for 1 h on day 4 immediately before stimulation. Normalized Ca^{2+} fluorescence traces for each condition are shown in Figure 8B. The calcitriol-pretreated chip exhibited a transient Ca^{2+} peak at ~ 160 s, later than the ~ 90 s peak of the ES-only condition, followed by a decline toward baseline, consistent with enhanced L-type channel availability during ES-induced depolarization, followed by inactivation or intracellular buffering. The mirtazapine-pretreated chip exhibited a continuously rising Ca^{2+} signal that did not return to baseline within the 400 s recording window, exceeding the drift observed in unstimulated controls. Together, these recordings demonstrate that pharmacological pretreatment and ES can be applied to the same chip and that condition-specific calcium traces can be resolved, illustrating the platform's potential for combined electrophysiological and pharmacological interrogation of neuronal signaling. Future studies will systematically vary drug concentration and pretreatment duration, with replication sufficient to support statistically robust dose–response relationships for drug-screening applications.

2.8. Automated Segmentation, Calcium Signal Extraction, and Analysis

As part of the platform's analysis toolkit and using the same fluorescence recordings described above (Figure 8B,C), an automated analysis pipeline was applied to characterize calcium dynamics at single-cell resolution. The pipeline integrates CalmAn motion correction,⁴⁸ Cellpose deep-learning segmentation,⁴⁹ and CTCF-based signal extraction with local annulus background subtraction, and its central advantage over manual ROI selection is the elimination of operator-dependent variability, particularly important in cortical cultures where functional

diversity across neurons is a biologically meaningful feature. The local annulus background subtraction strategy, which estimates background fluorescence from an annular region surrounding each cell, more effectively accounts for nonuniform illumination and local neuropil contamination than the global background correction used in the manual approach, resulting in smoother traces with improved signal-to-noise ratio.

Raw fluorescence images showed widespread calcium transients across the neuronal population (Figure 9A-i), and automated segmentation with a fine-tuned Cellpose model identified 313 individual cells within the field of view, generating masks that delineated cell boundaries, including closely apposed neurons, for downstream region-of-interest analysis (Figure 9A-ii) compared with the limited subset feasible through manual ROI selection in FIJI.⁵⁰ Normalized fluorescence traces ($\Delta F/F_0$) were extracted from all segmented cells over a 400 s recording (10 frames per second), revealing marked heterogeneity in spontaneous activity, with only a subset of cells displaying prominent calcium transients above baseline fluctuations (Figure 9B). Quality filtering on mean fluorescence intensity, peak amplitude, and signal standard deviation retained the 30 most active cells ($\sim 10\%$ of detected cells); the mean $\Delta F/F_0$ trace of this subset showed a gradual rise followed by sustained activity, and the $\pm$ SD envelope indicated substantial variability in event timing and amplitude across cells. This 10% retention rate, facilitated by a custom interactive visualization tool enabling real-time threshold adjustment and manual curation, itself highlights the substantial heterogeneity in primary cortical neuron cultures under ES. A 15-frame rolling average was applied to suppress high-frequency noise while preserving the temporal structure of the events (Figure 9C). To characterize functional heterogeneity across the full population, correlation-based hierarchical clustering (correlation distance

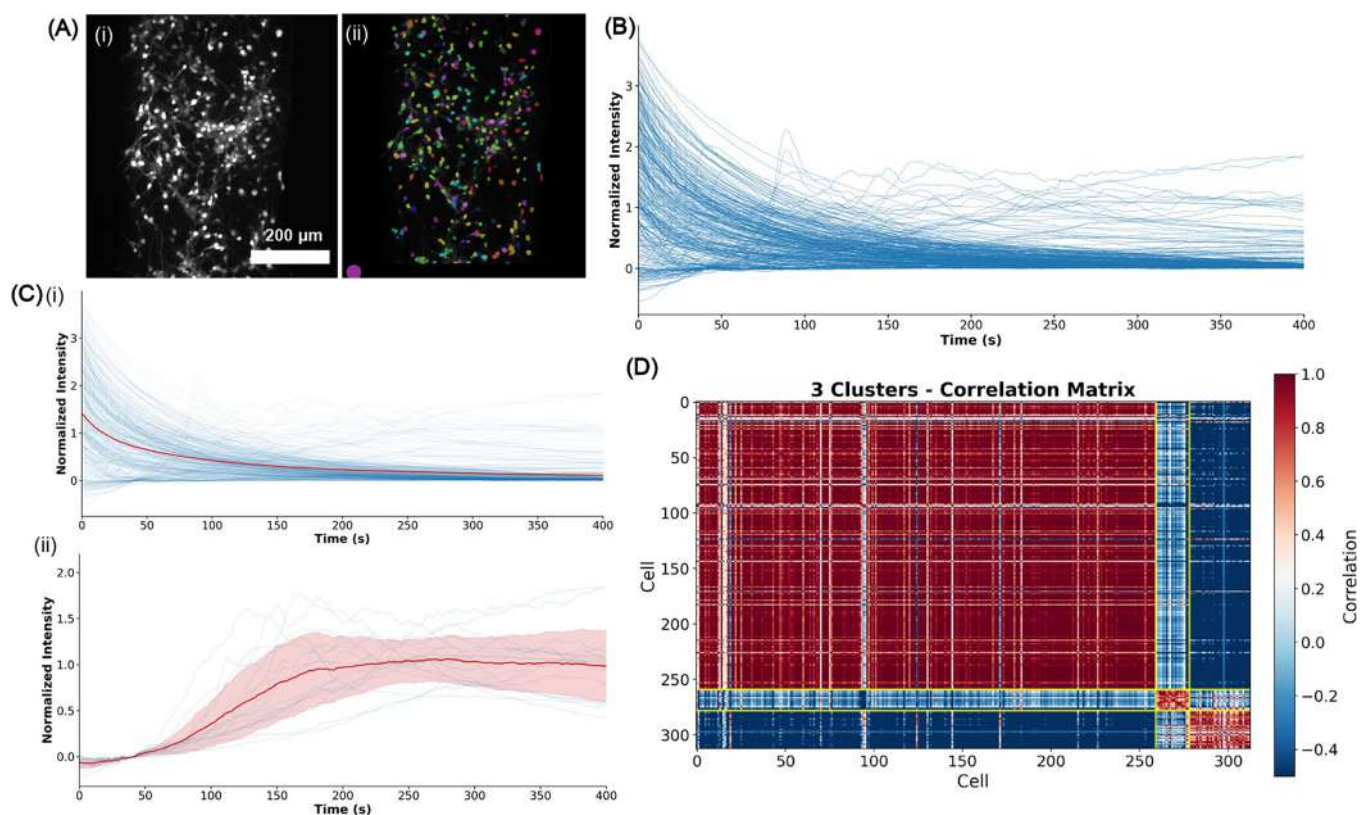


Figure 9. (A) Cell detection and segmentation: (i) raw fluorescence image; (ii) segmentation masks generated by the Cellpose model, identifying individual cells ($n = 313$); scale bar 200 μm . (B) Normalized intensity traces extracted from all segmented cells, showing spontaneous calcium activity over 400 s recording; background was estimated using a local-annulus method. (C) Signal summary and filtering: (i) population mean; (ii) the 30 most active cells retained after thresholding (mean ≥ 0.197 , peak ≥ 1.148 , late-activity index ≥ -0.032), with the population mean $\pm$ SD overlaid. (D) Correlation-based hierarchical clustering of all detected cells identified three functional groups; the correlation matrix, ordered by cluster assignment, shows clear block structure (within-cluster $r > 0.5$; yellow lines mark cluster boundaries). Cluster sizes: cluster 1, $n = 259$ (82.7%); cluster 2, $n = 19$ (6.1%); and cluster 3, $n = 35$ (11.2%). Cluster 1 represents typical spontaneous activity; clusters 2 and 3 capture early-burster and late-activator profiles, respectively.

$1 - |r|$, average linkage) was performed on all detected cells. Reordering the correlation matrix by cluster assignment revealed clear block structure (within-cluster $r > 0.5$), identifying three functional groups: a dominant cluster of typical spontaneous activity ($n = 259$; 82.7%) and two smaller clusters with distinct temporal patterns, an early-burster group ($n = 19$; 6.1%) and a late-activator group ($n = 35$; 11.2%) (Figure 9D).

The scalability of this pipeline makes it well-suited to high-throughput studies of pharmacological interventions or disease-related alterations in network activity, where analyzing hundreds to thousands of cells per condition is desirable. In future work, unsupervised clustering of the underlying distance matrices could link calcium activity patterns to morphological or molecular markers. Several limitations should be noted for prospective users: Cellpose segmentation may under-segment neurites or cells that overlap in densely packed regions,⁴⁹ the quality-filtering thresholds used here were set empirically and may benefit from data-driven optimization, and correlation distance captures zero-lag linear co-activation between traces but is insensitive to temporal lags and to nonlinear or distributional differences in activity—complementary measures such as lagged cross-correlation, mutual information, or distribution-based distances could further enrich the characterization of functional connectivity.

2.9. Electrical Stimulation of 3D-Cultured Non-excitable Osteocytes (OCY454)

As a final validation of platform utility across electrical phenotypes, extending beyond electrically excitable neurons to a non-excitable cell type, OCY454 cells were encapsulated in type I collagen (2.5 mg/mL) and electrically stimulated daily from day 8 to day 15 using a 200 V, 60 kHz sinusoidal waveform, generating an analytically estimated field strength of 4.68×10^3 V/m in the collagen region (S5.4). The 60 kHz sinusoidal waveform was selected to maximize capacitive transmission through the PDMS dielectric barrier, which attenuates displacement current at lower frequencies, while remaining within the range reported to enhance osteoblast-like cell proliferation and extracellular matrix formation.⁵¹ Samples were fixed on day 8 and day 15, fluorescently labeled for nuclei (DAPI) and sclerostin, and imaged by confocal microscopy (Figure 10A). Despite equivalent target seeding densities, ES-treated cultures contained fewer cells than controls on day 8 (Figure 10B; $p < 0.001$), most likely reflecting variability in cell encapsulation efficiency rather than a stimulation effect. By day 15, ES-treated cultures showed an approximately 8-fold increase in cell numbers compared to a 2-fold increase in controls, suggesting a positive effect of daily ES on proliferation over time. Sclerostin expression per cell showed time- and treatment-

