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Finite element modelling of a cellular electric microenvironment

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TITLE:

Finite element modelling of a cellular electric microenvironment

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SUMMARY:

This paper presents a strategy for building finite element models of fibrous conductive materials exposed to an electric field (EF). The models can be used to estimate the electrical input that cells seeded in such materials receive and assess the impact of changing the scaffold's constituent material properties, structure or orientation.

ABSTRACT:

Clinical studies show electrical stimulation (ES) to be a potential therapy for the healing and regeneration of various tissues. Understanding the mechanisms of cell response when exposed to electrical fields can therefore guide the optimization of clinical applications. In vitro experiments aim to help uncover those, offering the advantage of wider input and output ranges that can be ethically and effectively assessed. However, the advancements in in vitro experiments are difficult to reproduce directly in clinical settings. Mainly, that is because the ES devices used in vitro differ significantly from the ones suitable for patient use, and the path from the electrodes to the targeted cells is different. Translating the in vitro results into in vivo procedures is therefore not straightforward. We emphasize that the cellular microenvironment's structure and physical properties play a determining role in the actual experimental testing conditions and suggest that measures of charge distribution can be used to bridge the gap between in vitro and

in vivo. Considering this, we show how in silico finite element modelling (FEM) can be used to describe the cellular microenvironment and the changes generated by electric field (EF) exposure. We highlight how the EF couples with geometric structure to determine charge distribution. We then show the impact of time dependent inputs on charge movement. Finally, we demonstrate the relevance of our new in silico model methodology using two case studies: (i) in vitro fibrous Poly(3,4-ethylenedioxythiophene) poly(styrenesulfonate) (PEDOT-PSS) scaffolds and (ii) in vivo collagen in extracellular matrix (ECM).

INTRODUCTION:

ES is the use of EFs with the aim of controlling biological cells and tissues. Its mechanism is based on the physical stimulus transduced to the cell when the biomolecules inside and surrounding it are exposed to an externally generated voltage gradient. Charged particles are engaged in an organized motion governed by Coulomb's law, generating drag forces upon the uncharged particles. The resulting fluid flow and charge distribution alter cell activities and functions such as adhesion, contraction, migration, orientation, differentiation and proliferation¹ as the cell attempts to adapt to the change in the microenvironmental conditions.

As EFs are controllable, non-invasive, non-pharmacological and shown to have an effective impact on essential cell behavior, ES is a valuable tool for tissue engineering and regenerative medicine. It has been successfully used to guide neural², skeletal³, cardiac muscle⁴, bone⁵ and skin⁶ development. Moreover, as it enhances iontophoresis⁷, it is used as an alternative or complementary treatment to conventional pharmacological ones. Its efficiency in pain management is still debated as higher quality clinical trials are awaited⁸⁻¹⁰. Nevertheless, no adverse effects were reported and it has the potential to improve patient welfare¹¹⁻¹⁵.

While only clinical trials can give a definitive verdict for the efficacy of a procedure, in vitro and in silico models are required to inform the design of predictable ES treatment as they offer stronger control over a wider range of experimental conditions. The investigated clinical uses of ES are bone regeneration^{16,17}, recovery of denervated muscles^{18,19}, axonal regeneration after surgery^{20,21}, pain relief²², wound healing²³⁻²⁵ and iontophoretic drug delivery²⁶. For ES devices to be widely introduced on all possible target applications, clinical trials have yet to establish stronger evidence for efficient treatment. Even in domains where both in vivo animal and human studies consistently report positive outcomes, the great number of reported methods coupled with too little guidance on how to choose between them and high acquisition price deters clinicians from investing in ES devices²⁷. To overcome this, the target tissue can no longer be treated as a black box (limit of in vivo experiments) but must be seen as a complex synergy of multiple subsystems (**Figure 1**).

Multiple ES experiments have been carried out in vitro over the years²⁸⁻³⁴. Most of these only characterize the ES through the voltage drop between the electrodes divided by the distance between them – a rough approximation of the electric field magnitude. However, the electric field itself only influences charged particles, not cells directly. Also, when multiple materials are interposed between the device and the cells, the rough approximation may not hold.

A better characterization of the input signal requires a clear view on how the stimulus is transduced to the cell. Main methods of delivering ES are direct, capacitive and inductive coupling^{35,36}. Devices for each method differ with electrode type (rod, planar or winding) and placement relative to the target tissue (in contact or isolated)³⁵. Devices used in vivo for longer treatments need to be wearable, thus the electrodes and most times the energy source are either implanted or attached to the skin as wound dressings or electroactive patches. The generated voltage gradient displaces charged particles in the treatment area.

As it impacts the resulting charged particle flow in the vicinity of the cells, scaffold structure is of utmost importance in the design of ES protocols. Different charge transport configurations arise if the platform material, synthesis technique, structure or orientation relative to the voltage gradient change. In vivo, the availability and movement of charged particles is impacted not only by cells but also by the collagen network and interstitial fluid composing the supporting ECM. Engineered scaffolds are increasingly used to better recreate natural cell microenvironments in vitro^{1,35}. Concurrently, the ECM is a complex natural scaffold.

Artificial scaffolds are based on metals, conducting polymers and carbon, engineered with a focus on balancing biocompatibility with electrochemical performance and long-term stability³⁶. One versatile scaffold type is the electrospun fibrous mat that offers a controllable nanoscale topography. This can be engineered to resemble the ECM, thus deliver similar mechanical cues that aid regeneration of a wide range of tissues³⁷. To significantly impact ES, the mats need to be conductive to some degree. However, conductive polymers are difficult to electrospin and blending with insulating carriers limits the conductivity of the resulting fibers³⁸. One solution is polymerizing a conductive monomer on the surface of a dielectric fiber, resulting in good mechanical strength and electrical properties of the end product³⁸. An example is coating silk electrospun fibers with the semi conductive PEDOT-PSS³⁹. The combination of mechanical and electromagnetic cues significantly accelerates neurite growth^{40–42}. Neurites follow scaffolds fibers alignment, and elongate more after exposure to an EF parallel to the fibers than to a vertical one⁴³. Similarly, alignment of fibrous scaffolds to the EF also promotes myogenic maturation³³.

The ECM is mainly composed of fibrous-forming proteins⁴⁴, out of those collagen type I being the major constituent in all animal tissues apart from cartilage (rich in collagen type II)⁴⁴. Tropocollagen (TC), triple helical conformation of polypeptide strands, is the structural motif of collagen fibrils⁴⁵. Transmission electron microscopy and atomic force microscopy images of collagen fibrils show a D-periodic banded pattern⁴⁶ explained by the Hodge & Petruska model⁴⁷ as regular arrays of TC gaps and overlaps⁴⁵. Tendons are composed of an aligned collagenous fibrillar matrix shielded by a non-collagenous highly hydrophilic proteoglycan matrix^{48,49}. Decorin is a small leucine-rich proteoglycan (SLRP) able to bind the gap regions of collagen fibrils and connect with other SLRPs through their glycosaminoglycan (GAG) side chains⁴⁹. Studies done on tendons show that their electrical properties change significantly when hydrated^{50,51}, charge transport mechanism changing from protonic to ionic as hydration level increases⁵¹. This suggests that electric conduction along a collagen type I fibril could be enabled by a Decorin-water coat,

with gap and overlap regions having different electrical conductivities and dielectric constants.

As identical recreation of the ECM by artificial scaffolds is improbable, the knowledge producing synergy between in vivo and in vitro enabled by translatable results seems to be at a dead end. In silico modelling not only re-enables translation between the two, but also adds important benefits in characterizing the unknown processes involved in ES. Comparing the in vivo observations with the in vitro can bring information on the coupling strength between the target tissue and the rest of the organism but does not uncover current knowledge limits. The unknown can be exposed by observing the difference between what is expected to happen based on the current knowledge and what happens. In silico experiments based on mathematical modelling allow splitting the process into known and unknown subprocesses. This way, phenomena not accounted for in the model come to light when in silico predictions are compared to in vitro and in vivo experiments.

Forming and testing hypotheses regarding the underlying mechanism(s) of how cells and tissues are affected by electrical fields is hindered by the great number of parameters⁵² that need to be tested separately. To define representative experimental conditions, the ES process must be split in subprocesses (**Figure 1**) and dominant input signals affecting cell behavior must be identified. Models representing fundamental physical effects of ES on cells describe the domain that couples the EF with the cell – that of charged particles⁵³. The behavior of particles exterior to the cell depends on the microenvironment and can be investigated separately from the cell. The dominant input signal for the cell is the subset of ES device outputs that causes the greatest degree of variability in the cell response. The smallest subset of the full experimental parameters that can generate variations in all the dominant cell input signals can be used to decrease the parameter space dimension and the number of test cases.

The input of the biological ES target model must be a subset of the output signals produced by the ES device that are useful in describing the physical effects of ES on cells. A simple bioreactor with direct coupling has the same structure as electrolytic electrochemical cells. Models of those show the primary (accounting for solution resistance), secondary (also accounting for faradic reactions) or tertiary (also accounting for ion diffusion) current density distribution. As complexity translates into computational cost, the simplest model is most suitable for parameter space explorations. Simulations of fibrous composites motivated by material properties⁵⁴ focus on bulk material properties as a result of complex micro-architecture, hence cannot describe local effects of EF exposure. Existing in silico models, motivated by ES, focus on the biological sample, be it a single cell immersed in a homogenous medium^{55–57}, or complex tissues with homogenous extracellular space⁵⁸. Charge and current density (**Figure 2**) can act as interface signals between models of the ES device and the biological sample, or between different components of the ES device. The proposed FEM based protocol uses the equations described in **Figure 2** and was used to study how scaffold dependent parameters can be used to modulate those two signals, independent of the EF generated by a direct coupling setup. Results stress that it is necessary to account for scaffold or ECM electrical properties when investigating how ES impacts target cells.

PROTOCOL:

1. Build the model in COMSOL

1.1. Open COMSOL and select Blank Model.

1.2. Parameters: In **Model Builder**, right click on **Global Definitions**, select **Parameters**, and add parameters according to **Table 1**.

1.3. Materials: Add materials with properties according to **Table 2**.

1.3.1. In the **Model Builder** under **Global Definitions**, right click **Material** and select **Blank Material**.

1.3.2. Add Material Properties: In the settings of the newly added material, expand **Material Properties > Basic Properties**, select **Relative Permittivity** and press the '+' symbol to add property. Repeat the step for **Electrical Conductivity**.

1.3.3. In the **Material Contents**, fill in the current material properties according to **Table 2**.

1.4. Component definition: In the **Home** tab of the top ribbon, left click **Add Component** and select **3D**. A new component node will appear in the **Model Builder**.

1.5. Geometry: In the **Model Builder**, right click **Geometry**, left click **insert**, double click on the **Full Model** and select the appropriate sequence (SC/SNC/RC/RNC/RNCd).

1.6. Alternatively, build the geometry from scratch following the subordinate steps.

1.6.1. In the **Model Builder**, left click **Geometry** under the current component node. The same **Geometry** node will be referred in the following subordinate steps. In the **Settings** window, change the Length unit to nm and make sure "Scale values when changing units" is left unchecked.

1.6.2. Geometry of scaffold

1.6.2.1. In the **Model Builder**, right click on **Geometry** and select **Block**.

1.6.2.2. In the **Settings** tab, fill in the Label "Scaffold extents".

1.6.2.3. Expand **Size and Shape** and fill in "Ws", "Ls", "Hs" for Width, Depth and Height.

1.6.2.4. Expand **Position** and change the **Base** to **Center**.

1.6.2.5. Expand **Selections of Resulting Entities**, select **New** and add "Scaffold Selection".

220

221 1.6.3. Extents of surrounding substance: In the **Model Builder**, under **Geometry**, right click

222 **Scaffold extents** and select **Duplicate**, and edit **Settings** tab.