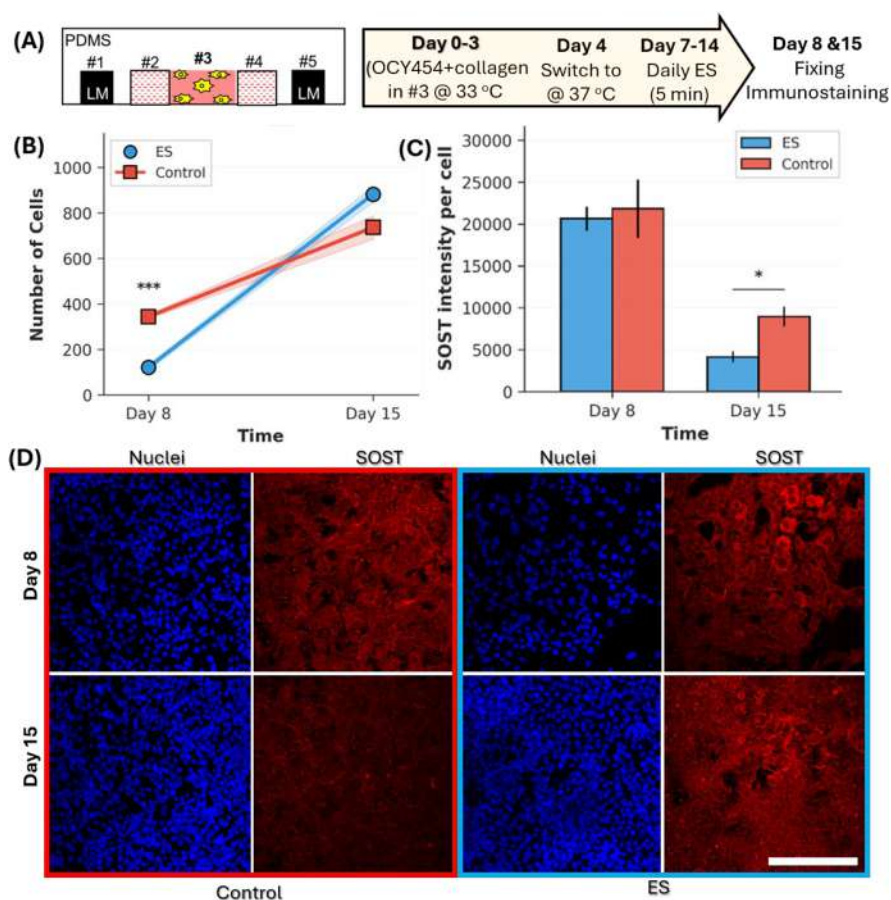


Figure 10. (A) Experimental design. (B) Cell number over time; ES-treated cultures (blue) contained fewer cells than control at day 8 ($***p < 0.001$), and both groups increased by day 15. (C) Sclerostin (SOST) expression per cell ($*p < 0.05$ at day 15). (D) Representative confocal fluorescence images of control (left) and ES (right) $n = 3$ chips per condition at days 8 and 15 (nuclei (blue); sclerostin (red)). Scale bars 200 μm .

dependent regulation (Figure 10C). At day 8, sclerostin levels were comparable between ES and control groups. By day 15, both groups exhibited substantial reductions in sclerostin; however, ES-treated cells showed a more pronounced decrease, with control cultures retaining approximately twice the sclerostin level of ES cultures ($p < 0.05$). Representative confocal images of ES-treated and unstimulated control cultures at day 8 and day 15 are shown in Figure 10D.

These findings situate the LM chip platform within a largely unexplored space: prior ES studies of bone cells have focused predominantly on osteoblasts rather than osteocytes. Work with human osteoblasts has shown that alternating electrical field stimulation modulates gene expression of differentiation markers, including osteocalcin and collagen type I, in a voltage-dependent manner,⁵¹ pulsed ES has been shown to enhance osteoblast adhesion, calcium signaling, and calcium mobilization,⁵² and capacitively coupled stimulation has been shown to enhance differentiation and mineralization of osteoblast-like cells.^{53,54} In the context of osteocytes, the effect of ES on sclerostin expression has not been reported previously. Because sclerostin inhibits bone formation by antagonizing canonical Wnt signaling through LRP5/6,^{55,56} the greater reduction in sclerostin observed in ES cultures is consistent with a shift toward a more pro-osteogenic signaling state, and it has been hypothesized that osteocytes serve as the primary

sensory cells for ES, orchestrating bone formation and remodeling through the same mechanotransduction pathways engaged by physical loading. As a platform-capability finding, this appears to be the first direct experimental evidence that ES modulates sclerostin expression in osteocytes. The stimulation conditions used (200 V, 60 kHz sine; $\sim 4.68 \times 10^3$ V/m in gel) reflect two converging considerations: the frequency-dependent transmission characteristics of the PDMS dielectric barrier, which favor higher frequencies for capacitive coupling, and the field-strength range reported in the literature to enhance osteoblast-like proliferation and extracellular matrix formation.^{51,57} This provides a rational design basis for the protocol we used in this work. These findings are preliminary ($n = 3$ chips per condition) and motivate a dedicated replicated study with dose-response characterization and downstream Wnt pathway readouts, including mineralization assays and active β -catenin quantification, to determine whether sclerostin downregulation translates into functional pro-osteogenic outcomes. Preliminary work on the chip was also performed with C3H10T1/2 cells, but these results are not reported here.

2.10. Engineering Limitations and Paths Forward

Taken together, the results above validate the platform's core capabilities, such as cleanroom-free fabrication, quantitatively characterized and reconfigurable field delivery, and functional

compatibility across excitable and nonexcitable cell types in 2D and 3D formats, while also reporting the constraints that should inform its adoption. The most significant constraint is the $\sim 150\ \mu\text{m}$ PDMS dielectric barrier set by the DLP-printed molds, which necessitates high applied voltages (200 V) for nonexcitable cell types. A thinner barrier is the most direct route to lower operating voltages, and the constraint here is throughput rather than achievability: the hybrid DLP/TPP chip demonstrates a $45\ \mu\text{m}$ barrier, which the analytical model predicts delivers ~ 3.3 -fold higher field at the same excitation (S10, Figures S6 and S7), but yield at that resolution is too low for routine device production. Reaching comparable thickness by DLP alone or raising the barrier permittivity with high- κ coatings, such as BaTiO_3 or ALD HfO_2 , would give the same gain at practical throughput. A related consequence of the barrier is frequency-dependent transmission: low-frequency protocols remain suboptimal for excitable cells, and stimulation parameters must be modeled per cell type rather than borrowed from direct-coupling literature. The instability of EGaIn above $\sim 1\ \text{V}$ confines the platform to capacitive mode and prevents low-impedance resistive stimulation; passivation coatings, alternative LM alloys, or electrochemically stable conductive hydrogels represent viable paths forward. Electrode resolution with DLP alone is limited to $\sim 200\ \mu\text{m}$, precluding single-cell or subcellular field targeting; the hybrid DLP/TPP route reaches $\sim 25\ \mu\text{m}$ but at throughput and yield that are too low for routine use. Field magnitudes were established by simulation and by the reproducibility of the measured barrier geometry rather than by direct electrical measurements; the reason for this is explained in S4. Although direct viability under the repeated regimen was not separately assessed for 50B11 by live/dead assay, the per-session and cumulative doses (5 min/day; $\sim 10\ \text{min}$ total across the two-day regimen) fall well within the non-cytotoxic range established for single-session stimulation (up to 60 min continuous; Figure SE). Also, OCY454 osteocytes stimulated daily from day 8 to day 15 continued to proliferate, indicating that repeated capacitive stimulation is tolerated over a multi-day culture period. Future work will focus on direct electrical characterization of the delivered field, thinner or higher-permittivity dielectric barriers, additional differentiation markers and neurite morphometry, viability under repeated stimulation, and functional readouts linking the observed calcium and molecular responses to downstream biological outcomes.

3. CONCLUSIONS

We have demonstrated a cleanroom-free workflow for fabricating PDMS microfluidic chips with capacitively coupled 3D liquid-metal (EGaIn) electrodes, using DLP-printed molds to achieve design-to-chip turnaround within a single day at $\sim 200\ \mu\text{m}$ electrode resolution, extendable to $\sim 25\ \mu\text{m}$ through a hybrid DLP/two-photon polymerization process. A $\sim 150\ \mu\text{m}$ PDMS dielectric barrier physically separates the electrodes from the culture medium, preventing the direct electrode–electrolyte contact and associated faradaic byproducts that limit conventional direct-coupled systems. Electrical-field distributions within the culture region were characterized by three complementary methods: an analytical lumped-circuit model, COMSOL, and ANSYS HFSS. The two finite-element solvers agreed to within $\sim 1.5\times$, and the analytical model matched their order of magnitude, together bracketing the field

magnitudes delivered to cells. On this validated foundation, using this platform, we observed stimulus-evoked calcium responses in 50B11 DRG neurons in 2D and 3D collagen and in primary cortical neurons, frequency-dependent upregulation of neuronal markers in FSK-differentiated 50B11 cells, and, in OCY454 osteocytes, changes in proliferation and sclerostin expression following daily stimulation. An automated Cellpose-based pipeline enabled unbiased single-cell calcium analysis across hundreds of cells, and interchangeable electrode geometries allowed stimulation to be biased toward uniform, localized, or directional field patterns without altering fabrication chemistry. Taken together, these results constitute the development and validation of an accessible, customizable platform technology for controlled 3D electrical stimulation of 2D and 3D culture systems. The biological observations reported here demonstrate that the platform is functional across electrically excitable and nonexcitable cell types and across culture formats. Having established and validated this capability, we intend the platform to serve the broader research community as a standardized, reproducible tool for designing future, adequately powered studies of how quantified 3D electrical fields govern cellular and tissue-level biological function.

4. METHODS

4.1. Design and Fabrication of LM-Chips

The process involves four steps: (i) surface modification of glass substrates and resin preparation, (ii) optical printing of the master mold, (iii) replica casting using PDMS, and (iv) injection of Ga-In solution into target channels of the chip.

- Glass substrates were modified before printing. Cleaned glass slides were submerged in piranha solution ($\text{H}_2\text{SO}_4\text{:H}_2\text{O}_2$, 7:3 v/v) for 30 min, then thoroughly rinsed with ethanol and distilled water, and dried under a stream of nitrogen. A solution containing toluene (Sigma-Aldrich) and 3-(trimethoxysilyl) propylmethacrylate (TMSPMA; Sigma-Aldrich) was prepared in a ratio of 8.5:1.5 v/v. Dried glass slides were fully submerged in the solution and incubated at $55\ ^\circ\text{C}$ overnight. Slides were then rinsed with ethanol and distilled water, dried, and stored at $4\ ^\circ\text{C}$ until use. This treatment forms a molecular acrylate layer that ensures proper adhesion of the 3D-printed structure to the substrate. Before printing, the glass slide was precut using a glass cutter and attached to the print head with double-sided tape. In parallel, the photo resin used for master mold fabrication was prepared. The resin consisted of poly(ethylene glycol) diacrylate (PEGDA, $M_n = 250\ \text{Da}$; Sigma-Aldrich) containing 0.4% (w/w) phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide (Irgacure 819; Sigma-Aldrich) as a photoinitiator, 0.4% (w/w) 2-isopropylthioxanthone (ITX; Sigma-Aldrich) as a photosensitizer, and 0.01% (w/w) 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO; Tokyo Chemical Industry) as a photo inhibitor. The stock solution was prepared in a 50 mL centrifuge tube wrapped in aluminum foil and vortexed to ensure thorough mixing, then stored at $4\ ^\circ\text{C}$ until use.
- A custom-assembled digital light processing (DLP) 3D printer was used for printing. This printer combines a continuous-wave 405 nm laser (iBEAM SMART 405, Toptica Photonics), a DLP development kit (DLP 9500 UV, 1080 p, Texas Instruments), and a motorized Z-stage with 50 mm travel (Thorlabs). A rotating diffuser removes laser speckle and converts the Gaussian beam into a uniform flat-top profile before the beam is expanded, collimated, and projected onto a 1920×1080 -pixel digital micromirror device ($\sim 10\ \mu\text{m}$ pixel pitch). A 3D model of the microfluidic chip, a positive replica

of the final chip, was designed in SolidWorks. Custom MATLAB scripts sliced the model into layers and exported each layer as a binary PNG mask. A LabVIEW program then uploaded these masks to the DLP, set the exposure time, and synchronized the DLP's exposure with the Z-stage's upward motion. During printing, the digitally modulated light patterns selectively cure layers of resin held in a vat above an oxygen-permeable PDMS window (maintaining an oxygen-inhibited "dead zone" at the interface). The LabVIEW control system coordinates illumination and stage movement, using a 20 μm layer height (z-step), 100 mW laser intensity, and 1 s exposure per layer; the projected light photopolymerizes each layer; the total number of layers is set by the target mold thickness.