223

224 1.6.3.1. Change the label to “Media extents”.

225

226 1.6.3.2. To each box in the **Size** and **Shape** section, add “med_ratio *” before the existent

227 parameter.

228

229 1.6.3.3. In the **Selections of Resulting Entities**, add New selection as “Media Selection”.

230

231 1.6.4. Geometry of a fiber (SC skip to 1.6.6)

232

233 1.6.4.1. Core (RC skip to 1.6.4.2)

234

235 1.6.4.1.1. In the **Model Builder**, right click **Geometry**, select **Cylinder**, and edit **Settings** tab as

236 follows.

237

238 1.6.4.1.2. Change the label to “Core”.

239

240 1.6.4.1.3. Expand **Size** and **Shape** and fill in “Rc” and “Lf” for Radius and Height.

241

242 1.6.4.1.4. Expand **Position** and fill in “-excess*Ws/2” and “-Lf/2” for x and y.

243

244 1.6.4.1.5. Expand **Axis** and change **Axis type** to y-axis.

245

246 1.6.4.1.6. Expand **Selections of Resulting Entities** and add “Core Selection”.

247

248 1.6.4.2. Coat (SNC skip to 1.6.5)

249

250 1.6.4.2.1. Slab (RC or RNC skip to 1.6.4.2.2; RNCd skip to 1.6.4.2.3)

251

252 1.6.4.2.1.1. In the **Model Builder** under **Geometry**, right click **Scaffold extents** and select

253 **Duplicate**. Make sure the duplicate (Scaffold extents 1) comes right after **Core** in the geometry

254 sequence.

255

256 1.6.4.2.1.2. In the **Settings** tab, change label to “Coat”.

257

258 1.6.4.2.1.3. In the **Settings** tab, expand **Selections of Resulting Entities** and create “Fiber

259 Selection”.

260

261 1.6.4.2.1.4. Skip to 1.6.5.

262

263 1.6.4.2.2. Homogenous

264

265 1.6.4.2.2.1. In the **Model Builder** right click **Geometry**, select **Cylinder** and edit its **Settings** tab as

266 follows.

267

268 1.6.4.2.2.2. Change the label to “Coat”.

269

270 1.6.4.2.2.3. Expand **Size** and **Shape** and fill in “Rf” and “Lf” for Radius and Height.

271

272 1.6.4.2.2.4. Expand **Position** and fill in “-excess*Ws/2” and “-Lf/2” for x and y.

273

274 1.6.4.2.2.5. Expand **Axis** and change axis type to y-axis (RC skip to 1.6.4.2.2.7).

275

276 1.6.4.2.2.6. Expand **Layers**, fill in “Rf-Rc” for **Thickness** and leave only “Layers on side” checked.

277

278 1.6.4.2.2.7. Expand **Selections of Resulting Entities** and add “Coat Selection” or, for RC, “Fiber

279 Selection” (RC skip to 1.6.5).

280

281 1.6.4.2.2.8. In the **Model Builder**, right click **Geometry** and select **Delete Entities**.

282

283 1.6.4.2.2.9. In the **Settings** tab, change **Geometric Entry Level** to **Domain** and choose **Coat**

284 **Selection** for Selection.

285

286 1.6.4.2.2.10. Remove all domains but domain 3 from the selection box (the core of the layered

287 cylinder).

288

289 1.6.4.2.2.11. Expand **Selections of Resulting Entities** and select **Coat Selection**.

290

291 1.6.4.2.2.12. Skip to 1.6.5.

292

293 1.6.4.2.3. Periodic array of two types

294

295 1.6.4.2.3.1. In the **Model Builder**, right click **Geometry** and select **Cylinder**. Right click the newly

296 created element and select **Duplicate**. Edit their **Settings** tab as follows.

297

298 1.6.4.2.3.1.1.1. Set Label to “Coat 1” / “Coat 2”.

299

300 1.6.4.2.3.1.1.2. Set radius to Rf.

301

302 1.6.4.2.3.1.1.3. Set height to “D*prop”/”D*(1-prop)”.

303

304 1.6.4.2.3.1.1.4. Expand position and fill in “-excess*Ws/2” for both on x and “-Lf/2”/”-

305 Lf/2+D*prop” on y.

306

307 1.6.4.2.3.1.1.5. Expand **Axis** and change axis type to y-axis.

308
309 1.6.4.2.3.1.1.6. Expand **Layers** section and fill in “Rf-Rc”; Make sure only the “Layers on side” box
310 is checked.
311
312 1.6.4.2.3.1.1.7. Expand **Selections of resulting entities** and create “Coat 1 Selection”/ “Coat 2
313 Selection”.
314
315 1.6.4.2.3.2. In the **Model Builder**, right click **Geometry** and select **Delete Entities**.
316
317 1.6.4.2.3.2.1. In the **Settings** tab, change **Geometric Entry Level** to **Domain** and select cylinder 2
318 (cyl2) and cylinder 3 (cyl3) from the graphics window.
319
320 1.6.4.2.3.2.2. Remove all domains but domain 3 from the selection box (the core of the layered
321 cylinders).
322
323 1.6.4.2.3.2.3. Expand **Selections of Resulting Entities** and create **Coat Selection**.
324
325 1.6.4.2.3.3. In the **Model Builder**, right click **Geometry**, expand **Transforms** and select **Array**. Edit
326 **Settings** tab as follows.
327
328 1.6.4.2.3.3.1. Select “Coat Selection” for Input objects.
329
330 1.6.4.2.3.3.2. Set **Array** type to Linear.
331
332 1.6.4.2.3.3.3. Set Size to “Lf/D”.
333
334 1.6.4.2.3.3.4. Set **Displacement** on y axis to “D”.
335
336 1.6.5. Geometry of an oriented fiber array
337
338 1.6.5.1. Core Array (RC skip to 1.6.5.2)
339
340 1.6.5.1.1. In the **Model Builder**, right click **Geometry**, **Expand Transforms** and select **Rotate**.
341
342 1.6.5.1.1.1. In the **Settings** tab, turn on the **Input objects Switch** and select **Core Selection** from
343 the dropdown list. Make sure Keep input objects box is not checked.
344
345 1.6.5.1.1.2. Expand **Rotation Angle** and fill in “theta” for Rotation.
346
347 1.6.5.1.1.3. Expand **Selections of Resulting Entities** and create “Fiber Selection”.
348
349 1.6.5.1.2. In the **Model Builder** right click **Geometry**, **Expand Transforms** and select **Array**.
350
351 1.6.5.1.2.1. Change label to “Core Array”.

352

353 1.6.5.1.2.2. Select **Core Selection** in the dropdown list for Input objects.

354

355 1.6.5.1.2.3. Expand **Size**, change **Array type** to Linear and fill in " $n_1 * (\theta \leq 45)$ "

356 $+ n_2 * (\theta > 45)$ " for Size.

357

358 1.6.5.1.2.4. Expand **Displacement** and fill in " $2 * t_{es} * R_c * \cos(\theta)$ ", " $2 * t_{es} * R_c * \sin(\theta)$ " for x

359 and y.

360

361 1.6.5.1.2.5. Expand **Selections of Resulting Entities** and select "Fiber Selection".

362

363 1.6.5.2. Coat Array (SNC skip to 1.6.5.3)

364

365 1.6.5.2.1. In the **Model Builder**, right click **Geometry**, **Expand Transforms** and select **Rotate**.

366

367 1.6.5.2.1.1. In the **Settings** tab turn on the **Input objects Switch** and select **Coat Selection** from

368 the dropdown list. Make sure "Keep input objects box" is not checked.

369

370 1.6.5.2.1.2. Expand **Rotation Angle** and fill in " θ " for Rotation.

371

372 1.6.5.2.1.3. Expand **Selections of Resulting Entities** and select "Fiber Selection".

373

374 1.6.5.2.2. In the **Model Builder**, right click **Geometry**, **Expand Transforms** and select **Array**.

375

376 1.6.5.2.2.1. Change label to "Coat Array".

377

378 1.6.5.2.2.2. Select **Coat Selection** in the dropdown list for Input objects.

379

380 1.6.5.2.2.3. Expand **Size**, change **Array type** to Linear and fill in " $n_1 * (\theta \leq 45)$ "

381 $+ n_2 * (\theta > 45)$ " for Size.

382

383 1.6.5.2.2.4. Expand **Displacement** and fill in " $2 * t_{es} * R_c * \cos(\theta)$ ", " $2 * t_{es} * R_c * \sin(\theta)$ " for x

384 and y.

385

386 1.6.5.2.2.5. Expand **Selections of Resulting Entities** and select "Fiber Selection".

387

388 1.6.5.3. Cut Scaffold

389

390 1.6.5.3.1. Unite Fibers: In the **Model Builder** right click **Geometry**, **Expand Booleans** and

391 **Partitions** and select **Union**.

392

393 1.6.5.3.1.1. Change label to "Fibers".

394

395 1.6.5.3.1.2. Turn on **Input objects switch** and select **Fiber Selection** from the dropdown list.

396
397 1.6.5.3.1.3. Make sure “Keep input objects” box is not checked.
398
399 1.6.5.3.1.4. Make sure “Keep interior boundaries” is checked.
400
401 1.6.5.3.1.5. Expand Selections of Resulting Entities and select Scaffold Selection from the
402 dropdown list.
403
404 1.6.5.3.2. Perform cut: In the **Model Builder** right click **Geometry**, expand **Booleans and**
405 **Partitions** and select **Intersection**.
406
407 1.6.5.3.2.1. Change label to “Scaffold”.
408
409 1.6.5.3.2.2. Turn on the **Input objects switch** and select **Scaffold Selection** from the dropdown
410 list.
411
412 1.6.5.3.2.3. Make sure “Keep input objects” box is not checked.
413
414 1.6.5.3.2.4. Make sure “Keep interior boundaries” is checked.
415
416 1.6.6. Ensemble Geometry
417
418 1.6.6.1. Define surrounding substance geometry.
419
420 1.6.6.1.1. In the **Model Builder**, right click **Geometry**, expand **Booleans and Partitions** and select
421 **Difference**. Continue to the **Settings** tab.
422
423 1.6.6.1.2. In the **Objects** to add dropdown list, select **Media Selection**.
424
425 1.6.6.1.3. In the **Objects to subtract** dropdown list, select **Scaffold Selection**.
426
427 1.6.6.1.4. Make sure “Keep input objects” and “Keep interior boundaries” boxes are both
428 checked.
429
430 1.6.6.2. Delete Media extents box.
431
432 1.6.6.2.1. In the **Model Builder**, right click **Geometry** and select **Delete entities**. Continue to the
433 **Settings** tab.
434
435 1.6.6.2.2. Select **Domain** from the **Geometric** entry level dropdown list.
436
437 1.6.6.2.3. Select **Media Selection** from the **Selection** dropdown list.
438
439 1.6.6.2.4. Remove “dif1” from the selection box.

1.6.7. Build geometry

1.6.7.1. Under the **Geometry** node, make sure the last element before the Cumulative Selections node is **Form Union and not Form Assembly**. If necessary, right click **Form Assembly** and change Action to “Form a union” in the Settings tab.

1.6.7.2. In the **Model Builder**, left click **Geometry** and select **Build All**.

1.7. Materials

1.7.1. In the **Model Builder** under the **Current** component node, right click **Materials** and select **Material Link**.

1.7.2. Repeat section five times for as many times as many materials are considered according to the complexity level.

1.7.3. Associate materials for each component in the following order: surrounding substance, coats, cores.

1.7.4. In the **Settings** tab, choose **Media/Scaffold** (for SC, SNC, RC)/Coat/Core Selection from the **Selection** dropdown list.