- iii. Replica casting. After completing the 3D printing process, the final mold was thoroughly washed with ethanol to remove any residual resin, adsorbed contaminants, or unwanted materials. The mold was irradiated with ultraviolet (UV) light for several minutes before casting polydimethylsiloxane solution (PDMS, Sylgard 184; Dow Corning Corporation). Briefly, the base and curing agent, mixed in a 10:1 ratio, were degassed, cast onto the PEGDA mold, and allowed to cure at 60 $^{\circ}\text{C}$ for 24 h. The cured PDMS layer was peeled off, trimmed, and holes for inlets and outlets were created using standard biopsy punches of various sizes to accommodate different channel designs. The assembly was designed to fit onto a 22 $\times$ 22 mm no. 1.5 glass slide or coverslip. Immediately before bonding, particulates were removed from the glass coverslip with adhesive tape (Scotch Magic Tape, 3M); no further cleaning or surface modification was applied. Both the PDMS and the glass substrates were then treated with oxygen plasma at 20 standard cubic centimeters per minute, 0.5 Torr, and 60 W (50 kHz) for 25 s, followed by a 5 s purge and a 30 s vent (Femto Science CUTE). The two surfaces were brought into conformal contact immediately after removing from the chamber to form the final PDMS chip. Post-bonding baking was performed at 100 $^{\circ}\text{C}$ overnight to promote a strong, irreversible bond. Bonding integrity was verified by a dye-fill leak check in which the electrode and central culture channels were filled with red and blue food coloring in DI water, respectively, and imaged on a stereomicroscope for 10 min; chips showing dye crossover across the PDMS dielectric wall, PDMS–glass delamination, or port leakage were discarded (S1, Figure S1 and Table S1a).

The DLP process imparts fine pixelation to printed features, reflecting the discrete micromirror array used for projection, while the TPP process imparts a fine periodic corrugation reflecting the line spacing of the raster scan path. As both master molds were printed directly onto smooth glass substrates, this texture was confined to the microstructures, while the surrounding planar regions retained the flatness of the underlying glass. Both masters are positive 2.5D reliefs; so, as in conventional soft lithography, the printed features were transferred to the PDMS as recessed channels while the unwritten planar field was transferred as the raised bonding face; any texture from the writing process therefore resides on the internal channel walls and ceiling rather than on the sealing plane. The corresponding areas of the PDMS replica were cast against a smooth surface, presenting a smooth bonding face that formed conformal contact and a reliable seal with the glass coverslip.

For the central channel of the LM chip designed for PCNs, the surface was pre-coated with 0.1 mg/mL poly-D-lysine (Gibco, A3890401) by incubating at 37 $^{\circ}\text{C}$ overnight. To improve collagen matrix adhesion to the PDMS surface in 3D chips, the central channel was first treated with a 2 mg/mL polydopamine (PD; Sigma-Aldrich, H8502) solution, incubated at room temperature for 24 h, and rinsed three times with PBS. The chip surfaces were then coated with 0.01% poly-L-lysine (Sigma-Aldrich, P4707) for 15 min at room temperature, washed three times with PBS, and subsequently treated with 0.15 mg/mL rat tail type I collagen (Advanced Matrix) for 1 h at room temperature.

Finally, the central channel was washed three times with PBS, dried, and sterilized under UV light for 45 min before introducing the cell suspension into the chip.

- iv. To create 3D liquid-metal (LM) microelectrodes in the chips, eutectic gallium–indium (EGaIn; 75.5% Ga and 24.5% In by weight, m.p. = 15.7 $^{\circ}\text{C}$; MSE Supplies) was injected into designated channels (Ch#1 and Ch#3 for 2D cell culture; Ch#1 and Ch#5 for 3D cell culture) using an air-tight glass syringe with a blunt needle at room temperature under low, regulated pressure. Upon injection, an oxide layer forms almost instantly on the liquid metal surface, providing mechanical stability within the channels while maintaining low viscosity (approximately twice that of water) and high electrical conductivity ($\sigma = 3.4 \times 10^6$ S/m).

In another experiment, multiscale molds were fabricated using a combination of DLP and TPP, offering an efficient and cost-effective alternative to traditional microfabrication techniques for producing intricate devices spanning micrometers to centimeters. The same resin formulation described earlier was used throughout to maintain consistency in material properties. Features larger than 50 μm were printed using DLP, which crosslinks entire resin layers simultaneously as the substrate moves upward, forming the structure layer by layer. For sub-micrometer features, TPP was employed on a custom platform integrating a wavelength-tunable Ti: sapphire femtosecond laser (690–1080 nm, 80 MHz, 140 fs) with a Zeiss microscope, beam-expansion optics, a polarization-based power-tuning system, and real-time imaging via an sCMOS camera. An automated visual basic program precisely controlled the stage movement, enabling accurate positioning for user-defined patterns. The quality of TPP printing was influenced by objective numerical aperture (NA), print speed, laser wavelength (800 nm), and power; for example, microchannels approximately 2 μm in width and height were fabricated using a 50 $\times$ objective (NA = 0.5) at 200 mW and 200 $\mu\text{m/s}$, whereas channels approximately 7 μm in width and 5 μm in height were produced using a 20 $\times$ objective (NA = 0.22) at 350 mW and the same speed. Wider channels were achieved by printing consecutive channels without gaps, and iterative optimization was essential to determine ideal parameters for each objective and resin. The printed microstructures were characterized using a HIROX digital microscope to verify channel dimensions and assess structural fidelity.

4.2. Operation of LM-Chips during Electrical Stimulation

The EF stimulation system consisted of a function generator, an amplifier, and the LM-chip. Electrical signals with user-defined waveforms, frequencies, and amplitudes were generated and subsequently amplified as required. These signals were interfaced with the microfluidic chip via a standard 24-gauge jumper wire that made direct contact with the LM electrodes within the chip's electrode channels (#1 and #5 for five-channel chips, and #1 and #3 for three-channel chips). The chip architecture features a noncontact electrode configuration in which the conductive liquid metal is separated from the cell culture channel by a thin dielectric barrier (~ 150 μm). This design enables the application of a precise, uniform electrical field within the central cell culture channel while physically isolating the electrodes from the culture medium.

Daily stimulation was initiated by connecting the male jumper wire, which was interfaced either directly to the function generator or to the external amplifier, to the designated LM electrode channel on the chip. For stimulation periods longer than 5 min, the connection was established, and stimulation was applied while the chip was placed inside a standard cell culture incubator. For shorter stimulation periods, the procedure was performed inside the biosafety cabinet, and for stimulations conducted during Ca^{2+} activity imaging, the electrical connection was established and maintained throughout the recording session.

4.3. Analytical Model

To estimate the electrical field reaching the cells under capacitively coupled stimulation, the chip was modeled as a one-dimensional series stack of electrode, PDMS, gel, PDMS, and electrode. Each layer was represented as a parallel resistor–capacitor element, with its resistance and capacitance derived from the layer's resistivity, relative permittivity, thickness, and cross-sectional area. Layer impedances were summed in series; the voltage across the gel was obtained by complex voltage division, and the field was taken as that voltage divided by the gel thickness. Because PDMS is effectively insulating, it dominates the stack impedance across the frequency range used here and blocks any steady-state current; the field in the gel, therefore, tracks the rate of change of the applied voltage rather than its instantaneous value. Field strength consequently scales with applied amplitude and with frequency, and decreases as PDMS thickness increases. Under sinusoidal drive, the field oscillates continuously, whereas under square-wave drive it appears as brief transients at the rising and falling edges and is negligible during the constant-voltage plateaus. Governing equations, material parameters, and model assumptions are provided in S3.

4.4. Cell Culture

The 50B11 cell line, an immortalized model of dorsal root ganglion (DRG) neurons, was purchased from the Biobank at Johns Hopkins University and was cultured in T-75 flasks containing neurobasal medium (NBM; Gibco, 21103049). The medium was supplemented with 10% fetal bovine serum (GeminiBio, S11150), 1% of 20% glucose, 0.25% of 0.2 M L-glutamine (Gibco, 25030081), and 2% B-27 supplement (Gibco, 17504044). 50B11 cells were first grown to 70% confluency. To prepare them for seeding, the cells were dissociated from the culture flask with 0.05% trypsin–EDTA (Gibco, 25300054) and subsequently pelleted by centrifugation at 1000 rpm for 5 min. The cell pellet was then resuspended in complete medium, and a cell density of 150,000 cells in 200 μ L was seeded into the central channels of LM-chips for 2D culture experiments. For 3D culture experiments, the central channel was first surface-modified by sequentially incubating it with 2 mg/mL polydopamine (prepared from dopamine hydrochloride; Sigma-Aldrich, H8502) for 24 h, 0.01% poly-L-lysine (Sigma-Aldrich, P4707) for 15 min, and 0.15 mg/mL rat tail type I collagen for 1 h, with PBS washes between each step. The chips were then washed, dried, and sterilized under UV irradiation for 45 min. 50B11 cells in suspension were then mixed with a 3 mg/mL rat tail type I collagen solution prepared according to the manufacturer's protocol. Briefly, stock collagen (10 mg/mL, rat tail tendon, ibidi, Germany) was diluted to 3 mg/mL using 1 $\times$ DMEM (Gibco, 11965092) and 10 $\times$ HBSS (Gibco, 14185052) to achieve a final salt concentration of 1 $\times$ and neutralized to pH 7.2–7.4 by adding a calculated amount of 1 M NaOH. Resuspension medium containing cells was then added and mixed until 10 μ L of the diluted collagen solution contained approximately 10,000 cells. This 10 μ L volume was pipetted into the modified central channel of the LM-chip to achieve a final density of $\sim$ 10,000 cells per chip. Then, the cell-laden chips were incubated at 37 $^{\circ}$ C for 30 min to induce gelation. Following gelation, culture medium was added to the side channels and replenished daily. Following an initial 24 h incubation period at 37 $^{\circ}$ C and 5% CO₂, the culture medium was replaced with medium supplemented with 75 μ M forskolin (FSK; Sigma-Aldrich, F6886) to induce neurite outgrowth under both seeding and encapsulation conditions.

Primary cortical neuronal cultures were established from embryonic day 15.5 CD1 mice (Charles River, 022; RRID: IMSR_CRL:022). Cortices were dissected in dissociation medium containing MgKyn, glucose, AP-V (Sigma-Aldrich, A5282), penicillin–streptomycin (Invitrogen, 15070063), and B27 supplement (Gibco, 17504044), pooled, and dissociated using cysteine (Sigma-Aldrich, C9768) and papain (Sigma-Aldrich, P3125). After the enzymes were washed out, the cell pellet was resuspended in OptiMEM (Gibco, 51985034) and then added to plating medium composed of NBM (Gibco, 21103049) containing GlutaMAX (Gibco, 35050061), fetal bovine

serum (Invitrogen, 50253931), and penicillin–streptomycin. The resulting cell suspension was seeded into a chip pre-coated with 0.1 mg/mL poly-D-lysine at a final density of 150,000 cells per chip. After 4 h, the plating medium was replaced in full with a growth medium consisting of neurobasal supplemented with GlutaMAX, penicillin–streptomycin, N2 (Gibco, 17502048), and B27 supplements.

OCY454 osteocyte-like cells were cultured in alpha-MEM (Gibco, 12571063) supplemented with L-glutamine (Gibco, 25030081), 1% anti–anti (Life Technologies, 15240062), and 10% fetal bovine serum (GeminiBio, S11150). Cells were maintained in surface-treated flasks (Thermo Fisher, 13019) at 33 $^{\circ}$ C in a humidified 5% CO₂ atmosphere. When cultures reached approximately 90% confluency, OCY454 cells were trypsinized (Life Technologies, 12605-010), resuspended in media, and encapsulated in a 2.5 mg/mL type I collagen solution. Collagen preparation followed the manufacturer's protocol: stock collagen (10 mg/mL, rat tail tendon, ibidi, Germany) was diluted to 2.5 mg/mL using 1 $\times$ DMEM (Gibco, 11965092) and 10 $\times$ HBSS (Gibco, 14185052) to maintain a final salt concentration of 1 $\times$ and neutralized to pH 7.2–7.4 by adding a calculated amount of 1 M NaOH. Resuspension medium containing cells was then added and mixed so that 10 μ L of the diluted collagen solution contained approximately 10,000 cells. This mixture was pipetted into channel 3 (Ch#3) of each chip and incubated at 33 $^{\circ}$ C for 30 min to allow collagen gelation, while channels 2 and 4 (Ch#2 and Ch#4) were filled with media and replenished daily. Chips were cultured at 33 $^{\circ}$ C for 3 days and subsequently maintained at 37 $^{\circ}$ C for the remainder of the experiment.

4.5. Cell Viability Assay

Cell viability following ES was assessed using a live/dead viability/cytotoxicity assay (Invitrogen, L3224). 50B11 cells were subjected to ES for 5 min, 15 min, or 1 h. Following stimulation, cells were stained with Calcein AM (1:2000) and ethidium homodimer-1 (1:1000) to identify live and dead cells, respectively. Cells were then washed with phosphate-buffered saline (PBS) and imaged using a Leica DMI6000 inverted fluorescence microscope.

4.6. Calcium Imaging and Analysis

To visualize intracellular Ca²⁺ signaling, cells were loaded using a Fluo-4 calcium imaging kit (Invitrogen). The loading solution was prepared by mixing 10 μ L of 100 $\times$ power load concentrate and 1 μ L of 1000 $\times$ Fluo-4 AM stock into 1 mL of cell culture medium. Existing medium was removed from the chip and replaced with 100 μ L of the Fluo-4 loading solution. The cells were then incubated for 15 min at 37 $^{\circ}$ C, followed by an additional 15 min at room temperature to allow for complete de-esterification of the dye. Time-lapse fluorescence imaging was performed on a Leica DMI6000 Inverted microscope (20 $\times$ objective). Image stacks were recorded at 10 frames per second for a total of 4000 frames. For each experiment, a baseline recording was captured for 10 or 30 s before the application of an electrical field (EF) stimulation. The recorded image stacks were analyzed using FIJI (ImageJ) software. To identify individual cells, a maximum-intensity projection of the time-lapse was created, and cell outlines were automatically traced using a fixed intensity threshold to define the regions of interest (ROIs). The mean fluorescence intensity of each ROI was measured for every frame in the stack, using non-cellular regions as the background for subtraction. For data normalization, the baseline intensity for each cell was calculated as the average fluorescence over the first 10 or 30 s before stimulation. The intensity at each subsequent time point was then divided by this baseline to generate a normalized fluorescence trace expressed as a fold increase. This normalized data was then exported as a CSV file for further analysis and plotting done in MATLAB.

The same fluorescence recordings were reanalyzed using an automated pipeline to enable comprehensive single-cell characterization of the entire neuronal population. Sample drift during acquisition was corrected using the CalmAn toolbox. Cells were segmented with a fine-tuned Cellpose model: the pretrained model was fine-tuned on

20 manually annotated frames (learning rate 4×10^{-4}), improving detection from 84 cells (pretrained) to 313 cells and generating binary masks as spatial ROIs for signal extraction. Fluorescence signals were quantified using corrected total cell fluorescence (CTCF) with local annulus background subtraction applied to each cell to account for spatially varying background fluorescence and neuropil contamination. Normalized fluorescence traces ($\Delta F/F_0$) were computed for all detected cells, and a 15-frame rolling average was applied to reduce high-frequency noise while preserving the temporal structure of the transients. Quality-based filtering on mean fluorescence intensity (≥ 0.197), peak amplitude (≥ 1.148), late-activity index (≥ -0.032), and signal standard deviation retained the 30 most active cells for detailed analysis. To assess functional heterogeneity across the network, correlation-based hierarchical clustering was performed on all detected cells: pairwise correlation distances ($1 - |r|$) between $\Delta F/F$ traces were clustered by average linkage, identifying three groups.

A majority cluster ($n = 259$, 82.7%) showed typical spontaneous activity, and two smaller clusters ($n = 19$, 6.1%; $n = 35$, 11.2%) showed early bursters and late-activator patterns. Clustering was validated by reordering the correlation matrix according to cluster assignments, revealing a clear block structure indicative of within-cluster co-activity distributions across all segmented cells, which was visualized as a symmetric distance matrix.

4.7. Immunofluorescence and Imaging

Cells cultured within chips were fixed with 4% formaldehyde in PBS for 15 min at room temperature, washed three times with PBS, and permeabilized with Triton X-100 in PBS for 10 min. Following permeabilization, nonspecific binding was blocked, and cells were incubated with primary antibodies targeting cell-type-specific markers. After washing to remove unbound primary antibodies, cells were incubated with fluorophore-conjugated secondary antibodies and the nuclear counterstain DAPI (Invitrogen, D1306) at 1 $\mu\text{g/mL}$. Specific conditions for each cell type are detailed below.

For 50B11 cells, permeabilization was performed with 0.25% Triton X-100. Cells were incubated overnight at 4 $^{\circ}\text{C}$ with primary antibodies against the neuronal markers PGP9.5 (chicken polyclonal, Invitrogen, PA1-10011; 1:500) and β III-tubulin (rabbit polyclonal, Invitrogen, PAS-85639; 1:400), diluted in PBS containing 5% horse serum (Gibco, 16050122). Secondary antibodies, Alexa Fluor 555 goat anti-chicken IgY (Invitrogen, A-21437; 1:1000) and Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen, A-21109; 1:300), were applied for 2 h at room temperature in the same buffer.

For OCY454 cells, permeabilization was performed with 0.2% Triton X-100. Nonspecific binding was blocked for 1 h at room temperature with 1% BSA, followed by three 5 min PBS washes. Cells were incubated overnight at 4 $^{\circ}\text{C}$ with primary antibodies against sclerostin (rabbit polyclonal, Proteintech, 21933-1-AP; 1:200), diluted in PBS containing 1% BSA, 0.05% Tween 20, and 0.1% Triton X-100, followed by Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, A-11037), with 5 min washes between steps.

Following the final washes, high-resolution Z-stack images were acquired using a Leica DMi8 confocal microscope equipped with a 25 $\times$ water-immersion objective.

4.8. Statistical Analysis

Quantitative data are presented as the mean $\pm$ standard deviation (SD) or the mean $\pm$ standard error of the mean (SEM), as indicated in each figure legend, which also states the number of biological replicates (n) for each experiment. Two-group comparisons between electrically stimulated and unstimulated control conditions, including the OCY454 osteocyte cell count and sclerostin comparisons at day 8 and day 15 ($n = 3$ chips per group), were performed using unpaired Student's t -tests. Because normality cannot be formally assessed at these small sample sizes, the underlying measures were assumed to be approximately normally distributed, and parametric tests were applied; exact p -values are reported, and results from $n = 3$

experiments are interpreted as preliminary. Comparisons involving more than two groups (the stimulation-duration viability series and the frequency–response experiment; $n = 5$ chips per condition) were evaluated using one-way ANOVA, followed by Tukey's post hoc test for pairwise comparisons. Differences in the frequency response of the two neuronal markers were assessed by two-way ANOVA (marker $\times$ frequency), with the interaction term testing for divergent frequency sensitivity. The pharmacological conditions (calcitriol and mirtazapine pretreatment) were performed as single replicates ($n = 1$) and are therefore reported descriptively, without statistical testing. Significance is denoted as $*p < 0.05$ and $***p < 0.001$ relative to the respective control. All analyses were performed in MATLAB (MathWorks).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsami.6c13015>.

Extended device fabrication and chip variants (DLP mold-printing parameters, photographs of PEGDA master molds and bonded PDMS chips, annotated CAD designs, dye-fill leak-check validation, batch yield and barrier-thickness reproducibility, and an itemized bill of materials with per-chip cost); hybrid DLP/two-photon-polymerization fabrication of the 25 μm and 10 μm channel variants with full TPP printing parameters; dielectric material properties (conductivity, relative permittivity, and layer dimensions) used in all field calculations; the rationale for the infeasibility of direct electrical-field measurement within the culture ROI, together with the electrode-continuity and barrier-thickness measurements used to validate field delivery; an analytical lumped-RC model of the capacitive stack for the field delivered to the culture channel, including governing equations, matching-frequency treatment of square-wave edges, and the 60 kHz sinusoidal case; finite-element electromagnetic validation (COMSOL and ANSYS HFSS) with depth-resolved field maps on six XY planes spanning the collagen gel channel and a three-method comparison of predicted magnitudes; tabulated in-channel electrical-field magnitudes for the three- and five-channel chips, with complete stimulation conditions (voltage, frequency, and duration), field estimates, and replicate counts for every experiment; simulated fields and measured calcium responses for three additional custom electrode geometries (peak field, field uniformity, and peak $\Delta F/F_0$); failure modes of the directly coupled liquid-metal chip (liquid-metal retraction above ~ 1 V and precipitate accumulation under sub-threshold drive); and strategies for reducing the PDMS dielectric barrier (analytical field-vs-thickness relationship, TBAF/NMP chemical etching, and silver-nanoparticle/PDMS composite electrodes) (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information

Cleanroom-free microfluidic chips with capacitively coupled 3D liquid metal electrodes as a platform technology for controlled electrical field stimulation of 2D and 3D cell cultures

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Contents of this Supporting Information

S1 – Extended fabrication: variants, validation, and yield (Figure S1; Tables S1a, S1b)

S2 – Hybrid DLP / two-photon polymerization fabrication (Figure S2; Table S2)

S3 – Material parameters (Table S3)

S4 – Infeasibility of direct field measurement within the ROI

S5 – Analytical model for electrical field

S6 – COMSOL and HFSS simulations (COMSOL/ANSYS HFSS; Figure S3)

S7 – Calculated electrical-field values and stimulation conditions (Tables S4, S5)

S8 – Custom electrode configurations (Figure S4; Table S6)

S9 – Directly-coupled LM chip: failed approach (Figure S5)

S10 – Strategies to reduce the PDMS dielectric barrier (Figures S6–S7)

S1. Extended fabrication: variants, validation, and yield. The DLP-printed PEGDA master molds for the standard three- and five-channel chips described in the main text are shown in Figure S1A and S1D, with their corresponding annotated CAD designs in Figure S1B and S1E, and the fabricated PDMS chips after replica casting and plasma bonding in Figure S1C and S1F; the three-channel device in Figure S1C is shown after EGaIn injection with male jumper wires inserted into the LM ports for electrical connection. A representative passing dye-fill image from a prototype PDMS device with a thinner dielectric wall is shown in Figure S1G, with food coloring confined to the LM channels (red) and central culture channel (blue), and no observable crossover across the 80 μm PDMS dielectric wall. While this prototype demonstrated isolation at an 80 μm wall, a 150 μm wall was selected for all reported devices for reliable, repeatable fabrication.