1.7.5. Expand the **Link** settings and choose appropriate material from the dropdown list.

1.8. Meshing

1.8.1. In the **Model Builder**, left click the **Mesh** node under the current component.

1.8.2. In the **Settings** tab, select **Normal** from the **Element** size dropdown list and left click **Build All**.

1.9. Physics: In the **Model Builder**, left click current component, select **Add Physics**, expand the AC/DC module in the **Add Physics** tab, select the **Electric Currents (ec)** module and click **Add to Component**.

1.9.1. Boundary conditions: In the **Graphics** tab, select the **xy view**.

1.9.1.1. Ground: In the **Model Builder**, right click on the **Electric Currents** node and select **Ground**.

1.9.1.2. Make sure the selection switch for the **Boundary Selection** is Active and left click on the highest surrounding substance face parallel to the xz plane to add **boundary 5** in the **Boundary Selection Box**.

1.9.1.3. Terminal: In the **Model Builder**, right click on the **Electric Currents** node and select **Terminal**.

1.9.1.4. Make sure the selection switch for the **Boundary Selection is Active** and left click on the lowest surrounding substance face parallel to the xz plane to add **boundary 2** in the **Boundary Selection Box**.

1.9.1.5. Expand the **Terminal** section and select **Voltage in the Terminal** type dropdown list; fill in V0 for **Voltage**.

1.10. Add complexity if needed: Save current component and go back to Step 1.4 to follow another complexity path (SC/SNC/RC/RNC/RNCd).

2. Perform simulation

2.1. Adaptive mesh refinement

2.1.1. In **Model Builder**, left click the model root node and select **Add Study** to open the **Add Study** tab, select **Stationary Study** and right click **Add Study** button.

2.1.2. Create a study step for each created component: under **Study** node right click **Step1: Stationary Study** and select **Duplicate**.

2.1.3. For each study step modify the settings tab with the details of the corresponding component.

2.1.3.1. Expand **Physics and Variables Selection**; in the **Solve** for column leave only the current component checked.

2.1.3.2. Expand **Study Extensions** and check **Adaptive** mesh refinement box.

2.1.3.3. Select current component's geometry from the dropdown list next to **Adaptation** in geometry.

2.1.4. In **Model Builder** right click **Study 1** and select **Compute**. This will generate adapted meshes for all the components' geometries with their current orientation angle.

2.2. Set orientation angle and perform a stationary study.

2.2.1. In **Model Builder**, under **Global definitions**, left click **Parameters** and change parameter **theta** to the fiber orientation angle desired for simulation.

2.2.2. For each component, in the **Model Builder** expand the component's node, right click

528 **Geometry** and select **Build all**.

529
530 2.2.3. In **Model Builder**, right click the adaptive mesh refinement study and select **compute**.

531
532 2.2.4. In **Model Builder** left click the model root node and select **Add Study** to open the **Add**
533 **Study** tab, select **Stationary Study** and right click **Add Study** button.

534
535 2.2.5. In **Model Builder**, under the newly added study, left click **Step 1**, expand mesh selection
536 and, for each component, select the meshes generated in the adaptive mesh refinement study.

537
538 2.2.6. Right click **Compute** button.

539
540 2.3. Set orientation angle, input signal and perform time dependent study.

541
542 2.3.1. In **Model Builder**, under **Global definitions**, left click **Parameters** and change parameter
543 **theta** to the fiber orientation angle desired for simulation.

544
545 2.3.2. In the **Model Builder**, left click the model root node and select **Add Study** to open the **Add**
546 **Study** tab. Select **Time Dependent Study**, left click **Add Study** button and edit **Settings** tab as
547 follows.

548
549 2.3.2.1. Set Times to “range(0, (2*pi/omega)/39, 2*pi/omega)”.

550
551 2.3.2.2. Expand **Physics and Variables Selection**; leave only the simulation component “Solve
552 for” check box checked.

553
554 2.3.2.3. Expand **Mesh** selection and select a mesh for the simulation component. Change the
555 other components’ meshes to **No mesh**.

556
557 2.3.2.4. Expand **Study Extensions** and check the **Adaptive** mesh refinement box; select the
558 simulation component’s geometry from the dropdown list.

559
560 2.3.3. Define time dependent boundary condition.

561
562 2.3.3.1. Under the current component node right click **Definitions** and under **Functions** select
563 **Waveform**.

564
565 2.3.3.2. In the **Settings** tab change **Function** name to “Input”.

566
567 2.3.3.3. Expand **Parameters**, set **Type** to **Sine**, **Angular frequency** to “omega”, **Amplitude** to V0.

568
569 2.3.3.4. For the simulation component, under its **Electric Currents** node, select **Terminal** and
570 change Voltage to “Input(t[1/s])”.

2.3.4. Right click **Compute** button.

3. Analysis

3.1. Charge density

3.1.1. In the **Model Builder** right click **Results** node, select **3D Plot Group** and edit **Settings**.

3.1.1.1. Change label to "Charge density".

3.1.1.2. Expand **Data** and select the **Parametric** study dataset in the **Data** set dropdown list.

3.1.1.3. Expand **Color Legend** and check "Show legends" and "Show maximum and minimum values".

3.1.2. Right click **Charge density** under the **Results** node in the **Model Builder** and select **Volume**; proceed to edit **Settings** tab.

3.1.2.1. Expand **Data** and select "From parent" from Data set dropdown list.

3.1.2.2. Expand **Expression** and fill in "ec.rhoq" in the Expression box.

3.1.2.3. Expand **Range** and check Manual color range box.

3.1.2.3.1. Set minimum to "-0.03" and maximum to "0.03".

3.1.2.4. Expand **Coloring and Style**, set **Coloring** to Color table, **Color table** to Wave, check **Color** legend box, check **Symmetrize color range**.

3.1.2.5. Right click **Volume in Model Builder** and select **Filter**.

3.1.2.5.1. In the **Settings** tab fill in " $\text{abs}(\text{ec.rhoq}) > 0.012$ " in the Logical expression for inclusion.

3.1.3. Left click on the **Plot** button to visualize results in the graphics window.

3.2. Current density

3.2.1. In the **Model Builder** right click the **Results** node, select **3D Plot Group** and edit the **Settings** tab.

3.2.1.1. Change label to "Current density".

3.2.1.2. Expand **Data** and select the **Parametric** study dataset in the Data set dropdown list.

3.2.1.3. Expand **Color Legend**, check “Show legends” and “Show maximum and minimum values”.

3.2.2. Right click **Current density** under the **Results** node in **Model Builder** and select **Arrow Volume**; proceed to edit **Settings** tab.

3.2.2.1. Expand **Data** and select “From parent” from Data set dropdown list.

3.2.2.2. Expand **Expression** and fill in “ec.Jx”, “ec.Jy”, “ec.Jz” in the Expression boxes for the x, y and z components respectively.

3.2.2.3. Expand **Arrow** positioning and fill in 20 for all coordinates number of points.

3.2.2.4. Expand **Coloring and Style**, set **Arrow length** to Normalized, **Arrow base** to Center, check **Scale** factor and set it to 85.

3.2.2.5. Right click **Arrow Volume** in **Model Builder** and select **Color Expression**.

3.2.2.5.1. In the **Settings** tab fill in “ec.normJ” in the Expression box.

3.2.2.5.2. Expand **Coloring and Style**, set **Color** table to Traffic, check **Color legend** and **Reverse color table**.

3.2.3. Click on the **Plot** button to visualize results in the graphics window.

REPRESENTATIVE RESULTS

The proposed model describes features of a composite mat with parallel fibers, immersed in a conductive substance and exposed to an externally generated electric potential gradient. Simulations show that accounting for the different components of a scaffold is important on a microscale and explore how change in alignment angle (input signal) of the fibers to the EF can generate variability in the current and charge density (output signals) in the vicinity of the fibers. **Five different geometrical complexity stages are presented, each having an effect upon the simulation result:** smooth conductive slab (SC), smooth slab with non-conductive embedded fibers (SNC), rough conductive composite (RC), rough composite with non-conductive embedded fibers (RNC), rough composite with non-conductive embedded fibers and two types of periodic coating (RNCd) (Error! Reference source not found.). Section 1.5 of the protocol presents the steps to importing the geometries in a project and section 1.6 shows how to build those step by step. The first two models do not account for surface morphology. SC and RC do not account for the fiber core dielectric properties. The RNC is the proposed model for nanofibrous artificial scaffolds, while RNCd is the proposed model for an ECM segment.

Minimization of computational cost was accomplished by reducing the ES device geometry to a model unit volume representing the microenvironment. While an ES device and scaffold’s width and length can easily be at the order of a few centimeters, the containing fibers’ diameter

is usually lower than a micron. Here, we use a scaffold cut comparable to the fiber diameter to reduce the computational cost induced by the aspect ratio and highlight the effect of the scaffold's fibrous nature on the electric microenvironment. The rest of the ES device is replaced with electric potential boundary conditions chosen so that a rough approximation for the magnitude of the electric field is 100 V/m, a frequently reported stimulation parameter. Moreover, a unit volume with five parallel fibers – as the one used in simulations, presented in Figure 3 – is assumed to be representative of a whole planar fibrous mat. Three types of fibers can be distinguished in a 1D array: interior central (with the longitudinal symmetry plane of the scaffold splitting them in half), interior transitory (with lateral surface surrounded by other fibers but with asymmetrical sides), and exterior (at the edge of the scaffold). Five is the minimum number of fibers required in order to include all the three types defined.

The model mesh element size requires special attention as it may impact simulation results and thus fail to expose important effects (Error! Reference source not found.). This is a general rule of the finite element method and an implication of the Nyquist-Shannon sampling theorem. The faster the essential simulation signals fluctuate in space the smaller the mesh elements need to be to produce a loyal representation of the phenomenon. On the other hand, the smaller the element, the greater the total number of model building blocks and the computational cost. The adaptive mesh refinement set up in section 2.1 is a good and facile method to balance those opposing objectives by decreasing the element size only where and as long as this operation produces a significant change.

A model that is too simplistic can fail presenting important effects (Error! Reference source not found.). Simulations show that accounting for surface morphology and scaffold component electrical properties is not redundant in predicting electric microenvironments. While surface morphology has a direct impact on the stationary EF (compare SC and SNC with RC, RNC and RNCd), a comparison between RC and RNC predictions shows that nonconductive fiber cores amplify this effect. From the point of view of modelling cellular electric microenvironments on nanofibrous scaffolds, the SC, SNC and RC models are thus sub-optimal. However, it is good practice to incrementally add complexity as comparisons between the different stages help indicate what features give rise to specific effects.

Model complexity impacts current and charge density change with fiber alignment to the EF. The proposed protocol helps highlight the effect (Error! Reference source not found.). While the SC model shows no variation in the proposed metrics when its alignment to the electric potential gradient is changed, the RNC model simulations predict a powerful contrast between the mat unit with fibers aligned to the EF and the one with fibers perpendicular (Error! Reference source not found.). When the non-conductive cores come in the way of the current flow, they form periodic dams that lead to alternating regions of high and low charge density.

Dynamic ES regimes can be simulated with time dependent studies. Videos in supplementary files show predictions made for a sinusoidal input voltage on a full artificial scaffold model (RNC), with fibers parallel or perpendicular to the electric potential gradient. Small currents along the fibers perpendicular to the EF appear when charge is released from the scaffold as the EF

magnitude decreases. This shows that stimulation could occur not only while the external EF is present, but also right after it is disconnected – See supplementary files for videos.