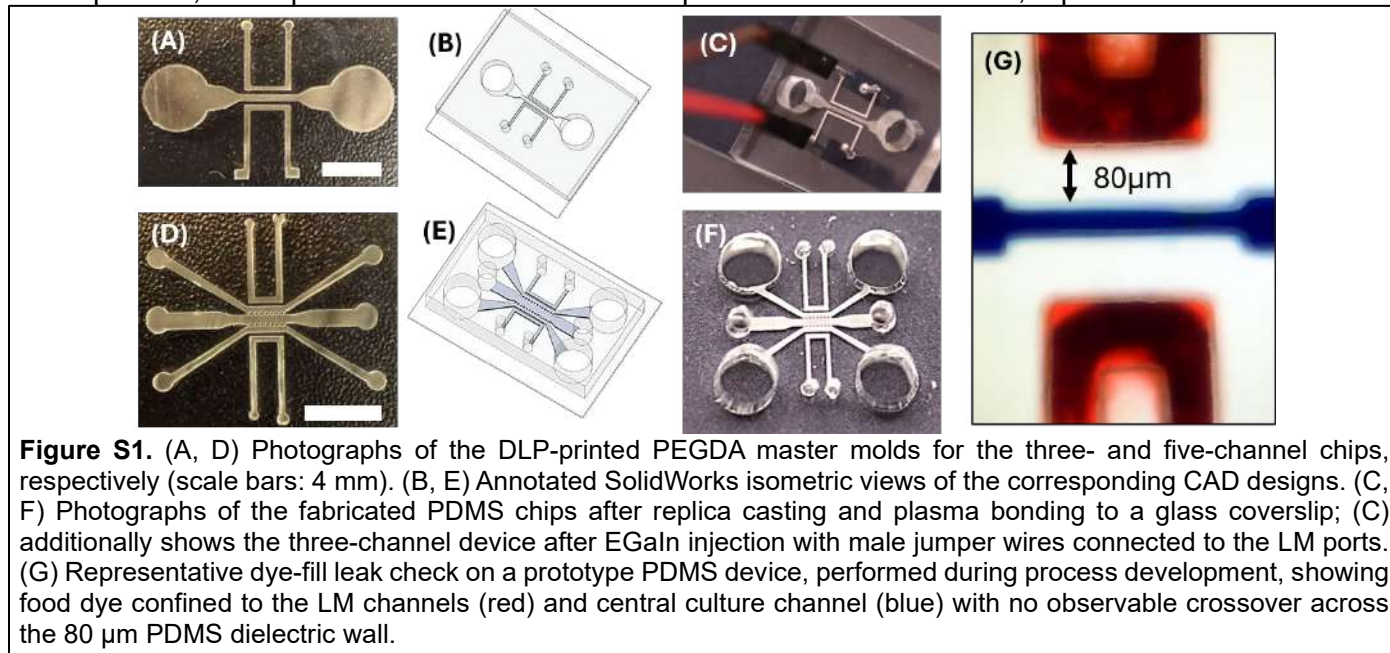


Table S1a. DLP printing parameters for PEGDA master mold fabrication.

Parameter	Value
Printer type	Custom-assembled DLP (405 nm continuous-wave laser)
DMD resolution	1920 × 1080 pixels (~10 μm pixel pitch)
Layer height (Z-step)	20 μm
Laser power	100 mW
Exposure time per layer	1 s
Resin composition	PEGDA ($M_n = 250$ Da) + 0.4 wt% Irgacure 819 + 0.4 wt% ITX + 0.01 wt% TEMPO
Nominal lateral resolution	~200 μm (LM electrode channels)
Post-print wash	Ethanol rinse, followed by UV post-cure
Substrate preparation	Piranha-cleaned glass + TMSPMA silanization
Print environment	Ambient temperature (~22 $^{\circ}\text{C}$), PDMS oxygen-permeable window

Bonding integrity was validated during process development by a dye-fill leak check, in which the LM channels and central culture channel were filled with red and blue food coloring in DI water, respectively, and imaged on a stereomicroscope for 10 min; chips were rejected for dye crossover across the PDMS dielectric wall, PDMS–glass delamination, or port leakage. Across this validation cohort of 47 chips fabricated in 6 independent batches over 4 months, 41 passed (87%), with failures from PDMS–glass delamination ($n = 3$), port leakage ($n = 2$), and dye crossover ($n = 1$). PDMS barrier thickness was measured at three locations per chip on 15 chips from three separate batches; mean thickness was 152 ± 5 μm , confirming batch-to-

batch reproducibility of the $\sim 150\ \mu\text{m}$ target. EGaln injection succeeded in 94% of the fabricated chips after quality control; the failures were due to incomplete filling from air entrapment.

Total fabrication time from CAD design finalization to functional chip averaged $18 \pm 2\ \text{h}$ across 10 independent runs. An itemized bill of materials and per-chip cost estimate is provided in Table S1b. Consumable cost is approximately \$3.00–\$5.00 per chip at the batch scale (10 chips per batch), compared with \$50–\$200 per chip and 2–4-week lead times for equivalent cleanroom-photolithography devices.

Table S1b. Bill of materials for fabrication of 10 LM chips (three-channel design).

Item	Vendor	Cat. No.	Qty per 10 chips	Cost / 10 chips
PEGDA (Mn = 250 Da)	Sigma-Aldrich	475629	1 mL	\$2.50
Irgacure 819	Sigma-Aldrich	511447	20 mg	\$1.20
ITX (2-isopropylthioxanthone)	Sigma-Aldrich	271969	20 mg	\$0.80
TEMPO	Tokyo Chemical Industry	T0634	0.5 mg	\$0.10
PDMS base + curing agent (Sylgard 184)	Dow Corning	4019862	30 g	\$10.00
EGalIn (75.5% Ga, 24.5% In)	MSE Supplies		0.2 mL	\$8.00
Glass coverslips (22×22 mm, No. 1.5)	VWR	48366-227	10	\$1.50
Biopsy punches (assorted)	Integra Miltex	Various	1 set	\$5.00
Jumper wires (24-gauge)	Generic		20	\$0.50
Piranha reagents (H_2SO_4 , H_2O_2)	Sigma-Aldrich	Various	Per protocol	\$3.00
TMSPMA	Sigma-Aldrich	440159	0.15 mL	\$0.40
Total (consumables only)				~\$26–\$36 (~\$3.30/chip)

S2. Hybrid DLP / Two-Photon Polymerization Fabrication. For the high-resolution multiscale chip shown in Figure 2 and S2, TPP was used to add microscale features to a DLP-printed base, enabling electrode and media-channel widths an order of magnitude below the $\sim 200\ \mu\text{m}$ resolution limit of DLP alone. The same PEGDA/Irgacure-819/ITX/TEMPO resin formulation was used in both printing modes to maintain chemical continuity and avoid interfacial delamination during PDMS replica casting. Two objective configurations were used: a 50×/NA 0.5 objective at 200 mW for highest-resolution features ($\sim 2 \times 2\ \mu\text{m}$ channels), and a 20×/NA 0.22 objective at 350 mW for medium-resolution features ($\sim 7 \times 5\ \mu\text{m}$ channels). Wider channels were produced by adjacent multi-pass printing with no inter-pass gap. Full TPP parameters are summarized in Table S2. Two variants were successfully fabricated and replicated into PDMS using this pipeline (Figure S2): a 25 μm central media channel and a 10 μm central media channel, each flanked by TPP-defined LM-electrode microchannels. EGaln was injected into the electrode microchannels at room temperature and remained stably confined within the TPP-defined geometry, with no intrusion into the central media channel.

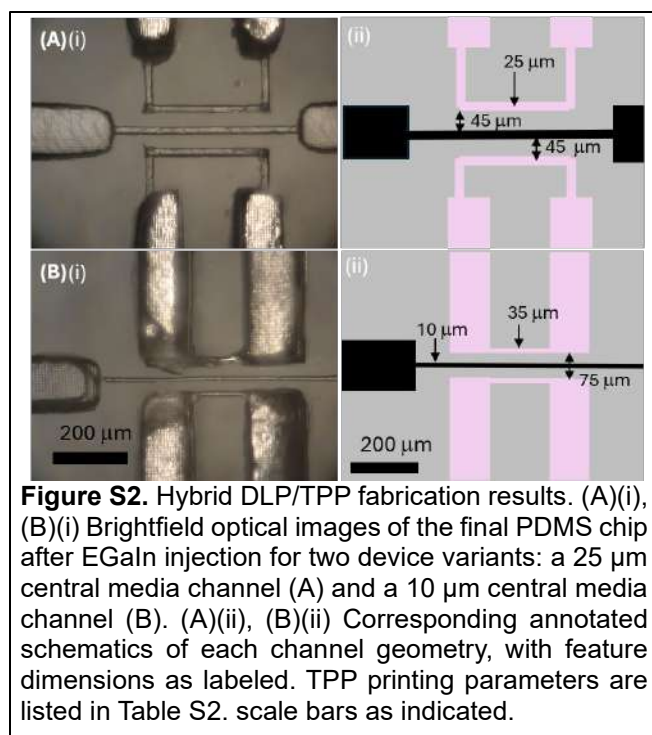


Table S2. Two-photon polymerization parameters to fabricate the high-resolution multiscale chips shown in Figure 2 and S2. Iterative optimization was required to identify ideal parameters for each objective and resin batch.

Parameter	Value/setting
Laser source	Wavelength-tunable Ti: sapphire (690–1080 nm), 80 MHz, 140 fs pulses
Operating wavelength	800 nm
Stage controller	Built-in XYZ stage of Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss, Germany)
Scan speed	200 $\mu\text{m/s}$ (all objectives)
High-resolution settings	50 $\times$ / NA 0.5, 200 mW, $\sim 2\ \mu\text{m} \times 2\ \mu\text{m}$ channels
Medium-resolution settings	20 $\times$ / NA 0.22, 350 mW, $\sim 7\ \mu\text{m} \times 5\ \mu\text{m}$ channels
Final electrode pitch	$\sim 25\ \mu\text{m}$ electrode width, $\sim 25\ \mu\text{m}$ media channel
Quality inspection	HIROX digital microscope

S3. Material Parameters. The material properties used in both analytical calculations and numerical simulations throughout this work are summarized in Table S3. Values for the collagen hydrogel in the five-channel chip were taken as $\sigma = 1/800\ \text{S/m}$ (0.00125 S/m) and $\epsilon_r = 80$, consistent with typical reports for hydrated collagen at physiological ionic strength.¹

Table S3. Material properties used in electrical field calculations.