FIGURE AND TABLE LEGENDS

Figure 1: Hierarchical block diagram of modelling – advantages and limitations of modelling with in vivo and in silico models. Block color marks blocks on the same hierarchical level. Lower rank blocks are included in higher rank ones. Block stroke color marks possibility to include the block into a certain type of model – coupling with other system blocks do not have yellow in their stroke, as they are not components for in vitro models. Bullets act like valves and signify controllability of the block. When a valve is ON, signal can pass through all arrow paths in the subordinate subsystems that have the color of the valve in their stroke. Interpretation of the diagram: the ES process is composed of the stimulation device and biological target, each with several inter-connected deterministic or stochastic sub-processes that cannot be separated in vivo or in vitro, thus they have no red or yellow valve. Stochastic processes also intervene on the interface between the simulation device and biological sample when they are both stimulated. An in vitro model decouples the system of interest (i.e., skin segment) from the rest of the organism. Thus, only intrinsic processes of the system of interest topped by stochastic processes of different nature can be observed. However, the different intrinsic processes involved cannot be stimulated and identified separately. The in silico models are parametric for known components – their behavior is expected to be of a certain shape – and non-parametric for the unknown – as there is no mechanistic reason to give credence to a certain extrapolation. All the in silico components can be simulated separately or in different combinations, allowing the portrayal of different hypothesis.

Figure 2: (A) Coulomb's Law (B) Electric potential field and mobile probe charge (C) Electric current (D) Charge density (E) Current density (F) Equation of continuity (G) Charge conservation law. (A) Electrically charged stationary particles q and Q interact electrostatically through Coulomb's force $\vec{F}_c(\vec{r}) = k \frac{qQ}{|\vec{r}|^2}$. **(B1)** Each charged particle Q generates a scalar field called electric potential at all positions \vec{r} in space: $V(\vec{r}) = k \frac{Q}{|\vec{r}|}$. The maximum work required to move another charged particle q from its position \vec{r}_q is the product between the charge q and the electric potential generated by Q at position \vec{r}_q . The electric potential field generated by multiple particles is the sum of the fields generated by each individual particle. **(B2)** A stationary field with fixed generator particles q and Q , acts with a $\vec{F}_c(\vec{r})$ upon a probe particle with positive charge q_p . In response, q_p moves to minimize its position's electric potential. To describe the motion of q_p , one can derive $\vec{F}_c(\vec{r})$ and the electric field $\vec{E}(\vec{r})$ from the electric potential field: $\vec{E}(\vec{r}) = -\nabla V = \frac{\vec{F}_c(\vec{r})}{q_p}$. **(C)** Multiple mobile positively charged probe particles uniformly released in a stationary electric field follow an organized motion. To track the charge configuration without tracking every particle, one can specify at every instant: **(D)** how space is occupied by particles, assigning a charge density $\rho(\vec{r}) = \epsilon_a \nabla \cdot \vec{E}(\vec{r})$ to each infinitesimal volume, according to Gauss's Law, and **(E)** how particles pass through the boundary surfaces between neighbouring infinitesimal volumes, assigning a current density $\vec{J}(\vec{r}) = \sigma \vec{E}(\vec{r})$ to each boundary according to

Ohm's Law. **(F)** Charge and current density evolve co-dependently according to the Equation of continuity, as non-uniform particle displacement leads to either accumulation or loss of particles in a certain volume. **(G)** Within an isolated system, the Charge conservation law prevails and there is no inflow or outflow of charged particles. **Notations used:** q, Q, q_p – charge and name of the charged particle; $|\vec{r}|$ – Euclidian norm of the position vector; k – Coulomb's constant; ∇ – gradient operator, ε_a – absolute permittivity of medium; σ – conductivity of medium.

Figure 2: Five different levels of complexity for a fibrous mat. SC – smooth with conductive embedded fibers, the simplest model, not accounting for surface morphology or different properties of the constituent components; SNC – smooth with non-conductive embedded fibers; RC – rough with conductive embedded fibers, accounting for surface morphology but not for different component properties; RNC – rough with non-conductive embedded fibers, full proposed model of nanofibrous artificial scaffolds; RNCd – rough with non-conductive embedded fibers coated with two different materials, full proposed model for a sheet of collagen fibers. Length unit used: nanometers.

Figure 3: Example results of the adaptive mesh refinement and the resulting charge density following the simulation. (Left) Automatically generated mesh with extra coarse tetrahedral elements; (Right) Initial mesh adaptively refined during stationary study; smaller elements are required for an accurate result in the areas where simulated signals have abrupt spatial changes.

Figure 4: Fiber alignment angle to electric potential gradient impacts EF strength in surrounding cell culture media when enough complexity is accounted for. SC, SNC, RC, RNC and RNCd are the different levels of complexity for the fibrous mat model presented in Figure 3. Vertical axis marks the alignment angle of the fibers to the electric potential gradient. Abstract electrodes featured – bottom side with high electric potential and top side with low electric potential.

Figure 6: Fiber alignment angle to electric potential gradient impacts space charge density in surrounding cell culture media when enough complexity is accounted for. SC, SNC, RC, RNC and RNCd are the different levels of complexity for the fibrous mat model presented in Figure 3. Vertical axis marks the alignment angle of the fibers to the electric potential gradient. Abstract electrodes featured – bottom side with high electric potential and top side with low electric potential.

Figure 7: Charge movement is influenced by scaffold fiber alignment relative to the EF. Both panels illustrate steady state RNC model predictions. On the left side the fibers are parallel to the EF, while on the right side they are perpendicular. The light red to blue color volume marks charge density, while the arrow volume marks current density orientation. The color of the arrows corresponds to the current density norm.

Table 1: Parameters used for simulation

Table 2: Material properties used in simulation

DISCUSSION:

The proposed protocol suggests a uniform modelling solution for natural and artificial scaffolds and highlights the need to consider the nanostructure of fibrous scaffolds when inspecting the effects of EF on cells seeded onto such materials. Although a coarse approximation for the EF intensity (electrode potential difference divided by the distance between the electrodes) would lead us to expect a field strength of 100 mV/mm, simulations predict stationary field strengths up to 30% higher in different areas of the mat (Error! Reference source not found.). This result should be of interest in ES experiment design and data interpretation, as cell death can be caused by too strong EFs. Exposing the electrical microenvironment would enable a direct correlation between ES and cellular development. While several studies present detailed morphology analysis of the used scaffolds^{33,43,59}, they do not investigate the interplay between the structure, electrical properties of the materials and the EF. This protocol can enable this link, as parameters such as fiber radius, coating layer thickness, distance between fibers and electrical properties of the component materials can be modified according to each experiment by changing the Global Definitions at steps 1.2 and 1.3. Hence, customized 3D spatially resolved charge and current density predictions can be made for both static and dynamic ES regimes.

Scaffold design optimization can be targeted through the RNC and RNCd models with wide parameter range explorations, scaling the proposed morphologies or parts of them. Alternatively, other scaffold configurations can be investigated with the proposed protocol by changing the Array types from Linear to Three-dimensional in section 1.6.5 and adapting Scaffold Geometry in section 1.6.2. However, scaffold optimization cannot be done without an objective. While for tissue engineering purposes the main focus is cell fate, a clearer picture on what stimuli are its main determinants is essential if its reliable control is desired. Charge and current density are good descriptors of cellular electric microenvironments as they show the interplay between the EF and the electrical properties of the different component materials of complex scaffolds such as ECM. The protocol shows how to compute predictions for those metrics given a nanofibrous scaffold geometry and highlights the importance of the alignment angle of the fibers with the EF. Predictions of charge and current density could then be linked to cellular development and thus scaffold and ES regimes may then be optimized for specific tasks.

Interestingly, a study shows that EF exposure generated mechanical stress more than double in strength in composite films with nanofibers perpendicular to the external EF compared to films with parallel alignment⁶⁰. The reported mechanical stress could be a result of Coulomb forces acting between charged fibers, predicted by the rough model simulations (RC, RNC, RNCd) (Error! Reference source not found.). While these simulations could be useful in investigating this hypothesis, it must be noted that the reported experimental results were obtained in a system with capacitive coupling, and the simulation presents direct coupling.

A limiting factor towards future possible uses of the protocol to estimate a cellular input signal is parameter uncertainty. Geometric uncertain parameters are coating layer thickness and distance between fiber cores. The first one could be inferred by finding the value that leads to a bulk impedance that can be experimentally validated. The second one can be extracted from high resolution material scans. Parameters describing the physical properties of the materials are also

affected by uncertainty. However, the electric conductivity and dielectric constant of exemplified materials differ far more than experimental measuring precision (Error! Reference source not found.). Therefore, the reported effects would be maintained despite moderate measurement errors.

The results show how not enough model complexity might hide relevant information. It is important to acknowledge that the protocol simulates a simplified version of the physical phenomenon taking place as it does not account for the different nature of materials involved in the process – conductor (electrodes), semiconductor (coating), dielectric (fiber cores) and electrolytic (surrounding substance) – that are able to influence charge transport. This issue can be accounted for in future model expansions by adding energy transfer delays at the interfaces (i.e., Faradic reactions) and ion transport delays within the electrolyte. Adding complexity should however be guided by experimental validation, as a simple model that reproduces most of what is observed is more useful than a remarkably accurate one that adds little more information but is deeply sensitive to many constituent parameters' uncertainty.

As the end goal of tissue engineering is to create bioreactors that not only mimic one or two aspects of in vivo environments, but replicate and control all cellular developmental cues⁶¹, electromagnetic and mechanical in silico models as well as models of heat transfer between bioreactor components will need to be combined. In a subsequent modelling phase, coupling phenomena between those interactions such as ohmic heating, electrolytic fluid flow, morphological scaffold deformations in response to electrical stimulation⁶⁰ and piezoelectricity⁶² can also be added. However, models should be merged only after each one has been experimentally validated. This way, we can gain a better understanding of each component's influence in the cellular microenvironment, and how stimuli can be optimized.

If the proposed model is experimentally validated, it can be combined with models of biological cells – Figure 1. Charge density patterns and modulations could asymmetrically influence specific ion pumps' activity, impact attachment to the fiber of proteins driving membrane adhesion⁶³ and hence guide migration, proliferation patterns and morphogenesis⁶⁴. Exploring those hypotheses is the way forward in understanding the mechanisms underpinning tissue and cell responses to ES.

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The authors have nothing to disclose.

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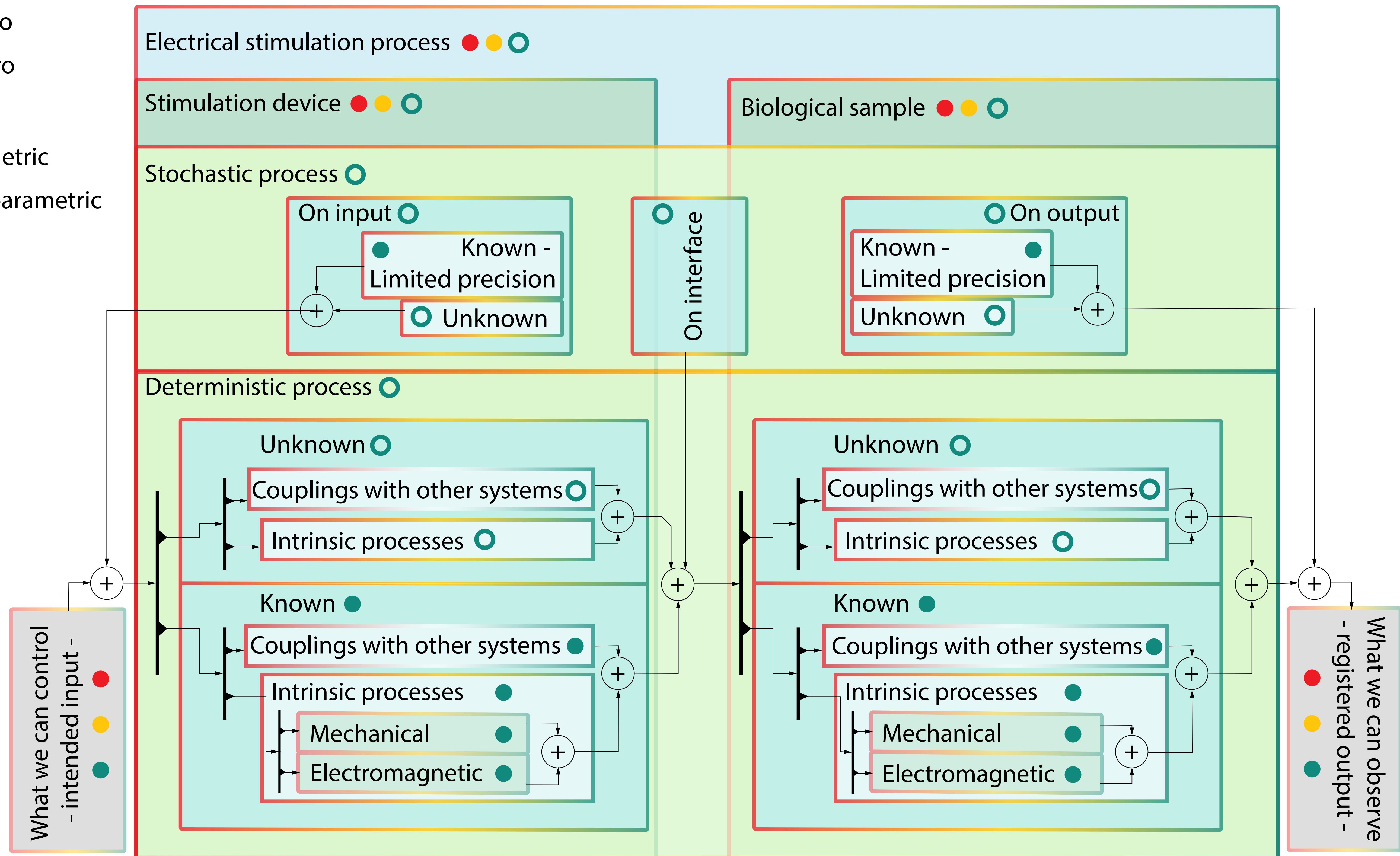
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1056

- In vivo
- In vitro
- In silico
- Parametric
- Non-parametric



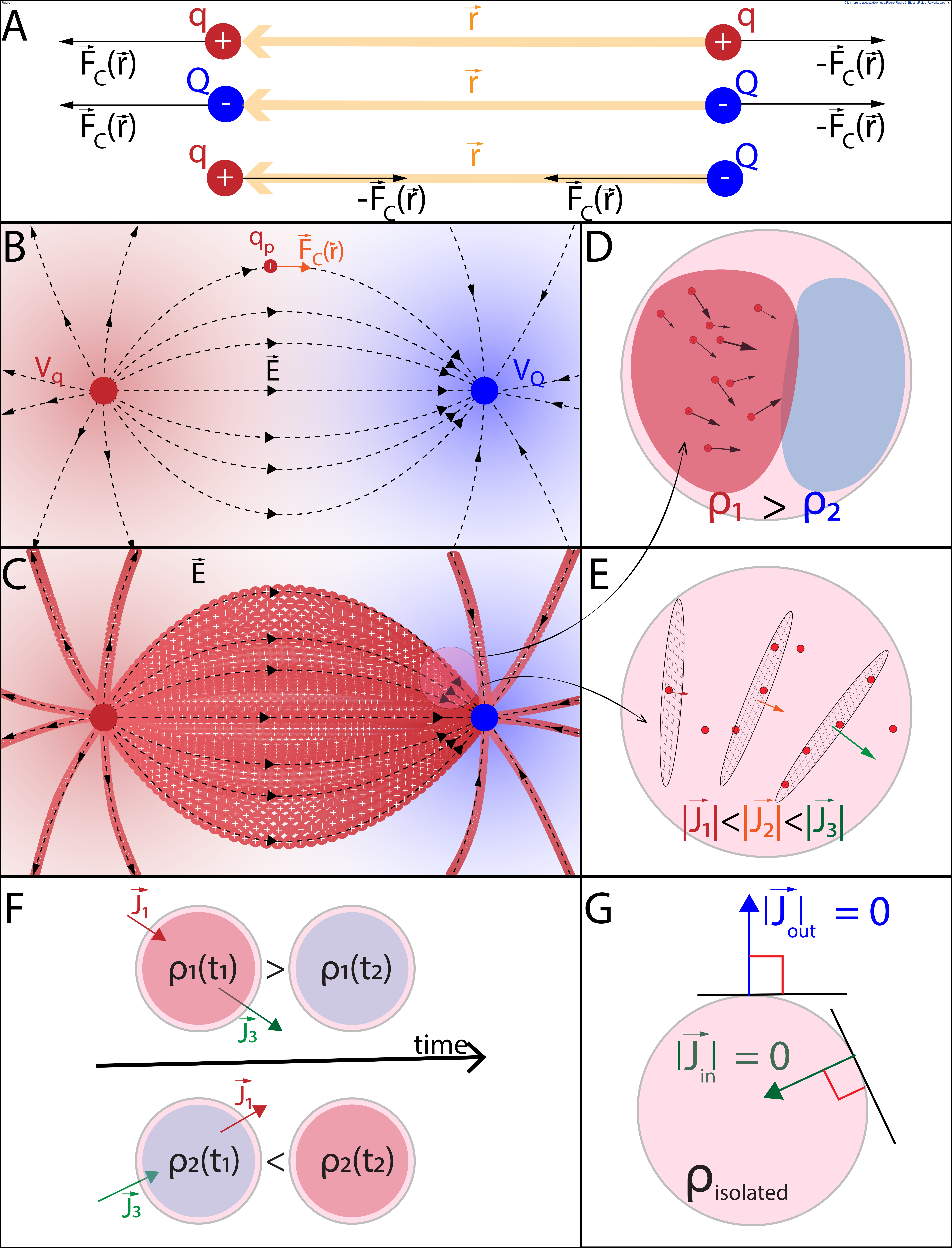
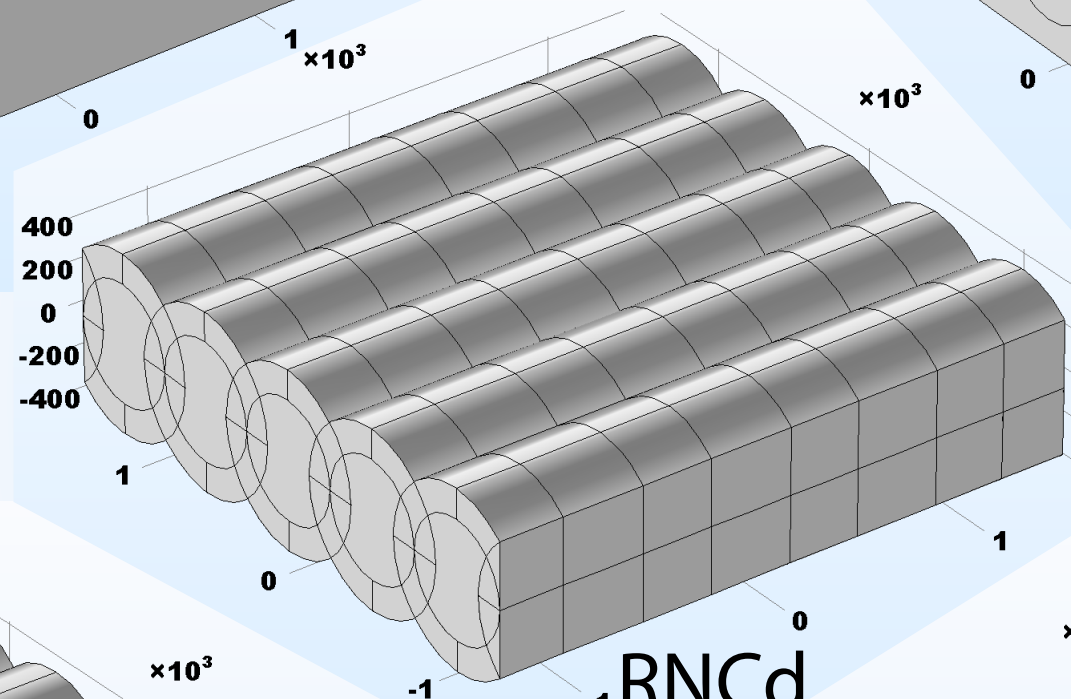
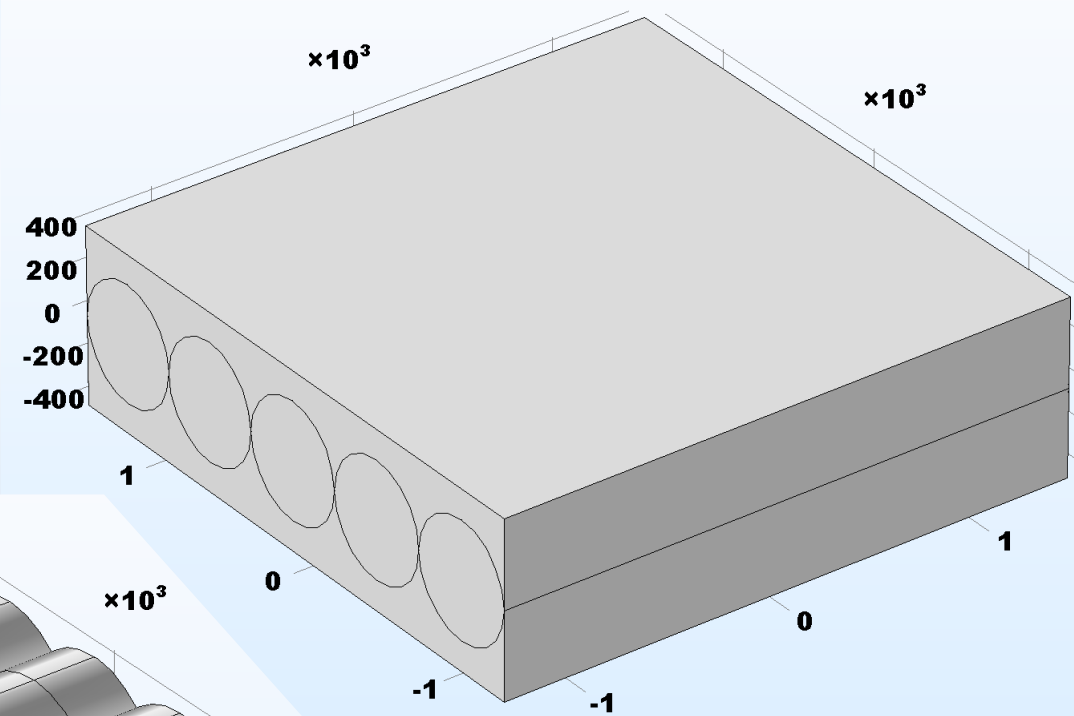
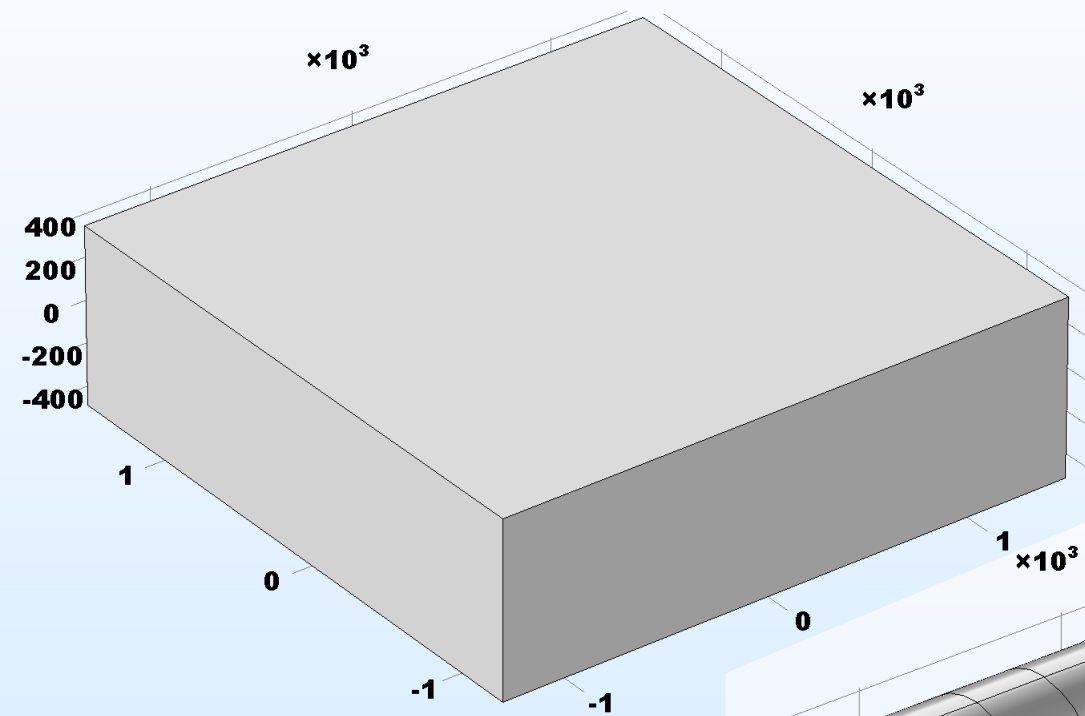