Material	Conductivity (S/m)	Relative Permittivity	Thickness along field width (μm)
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PDMS	$\sim 10^{-14}$ to 10^{-12}	2.5–3.0	$\sim 152 \pm 5 \mu\text{m}$
Culture Media	1.5	~ 80	200–400
Collagen Hydrogel	1.25×10^{-3}	~ 80	500
Liquid Metal (EGaIn)	3.4×10^6	N/A (conductor)	200
Glass Substrate	$\sim 10^{-12}$	4.0–7.0	$\sim 170 \mu\text{m}$ (No. 1.5 coverslip)

S4. Infeasibility of direct field measurement within the ROI. Direct measurement of $|E|$ would require positioning two microelectrodes at a known distance within the ROI and recording the potential difference between them with a voltage-measuring instrument, yielding $|E| \approx \Delta V/d$. This is infeasible in the present geometry for the following reasons. The electrodes are far more conductive than the collagen (1.25×10^{-3} S/m) and locally short the field they sample, most severely in the gel, whose high impedance relative to the media (1.5 S/m) produces the concentration exploited in the five-channel design. Insertion through the 300 μm collagen also creates a fluid track that backfills with culture medium, which is roughly three orders of magnitude more conductive than the gel, thereby altering local current by an amount that varies between insertions. And the spreading resistance of a micron-scale tip ($\sim 200 \text{ M}\Omega$) with instrument and cable capacitance forms a low-pass filter (corner $\sim 1.6 \text{ kHz}$) that cannot pass the sub-25 ns drive edges (spectral content to $\sim 14 \text{ MHz}$), recording only the 10 Hz envelope rather than the transient response resolved by the HFSS solver. Non-contact methods are also unsuitable: electro-optic probes have sensitivity floors above the 27-108 V/m three-channel range, voltage-sensitive dyes and voltage indicators report the transmembrane potential rather than the bulk field, and magnetic resonance electrical impedance tomography is limited to millimeter resolution.

Field delivery was therefore validated through the fabricated quantities that determine it. All EGaIn electrode lines measured below 1Ω by two-terminal resistance confirming continuous conductor formation; this value is at the resolution limit of the method and reflects fabrication consistency rather than an intrinsic resistance. As it is negligible relative to the series impedance of the PDMS and gel, the field magnitude is governed principally by the PDMS barrier thickness, $152 \pm 5 \mu\text{m}$ (coefficient of variation 3.3%; $n = 15$ chips, three batches; S1). Since field magnitude varies inversely with barrier thickness, the 3.3% thickness CV translates directly into an $\approx 3\%$ bound on chip-to-chip field variation at a given applied voltage. Together, these indicate reproducible field delivery across chips, consistent with the models in S6.1 and S6.2.

S5. Analytical Model for Electrical Field Calculations. An analytical model was developed to estimate the electrical field (E-field) distribution within the layered microfluidic chip structure. The model treats each material layer as a parallel RC circuit element, accounting for both resistive and capacitive contributions to the overall impedance. This approach enables frequency-dependent analysis, which is essential for capacitively coupled stimulation systems.

S5.1 Define Basic Layer Properties. The fundamental electrical properties for each distinct layer in the microfluidic device are calculated as follows:

Resistance (R_i): The resistance of the i^{th} layer is calculated using its resistivity (ρ_i), thickness (l_i), and cross-sectional area (A):

$$R_i = \rho_i \frac{l_i}{A} \quad 1$$

Capacitance (C_i): The capacitance of the i^{th} layer is calculated using its relative permittivity (ϵ_{ri}), thickness (l_i), area (A), and the permittivity of vacuum ($\epsilon_0 \approx 8.854 \times 10^{-12} \text{ F/m}$):

$$C_i = \frac{\epsilon_0 \epsilon_{ri} A}{l_i} \quad 2$$

S5.2 Calculate Layer and Total Impedance. Impedance (Z) is the frequency-dependent opposition to alternating current (AC). It is a complex value comprising both resistance and reactance. For each layer modeled as a resistor and capacitor in parallel:

$$\frac{1}{Z_i} = \frac{1}{R_i} + j\omega C_i \quad 3$$

Where $\omega = 2\pi f$ is the angular frequency, and j is the imaginary unit. The full impedance of a layer is:

$$Z_i = \frac{R_i(1 - j\omega C_i R_i)}{1 + \omega^2 C_i^2 R_i^2} \quad 4$$

The total impedance of the system is the sum of the individual complex impedances of all layers connected in series:

$$Z_{total} = \sum_i Z_i \quad 5$$

S5.3 Electrical Field Strength for Sinusoidal Waveforms. The voltage drop across a layer of interest is determined by voltage division:

$$V_i = \frac{Z_i}{Z_{total}} \cdot V_{in}$$

The magnitude of the electrical field is the magnitude of the complex voltage drop divided by the layer thickness:

$$|E_i| = \frac{|V_i|}{l_i}$$

S5.4 Estimation for Non-Sinusoidal Waveforms. For non-sinusoidal waveforms such as square waves or linear ramps, the electrical field is primarily generated during the transient phases (rising and falling edges). The field can be estimated by determining a "matching frequency" that represents the ramp's rate of change:

$$f = \frac{1}{2\pi\tau}$$

Where τ is the rise time or fall time of the ramp. This matching frequency is then used to calculate the corresponding angular frequency ($\omega = 2\pi f$) and applied to the impedance equations above. For our square-wave stimulation with a <25 ns rise time, the matching frequency is approximately 6.37 MHz.

This approach provides a correct order-of-magnitude estimate for the peak E-field strength during the rising periods, though it does not capture the complete time-domain waveform shape. The system equations are designed for sinusoidal inputs; rather than solving complex time-domain ODEs for arbitrary waveforms, the rise time is converted to estimate a dominant frequency component that captures the system's essential behavior. This enables AC impedance analysis at that frequency, simplifying calculations while still capturing the frequency-dependent behavior of the capacitive and resistive elements. At 6.37 MHz, impedance analysis reveals that the PDMS layers dominate the system's impedance due to their low conductivity, while the culture media contribute a much smaller impedance.

For comparison, under sinusoidal stimulation, the same frequency-domain (AC) impedance framework can be applied directly at the drive frequency (here, 60 kHz) without converting a rise time. Using a 60 kHz sine wave with 200 V (peak) applied to the five-channel chip, the electrical-field magnitude in the gel is approximately 4.68×10^3 V/m, consistent with the gel remaining the highest-impedance layer in the stack.

S6. COMSOL and HFSS simulations. The analytical voltage-divider model treats the chip as a 1D element stack and is most appropriate for order-of-magnitude estimates. To recover spatially resolved field magnitudes, time-dependent finite-element simulations were performed in COMSOL Multiphysics (AC/DC Module, Electric Currents) and cross-checked against ANSYS HFSS (Transient solver). Geometry, material properties, and excitation waveforms are specified in the main-text Methods and Table S3. Only the SI-unique post-processing, the depth-resolved analysis of the five-channel gel channel, is presented below.

S6.1 Depth-resolved field uniformity in the five-channel gel channel. The main-text COMSOL summary reports the bulk electrical-field magnitude in the gel region of the five-channel chip, but 3D cell-culture

experiments rely on cells distributed throughout the full 300 μm thickness of the collagen gel. To verify that cells at every depth experience comparable stimulation, $|E|$ was extracted on six XY planes spanning the gel channel from the bottom PDMS interface ($Z = 0 \mu\text{m}$) to the top ($Z = 300 \mu\text{m}$), all rendered on a common color scale (1690–1790 V/m) so slices can be compared without rescaling.

The bulk field is essentially uniform across the gel volume at $\sim 1700 \text{ V/m}$ (Figure S3), consistent with the analytical and ANSYS HFSS estimates summarized in the main text. Edge enhancement is confined to the two interface planes at $Z = 0$ and $Z = 300 \mu\text{m}$, where the gel directly contacts the PDMS dielectric: at these planes, $|E|$ rises to $\sim 1780 \text{ V/m}$ within $\sim 30 \mu\text{m}$ of the lateral boundaries ($X \approx \pm 250 \mu\text{m}$, adjacent to the LM electrode columns), while the central 80 % of each slice remains within $\sim 1 \%$ of the bulk value. Across the four interior planes ($Z = 50, 100, 200$, and $250 \mu\text{m}$), the field is uniform within 1–2 % over the full XY extent, with no detectable edge enhancement. Except for a sub-30 μm layer at the gel–PDMS interfaces, every cell embedded in the gel sees essentially the same field magnitude to within the numerical resolution of the simulation. The boundary-adjacent enhancement at $Z = 0$ and $300 \mu\text{m}$ is a fringing artifact at the gel–PDMS edge and does not represent a meaningful population of cells exposed to elevated fields, since the gel-loading protocol distributes cells throughout the bulk rather than at the dielectric surfaces.

S6.2 Comparison of analytical, COMSOL, and ANSYS HFSS predictions.

The two finite-element solvers agree to within $\sim 1.5\times$ of each other (COMSOL $1.72\text{--}1.79 \times 10^3 \text{ V/m}$; ANSYS HFSS $2.60\text{--}2.63 \times 10^3 \text{ V/m}$, $10 \text{ V} / 10 \text{ Hz}$ square wave; Table S4). The analytical estimate ($1.06 \times 10^3 \text{ V/m}$) reproduces the correct order of magnitude, underestimating COMSOL by $\sim 1.6\times$ and HFSS by $\sim 2.5\times$. The deviation between the analytical model and the FEM solvers reflects effects the lumped-circuit model intentionally omits: 3D fringing at material boundaries, non-uniform current paths that redistribute voltage across the layered structure, finite electrode dimensions and ohmic losses, and asymmetry introduced by surrounding layers. The analytical model, therefore, remains useful for parametric scaling, while COMSOL and ANSYS HFSS provide the spatially resolved magnitudes used for quantitative validation.