Figure 3

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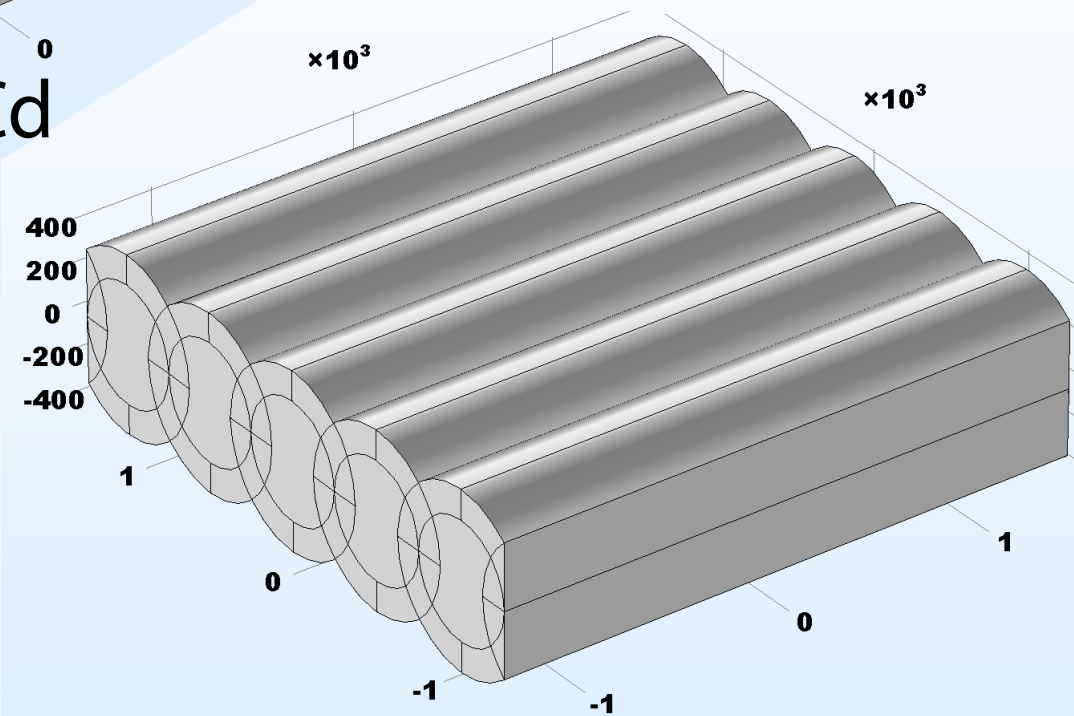
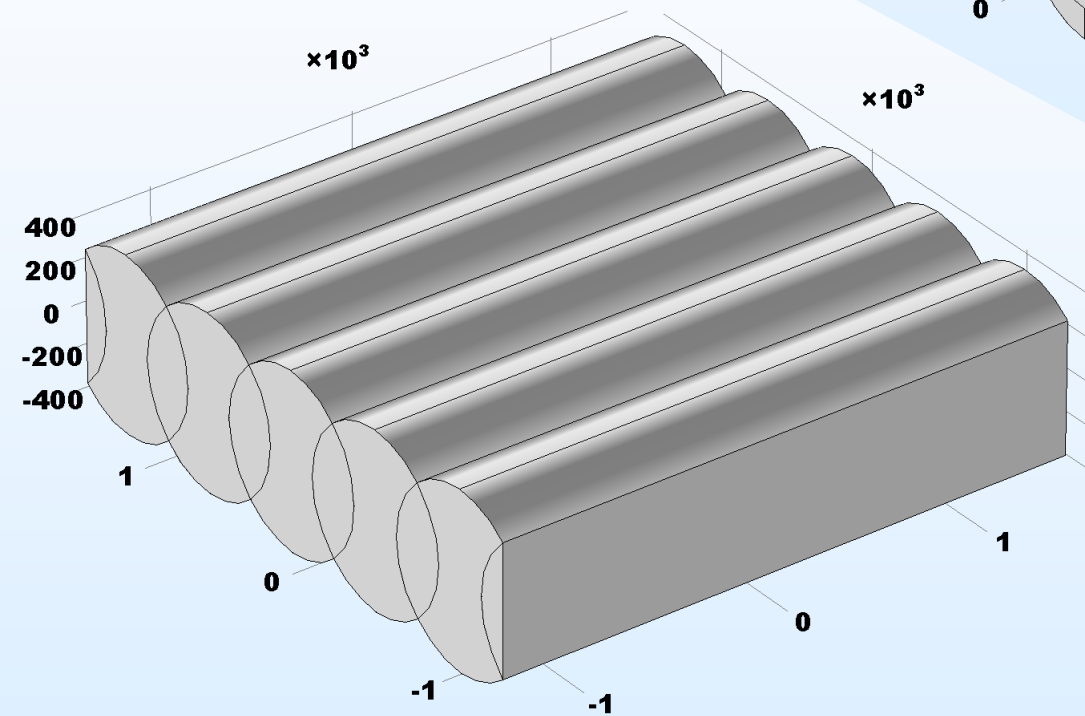
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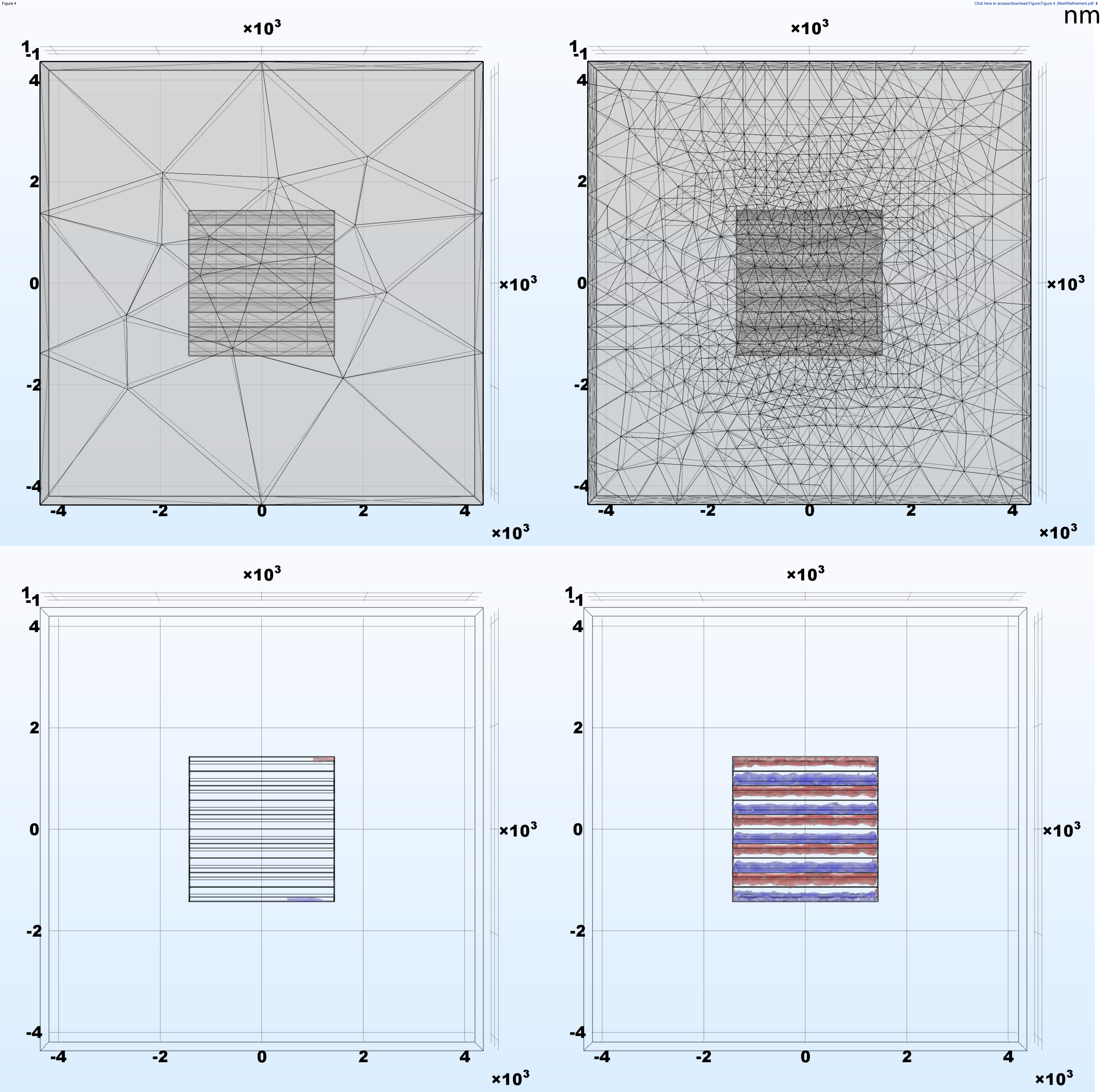


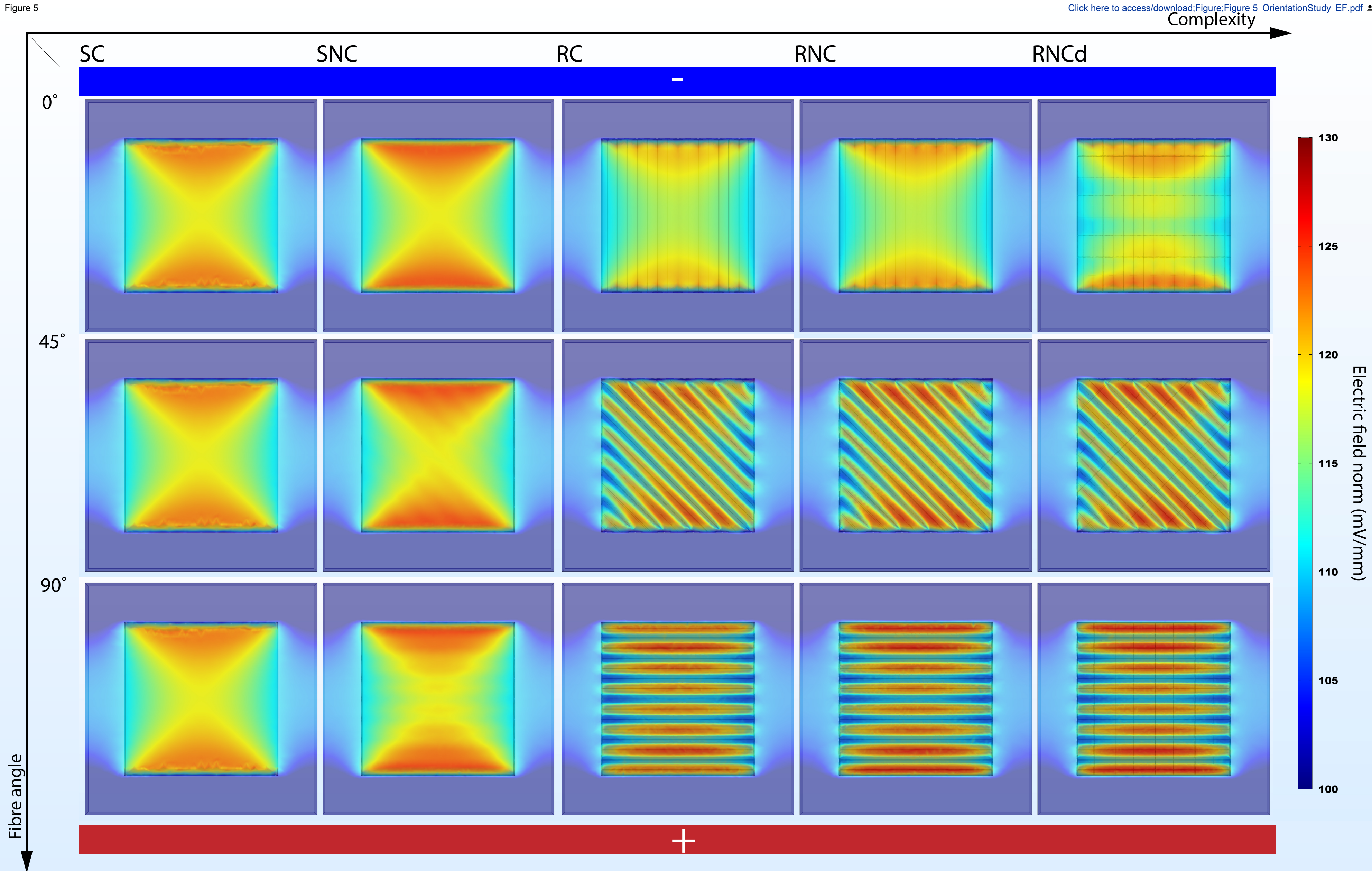
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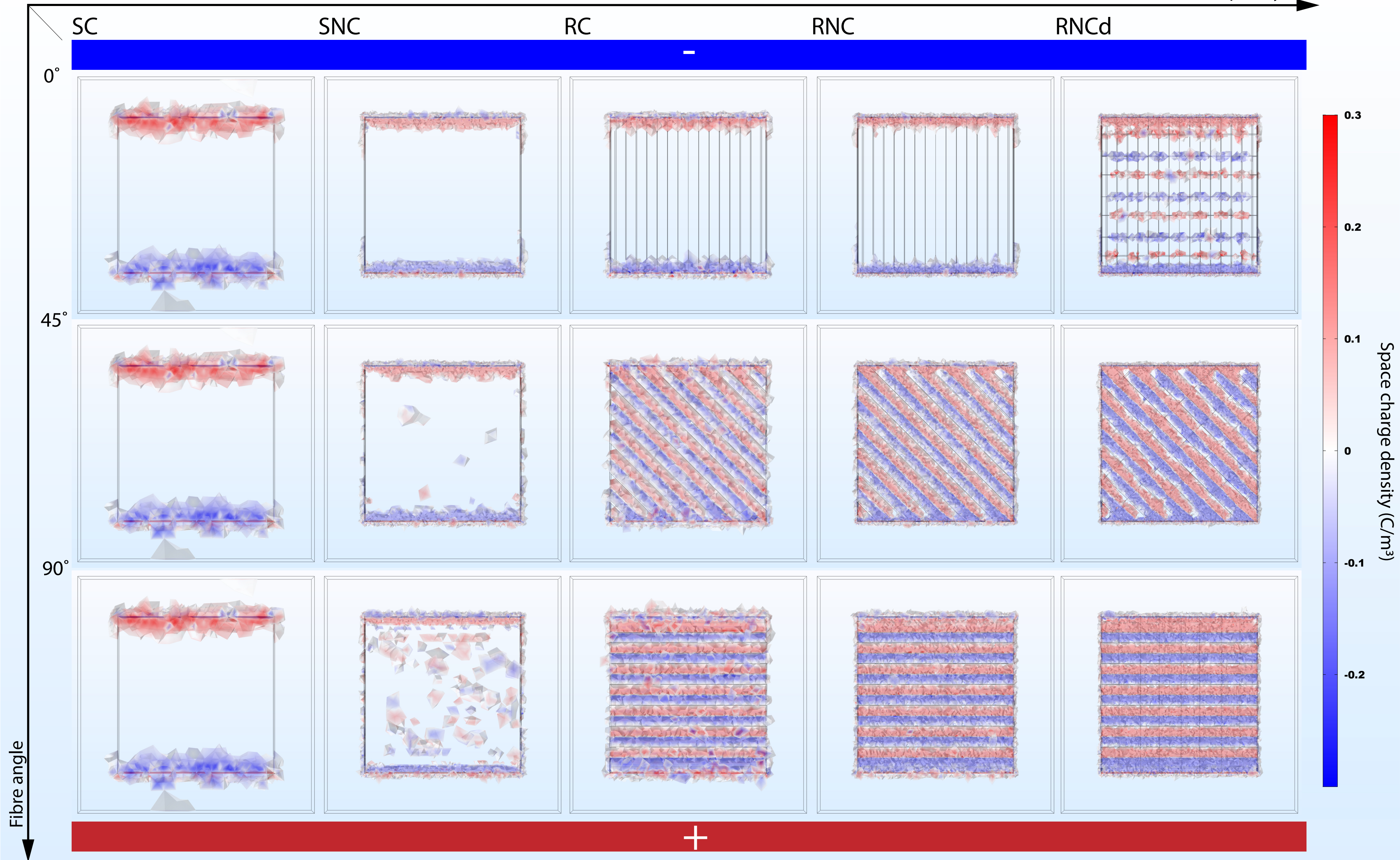
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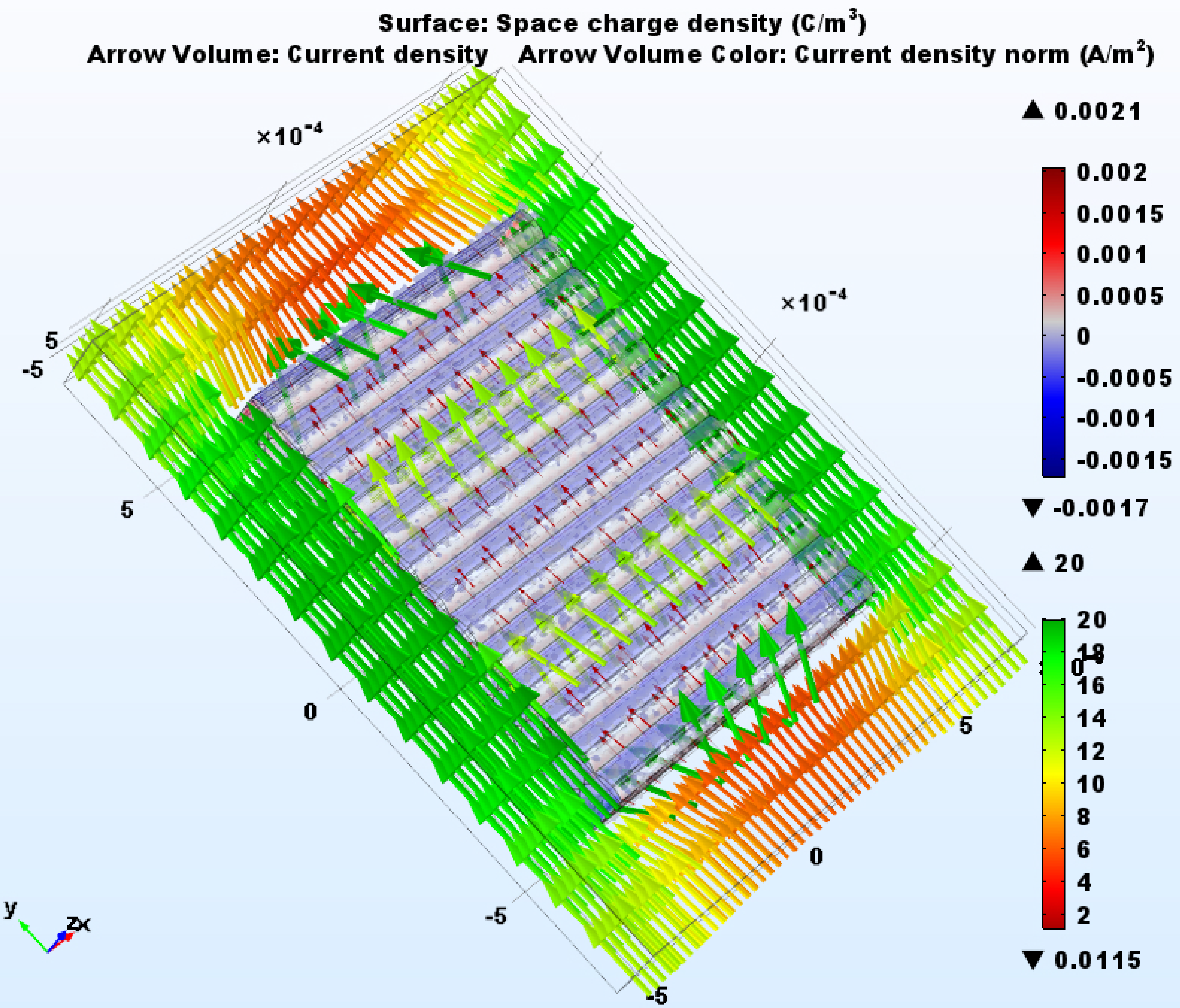
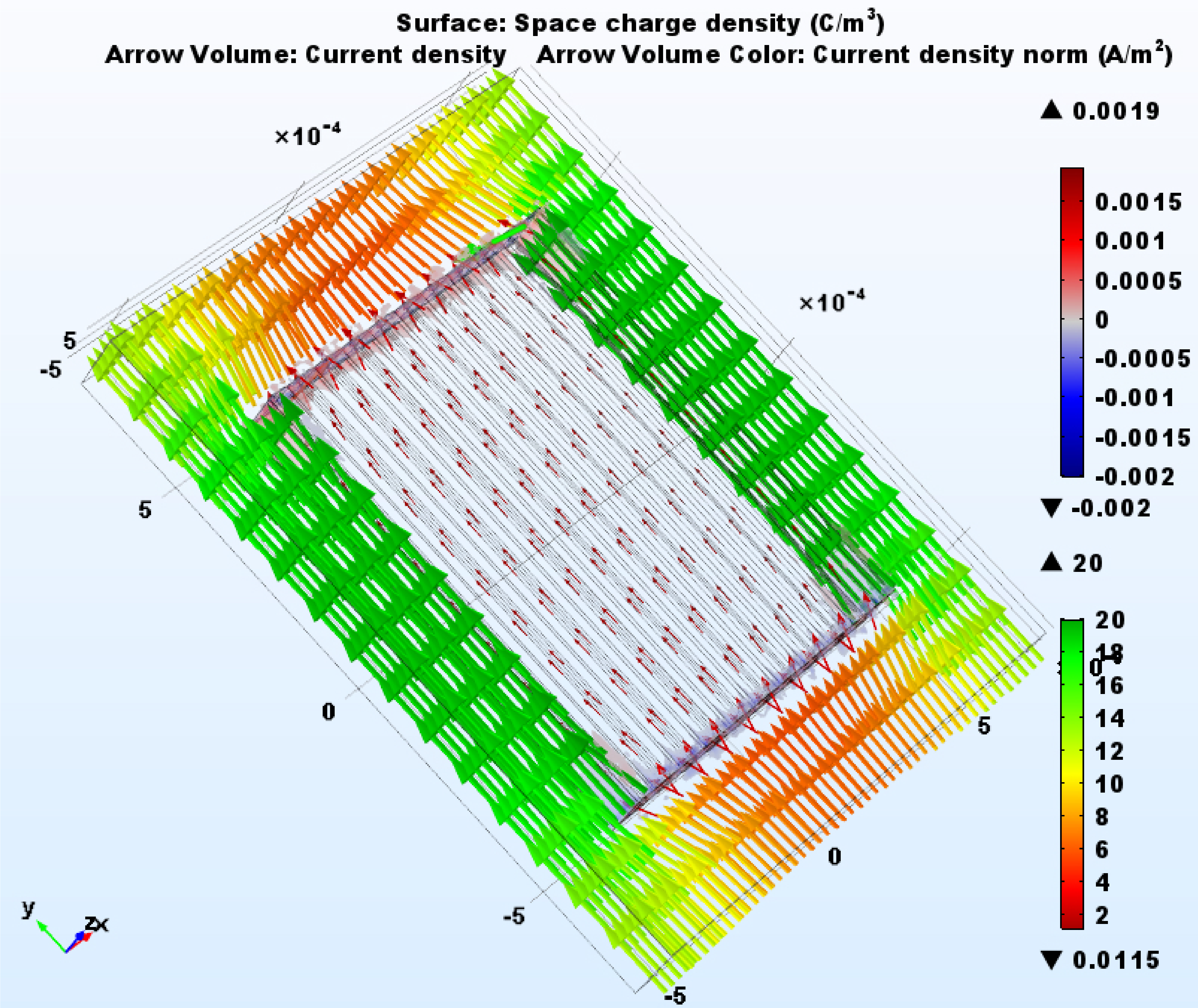