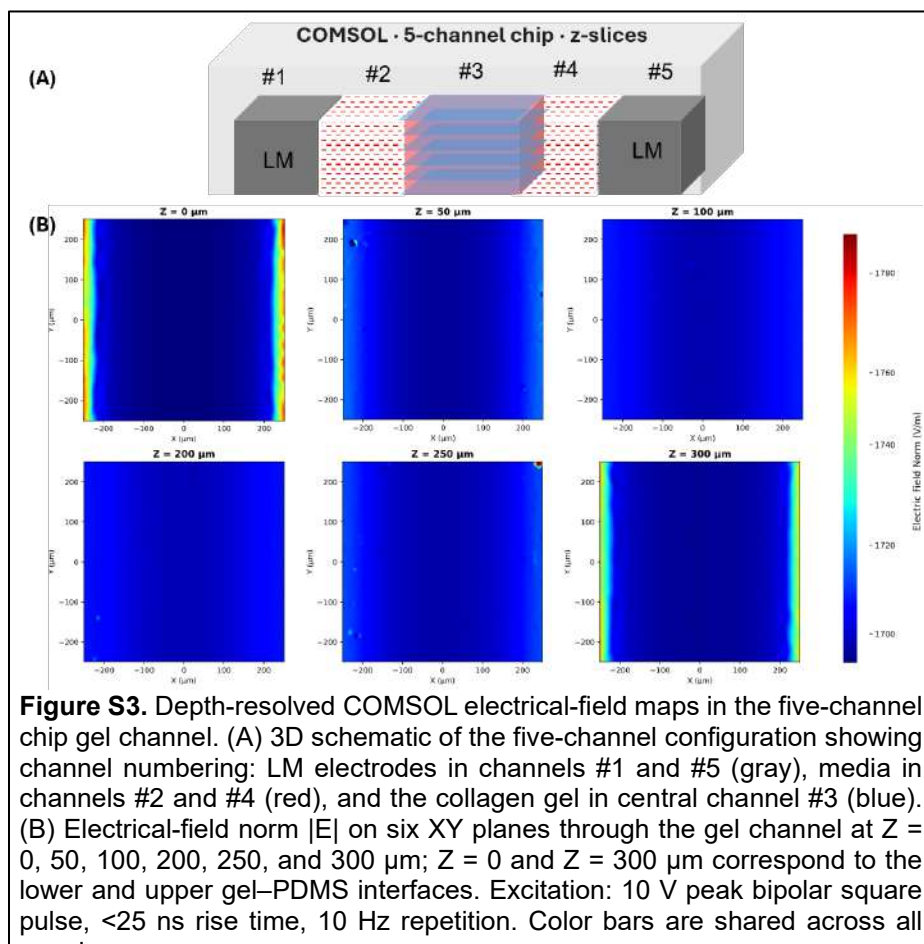


Figure S3. Depth-resolved COMSOL electrical-field maps in the five-channel chip gel channel. (A) 3D schematic of the five-channel configuration showing channel numbering: LM electrodes in channels #1 and #5 (gray), media in channels #2 and #4 (red), and the collagen gel in central channel #3 (blue). (B) Electrical-field norm $|E|$ on six XY planes through the gel channel at $Z = 0, 50, 100, 200, 250$, and $300 \mu\text{m}$; $Z = 0$ and $Z = 300 \mu\text{m}$ correspond to the lower and upper gel–PDMS interfaces. Excitation: 10 V peak bipolar square pulse, $<25 \text{ ns}$ rise time, 10 Hz repetition. Color bars are shared across all

S7. Calculated Electrical Field Values. Table S4 summarizes the electric field values calculated by each method for both chip configurations.

Table S4. Summary of electric field magnitudes in the cell culture region.

Configuration	Stimulation	Analytical	COMSOL	ANSYS HFSS
three-channel (Media)	10V, 10Hz square	21.25 V/m	27–108 V/m	40–100 V/m

five-channel (Gel)	10V, 10Hz square	$\sim 1.06 \times 10^3$ V/m	$1.72\text{--}1.79 \times 10^3$ V/m	$2.6\text{--}2.63 \times 10^3$ V/m
five-channel (Gel)	200V, 60kHz sine	$\sim 4.68 \times 10^3$ V/m	—	—

Table S5 provides the complete set of electrical stimulation conditions used across all cell-type experiments, the field estimate used to design each experiment, and the number of independent biological replicates.

Table S5. Complete stimulation conditions, field estimates, and biological replicates for all experiments reported in the main text.

Cell type/readout	Chip	Waveform	Schedule	E ROI	n (chips)	Statistical status
50B11 (2D, viability)	three-channel	10 V, 10 Hz square	5 / 15 / 60 min, single dose	27–108 V/m (COMSOL)	5 per condition	Confirmed (ANOVA)
50B11 (2D, Ca ²⁺)	three-channel	10 V, 10 Hz square	During recording	27–108 V/m (COMSOL)	5 per condition	Confirmed
50B11 (2D, Neuronal markers)	three-channel	10 V, 10/100/1k/1M Hz	5 min/day $\times$ 2 days	Frequency-dependent	5 per condition	Confirmed
50B11 (3D, Ca ²⁺)	five-channel	10 V, 10 Hz square	During recording	$(1.7 - 2.6) \times 10^3$ V/m (COMSOL)	3 per condition	Confirmed
PCN (Ca ²⁺ , ES only/control)	three-channel	10 V, 10 Hz square	During recording	27–108 V/m (COMSOL)	3 chips	Confirmed
PCN (calcitriol/mirtazapine)	three-channel	10 V, 10 Hz square	During recording	27–108 V/m (COMSOL)	1 per drug condition	Preliminary
OCY454 (3D, sclerostin)	five-channel	200 V, 60 kHz sine	5 min daily, Day 8-15	$\sim 4.68 \times 10^3$ V/m (analytical)	3 per condition	Preliminary
50B11 (electrode geometries)	three-channel (custom)	10 V, 10 Hz square	During recording	Geometry-dependent	3 per geometry	Confirmed

S8. Custom electrode configurations. The three custom electrode geometries (symmetric partial-coverage, V-shaped converging, asymmetric same-side) were simulated in COMSOL using identical material properties, boundary conditions, and 10 V peak / 10 Hz square-wave excitation as the standard chip. Field profiles were extracted along the centerline of the media channel over ± 2 mm from the channel center (Figure 4B). Field uniformity is reported as the coefficient of variation of |E| over a central ROI of ± 2 mm, applied identically to all three geometries (Table S6).

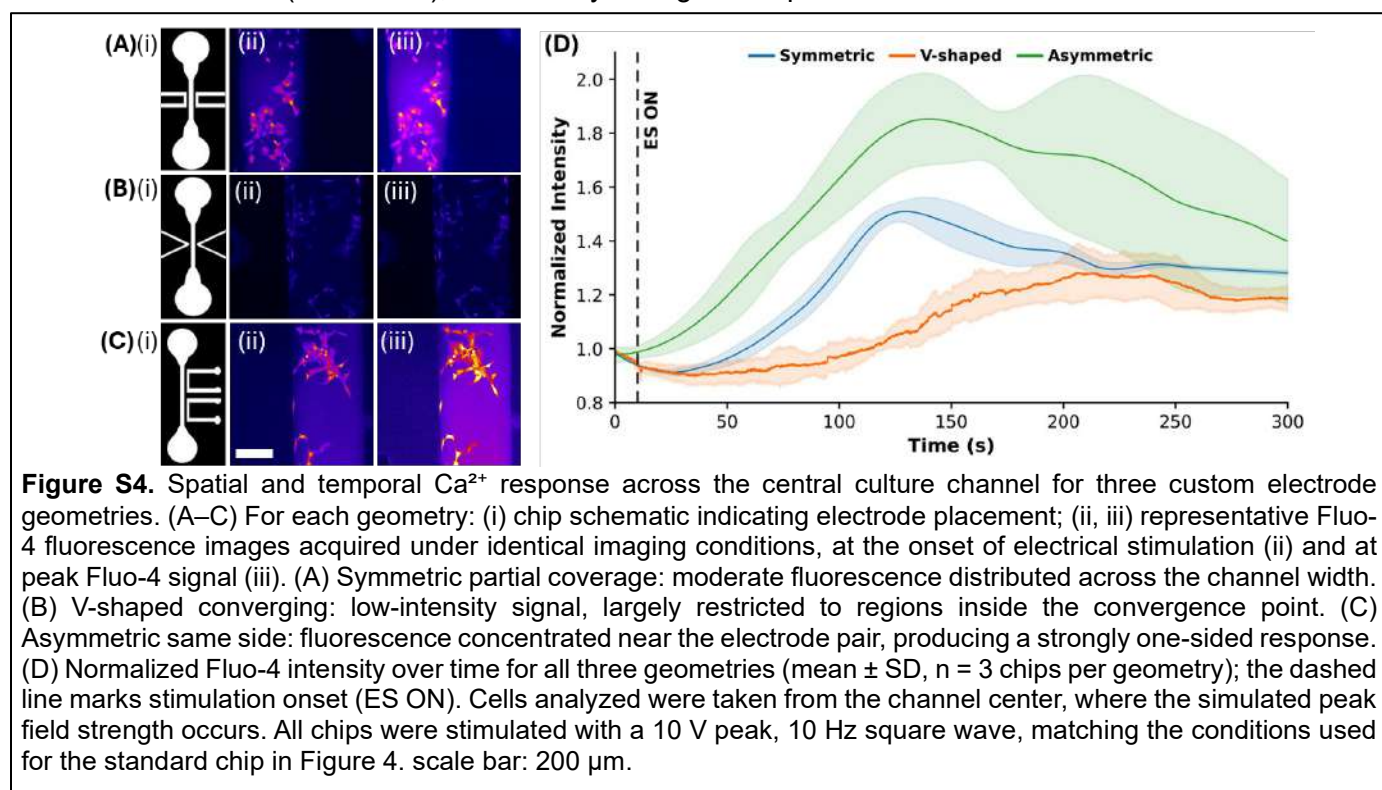
Table S6. Quantitative comparison of electrode geometry performance (n = 3 chips per geometry).

Electrode Geometry	Peak Field (V/m, COMSOL)	Field uniformity, CV (%)	Peak $\Delta F/F_0$ (mean $\pm$ SD)	Spatial Pattern
Symmetric opposing (partial coverage)	~ 60 (flat plateau across the center)	$\sim 2\text{--}3\%$	1.5 ± 0.18	Uniform across channel
V-shaped converging	~ 58 (narrow peak at the convergence point)	$\sim 30\text{--}35\%$	1.3 ± 0.31	Localized to convergence zone

Electrode Geometry	Peak Field (V/m, COMSOL)	Field uniformity, CV (%)	Peak $\Delta F/F_0$ (mean $\pm$ SD)	Spatial Pattern
Asymmetric same side	~310 (peak at the center)	~31%	1.85 $\pm$ 0.22	Concentrated, electrode-proximal

Chips of each geometry were then stimulated under the same conditions and imaged with Fluo-4 to compare the measured Ca^{2+} response against the predicted field distribution ($n = 3$ chips per geometry). Ca^{2+} responses closely reflected the predicted field distributions. Symmetric opposing electrodes produced a moderate, relatively uniform calcium response across the channel. The asymmetric same-side configuration yielded the strongest signal intensity, concentrated on the electrode-proximal side of the channel. The V-shaped configuration produced a weaker and noticeably delayed response outside the convergence region compared with inside, consistent with the simulated rapid spatial falloff in field magnitude. Representative fluorescence images at stimulation onset and at peak signal are shown in Figure S4A–C, and mean traces for all replicate chips in Figure S4D. Peak field magnitudes, field uniformity, and peak normalized fluorescence are summarized in Table S6.

These measurements support the use of electrode layout as an independent design parameter, biasing stimulation toward broad uniform activation (symmetric opposing), localized spatial patterning (V-shaped), or directional activation (same-side), without any change to chip fabrication.



S9. Directly-Coupled LM Chip — Failed Approach.

Figure S5 documents the directly coupled architecture explored prior to the capacitively coupled design reported in the main text. This section is included for transparency and to provide a rationale for adopting capacitive coupling exclusively. The directly coupled chip incorporated a 30 μm wide PDMS-microgroove array ($\sim 200\ \mu\text{m}$ in length) connecting the central culture channel ($\sim 700\ \mu\text{m}$ wide, #2) to the LM electrode region (200 μm wide, #1, #3). Chip dimensions and injection pressures were optimized to prevent LM intrusion into the microgrooves or culture channel. Inner surfaces were coated with 2 mg/mL polydopamine for 24 h to render them hydrophilic and ensure media continuity between electrodes and culture channel. While the spontaneous formation of a thin gallium oxide passivation layer provides beneficial mechanical stability, allowing the liquid to retain complex shapes, this interface becomes unstable under active electrical stimulation, leading to two distinct failure modes.

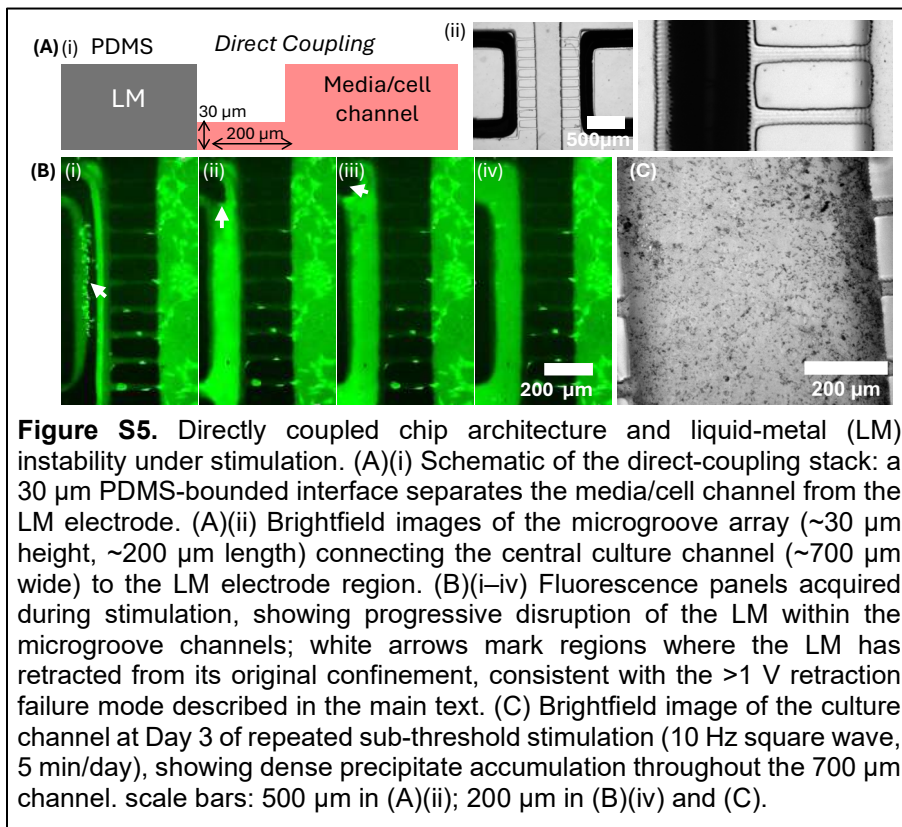


Figure S5. Directly coupled chip architecture and liquid-metal (LM) instability under stimulation. (A)(i) Schematic of the direct-coupling stack: a 30 μm PDMS-bounded interface separates the media/cell channel from the LM electrode. (A)(ii) Brightfield images of the microgroove array ($\sim 30\ \mu\text{m}$ height, $\sim 200\ \mu\text{m}$ length) connecting the central culture channel ($\sim 700\ \mu\text{m}$ wide) to the LM electrode region. (B)(i-iv) Fluorescence panels acquired during stimulation, showing progressive disruption of the LM within the microgroove channels; white arrows mark regions where the LM has retracted from its original confinement, consistent with the $>1\ \text{V}$ retraction failure mode described in the main text. (C) Brightfield image of the culture channel at Day 3 of repeated sub-threshold stimulation (10 Hz square wave, 5 min/day), showing dense precipitate accumulation throughout the 700 μm channel. scale bars: 500 μm in (A)(ii); 200 μm in (B)(iv) and (C).

S9.1 Failure Mode 1: LM retraction (voltages $> \sim 1\ \text{V}$).

When applied voltages exceeded $\sim 1\ \text{V}$, the LM retracted within microchannels within seconds, thereby eliminating the electrode and rendering the device non-functional (Figure S5B(i-iv), see white arrow). This is consistent with the electrochemical stripping of the gallium oxide passivation layer: Khan et al.² showed that a reductive potential of $\sim 1\ \text{V}$ applied to EGaIn in an aqueous electrolyte removes the surface oxide, restoring the high native surface tension ($\sim 500\ \text{mN/m}$) and triggering capillarity-driven withdrawal of the metal from microchannels. Hillaire et al.³ further demonstrated that applied potentials as low as 0.8 V can reduce the effective interfacial tension of EGaIn to near zero in electrolyte, causing dramatic morphological instability.

S9.2 Failure Mode 2: Precipitate accumulation (sub-threshold voltages).

At voltages below the retraction threshold, repeated daily stimulation (10 Hz square wave, 5 min/day over 7 days) progressively darkened the 700 μm culture channel as dense precipitates accumulated (Figure S5C). Precipitates were first apparent by the second-to-third stimulation session and were distributed throughout the channel, including regions distant from the groove interface, indicating release into the bulk medium rather than localized interfacial deposition. Their onset coincided with a progressive loss of adherent cells in the affected regions. Fragmented gallium oxide, unoxidized metal microdroplets, and cytotoxic indium ions have been reported in aqueous physiological media under similar conditions.^{4, 5} The progressive nature of this degradation indicates that sub-threshold electrochemical stress

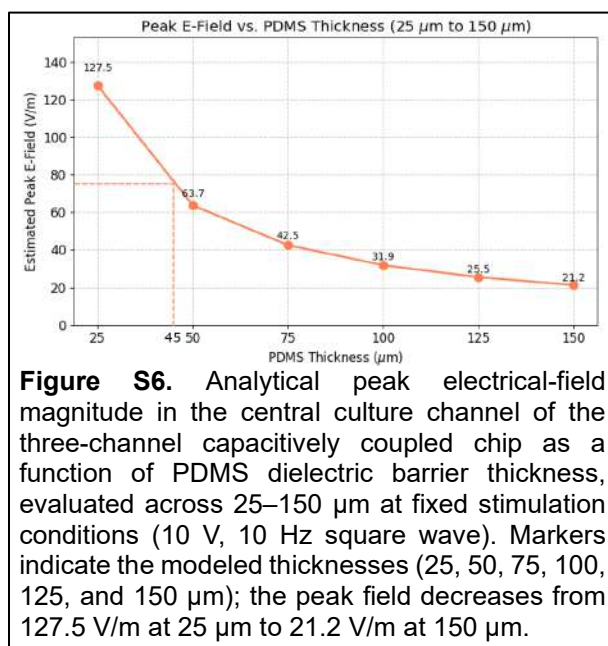


Figure S6. Analytical peak electrical-field magnitude in the central culture channel of the three-channel capacitively coupled chip as a function of PDMS dielectric barrier thickness, evaluated across 25–150 μm at fixed stimulation conditions (10 V, 10 Hz square wave). Markers indicate the modeled thicknesses (25, 50, 75, 100, 125, and 150 μm); the peak field decreases from 127.5 V/m at 25 μm to 21.2 V/m at 150 μm .

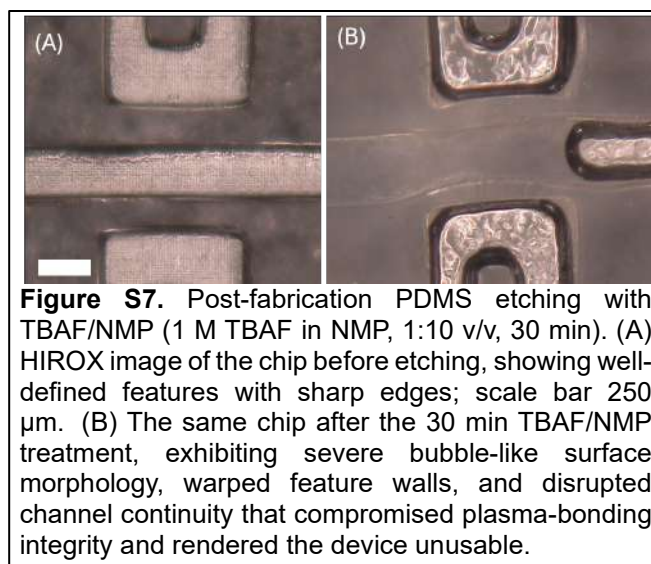
accumulates over repeated stimulation cycles, aligning with documented nonuniform oxide dissolution under modest applied potentials⁴ and time-dependent Ga^{3+} release following square-root-of-time kinetics in physiological buffers⁵.

Quantitative viability and compositional assays were not pursued for this configuration. The effect was progressive and reproducible across replicates, and its severity (precipitate accumulation with visible cell loss under sub-threshold drive) precluded direct coupling for prolonged culture and motivated the move to non-contact capacitive coupling. Because the degradation accumulates over repeated cycles, it cannot be mitigated by reducing voltage alone. Together, these failure modes establish that directly coupled LM electrodes are unsuitable for prolonged cell culture applications under the stimulation conditions used here and motivate the exclusive adoption of the capacitively coupled architecture.

S10. Strategies to reduce the PDMS dielectric barrier. In the capacitively coupled architecture, the peak electrical field magnitude in the central culture region scales inversely with PDMS dielectric barrier thickness. Figure S6 shows the analytical solution for the peak field across barrier thicknesses of 25–150 μm in the three-channel chip at fixed stimulation conditions. The field falls from 127.5 V/m at 25 μm to 21.2 V/m at 150 μm , a ~ 6 -fold drop over the modeled range. Reducing the barrier to 50 μm increases the peak field 3-fold (21.2 $\rightarrow$ 63.7 V/m); reducing further to 25 μm yields an additional $\sim 2\times$ gain. This inverse dependence on dielectric thickness motivates reducing the barrier to a value below the minimum imposed by DLP printer resolution. Two strategies were explored: post-fabrication chemical etching with TBAF/NMP (S10.1) and incorporation of silver nanoparticles into the PDMS matrix to raise its dielectric permittivity (S10.2). Both were ultimately abandoned due to technical challenges described below; thus, for this work, the final chips with ~ 150 μm PDMS barrier were used.

S10.1 Post-fabrication PDMS etching with TBAF/NMP. The tetra-*n*-butylammonium fluoride (TBAF)/1-methyl-2-pyrrolidinone (NMP) etch protocol followed the formulation of Wang et al., in which NMP serves as a co-solvent that minimizes PDMS swelling during etching.⁶ Etching was performed for 30 min at room temperature on plasma-bonded PDMS-on-glass chips. The resulting etched channel widths ranged from approximately 170 to 270 μm across nominally identical chips, with substantial variability within and across chips (Figure S7B). Etched regions also developed a textured, bubble-like surface morphology that prevented reliable plasma-bonding. This combination of non-uniform etching and degraded bonding surface made the protocol unsuitable for reproducible chip fabrication.

S10.2 Silver nanoparticle / PDMS composite. Silver (Ag) nanoparticles (0.5 wt%) were incorporated into PDMS to increase the effective dielectric permittivity of the barrier. In a series capacitive stack, the field magnitude in the cell-culture region scales with the relative permittivity of the dielectric layer, so raising ϵ of the PDMS barrier would proportionally raise the in-channel field without requiring higher applied voltages or thinner walls. Loading PDMS with conductive nanoparticles is an established route to large increases in permittivity, particularly as the filler approaches the percolation threshold.⁷ In practice, gravitational sedimentation during thermal cure produced macroscopic concentration gradients across each slab, resulting in spatially non-uniform dielectric properties and poor chip-to-chip reproducibility.



References

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