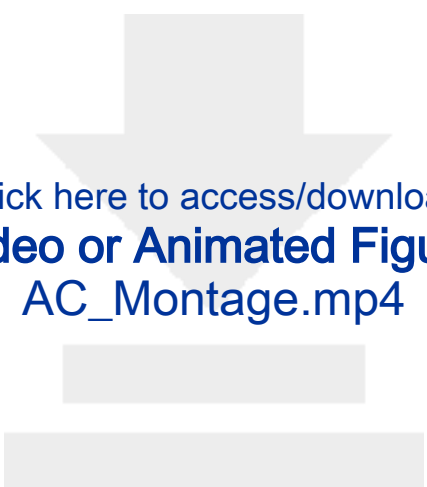
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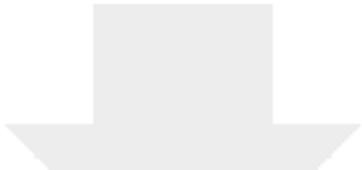









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Name	Expression	Description
Ws	$10 \cdot Rc \cdot med_ratio$	Scaffold width
Ls	$10 \cdot Rc \cdot med_ratio$	Scaffold length
Hs	$2 \cdot Rf$	Scaffold height
med_ratio	1.5	Ratio cell culture media to scaffold
Rc	278.5[nm]	Fiber core radius
r	1.5	Fiber core to coat ratio
Rf	$Rc \cdot r$	Fiber with coat radius
theta	90[deg]	Fiber orientation angle
Lf	$1.3 \cdot (Ls \cdot \cos(\theta) + Ws \cdot \sin(\theta))$	Fiber length
tes	1	Ratio fibre core radius to distance between fibres
n_1	$2 \cdot (\text{fix}((Ws / (2 \cdot \cos(\theta)) - Rf) / (2 \cdot tes \cdot Rc)) + 3) \cdot (\cos(\theta) \neq 0) + 1 \cdot (\cos(\theta) == 0)$	Max number of fibers if $\theta \leq 45$
n_2	$2 \cdot (\text{fix}((Ls / (2 \cdot \sin(\theta)) - Rf) / (2 \cdot tes \cdot Rc)) + 3) \cdot (\sin(\theta) \neq 0) + 1 \cdot (\sin(\theta) == 0)$	Max number of fibers if $\theta > 45$
excess	$1.2 + 0.3 \cdot \text{abs}(\sin(2 \cdot \theta))$	First fiber relative offset from scaffold
D	$Lf / 5$	Coat periodicity
prop	0.46	Length of first coat relative to periodicity D
E	100[mV/mm]	Electric field magnitude
V0	$E \cdot Ls \cdot med_ratio$	Terminal Voltage
omega	500[Hz]	Time dependent study Voltage frequency
p_sigma	0.5	Second coating relative conductivity
p_eps	1.5	Second coating relative dielectric constant

Table 2

	Culture Media	PEDOT:PS S 1	PEDOT:PS S 2	Collagen Hydrated 1	Collagen Hydrated 2	Silk Fibroin	Collagen Dry
Electrical Conductivity (S/m)	1.7014	1.00E-01	p_sigma * 0.1	2.00E-05	p_sigma * 2e-5	1.00E-08	2.50E-08
Relative Permittivity	80.1	2.2	p_eps * 2.2	9.89	p_eps * 9.89	7.81E+00	4.97

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Comsol multiphysics 5.2 AC/DC module	COMSOL	-	FEM modelling software

Dear editorial board,

Thank you for recruiting additional reviewers. All the comments were addressed and gave us a better understanding of the reader's experience. They helped us rewrite key sections of the manuscript to convey our message in a clearer, more concise manner. We hope that you, as well as the reviewers, will be pleased by the evolution of our manuscript.

Please find below our responses to the editorial and reviewers' comments.

Yours Sincerely,
Miruna Verdes

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript was proofread, and a few errors were corrected. Also, a short paragraph was modified to improve its readability.

“Multiple ES experiments aiming to shed light on underlying mechanisms have been carried out in vitro. EF exposure is reported through parameters describing the magnitude of the generated voltage gradient and the stimulus delivery device. However, to accomplish their purpose, the target system (i.e. group of cells) input signal must also be investigated.”

was changed to:

“Multiple ES experiments have been carried out in vitro over the years. Most of these only characterize the ES through the voltage drop between the electrodes divided by the distance between them – a rough approximation of the electric field magnitude. However, the electric field itself only influences charged particles, not cells directly. Also, when multiple materials are interposed between the device and the cells, the rough approximation may not hold.”

2. Please ensure that the references are numbered in order of appearance. See line 83.

References were checked and reordered.

Reviewers' comments:

We thank both reviewers for taking the time to read, understand and react honestly to our manuscript. We found their comments and questions very useful, as they helped identify key messages that were not clearly enough presented in our manuscript. Both sets of questions and comments contributed to our improvement of this manuscript.

Reviewer #3:

Manuscript Summary:

Overall this is a good manuscript and serves a much needed issue in the field.

1) cellular micro environment's structure and physical properties play a determining role in the actual experimental testing conditions. Hypothesis is very general and already been showed before. It would be good if the authors could be a bit more specific to shed light on the strength of the work presented.

We thought that for the discussion of this point it is relevant to bring the statement back to the context: “**However, the advancements in in vitro experiments are difficult to reproduce directly in clinical settings. Mainly, that is because the ES devices used in vitro differ significantly from the ones suitable for patient use. Translating the in vitro results into in vivo procedures is therefore not straightforward.** We hypothesize that the cellular microenvironment’s structure and physical properties play a determining role in the actual experimental testing conditions **and that measures of charge distribution can be used to bridge the gap between in vitro and in vivo.**”

In other words, the path from the electrodes to the cell, including microenvironment structure and its electrical properties (conductivity, permittivity) are not reported, investigated or accounted for in most electrical stimulation in vitro/in vivo studies although they differ significantly. Our hypothesis is that this is (at least partially) why in vitro results are not fully reproduced in vivo, thus the gap between in vitro and in vivo that hinders progress. Our solution to this problem is to encourage the use of charge distribution measures rather than simply reporting the electric field strength – which does not directly impact the cell.

To better express this, we made the following modifications to the abstract segment:

“However, the advancements in in vitro experiments are difficult to reproduce directly in clinical settings. Mainly, that is because the ES devices used in vitro differ significantly from the ones suitable for patient use, and the path from the electrodes to the targeted cells is different. Translating the in vitro results into in vivo procedures is therefore not straightforward. We emphasize that the cellular microenvironment’s structure and physical properties combined play a determining role in the actual experimental testing conditions and suggest that measures of charge distribution can be used to bridge the gap between in vitro and in vivo.”

2) Why the in vitro and in vivo scenarios are not similar?

In vivo and in vitro devices are similar in principle: an electric field accelerates and redistributes charged particles within and around the targeted cells. However, the electrodes and the interface between the electrodes and the cells often differ. This means that for the same potential difference applied on the electrodes a different stimulus is actually applied to the cells in vitro versus the ones in vivo. For instance, the most widespread in vitro experimental setup uses Ag/AgCl electrodes in culture medium, while dressings with soft metal electrodes embedded in hydrogel are attached to intact and damaged skin in the treatment of chronic ulcers in vivo. The translation between the in vitro study and in vivo therapy is not straightforward, and a voltage drop enhancing keratinocyte proliferation in vitro might actually be damaging in vivo.

The above changes to the abstract should also clarify this.

I believe the hypothesis would be more insightful if they were both collagen fibers in vitro and in vivo.

In the present study, we focus on a segment of the interface between the electrodes and the cells: the extracellular matrix. We explore how different fibre types (collagen vs. PEDOT:PSS silk fibroin) transduce the electrical stimulus at different orientations to the electric field.

The study of the influence of different electrode materials or shapes, or electrochemical reactions between the electrodes and culture medium/hydrogels is however beyond the scope of the model presented. **The electrodes are not included in the model, but replaced with boundary conditions for the electric potential. These boundary conditions are chosen so that a rough approximation for the magnitude of the electric field ($\Delta V/d$) is 100 V/m, a frequently reported stimulation parameter.**

This means that the feature that makes the RNCd model representative of an in vivo microenvironment is the structure of the fibers, characteristic for collagen fibers.

While we are aware that collagen scaffolds are also used for in vitro experiments, we consider collagen fibers to be representative for in vivo microenvironments. On the one hand, they make up the majority of the fibrous extracellular matrix. On the other hand, other fibre types are often used as replacements as they solve sourcing issues and mechanical limitations imposed by collagen (Collagen fibers available for scaffolds are either extracted from animal tissues and pose an interspecies disease transmission risk, or have low yield (cell synthesized collagen in vitro) and pose stability issues (artificial synthesis)¹). Our results show that those other fibers are not a perfect replacement for the collagen fibers – regardless to whether the collagen fibers are in vivo or in vitro. We believe this result to also be insightful, especially for scientists developing and optimizing fibrous scaffolds.

The following highlighted modifications were made in the results section to clarify the components included in the model:

“Minimization of computational cost was accomplished by reducing the ES device geometry to a model unit volume representing the microenvironment. While an ES device and scaffold’s width and length can easily be at the order of a few centimeters, the containing fibers’ diameter is usually lower than a micron. Here, we use a scaffold cut comparable to the fiber diameter to reduce the computational cost induced by the aspect ratio and highlight the effect of the scaffold’s fibrous nature on the electric microenvironment. The rest of the ES device is replaced with electric potential boundary conditions chosen so that a rough approximation for the magnitude of the electric field is 100 V/m, a frequently reported stimulation parameter.”

As such, the comparison between complete in vitro and in vivo ES devices is outside the scope of this manuscript.

3) Comments:

* Starting line 133 it talks about silico modeling and the benefits of it, but it doesn't reason why its such a good model or references any papers for it.

In the paragraph ending at line 133: “The unknown can be exposed by observing the difference between what is expected to happen based on the current knowledge and what happens. In silico experiments based on mathematical modelling allow splitting the process into known and unknown subprocesses. This way, phenomena not accounted for in the model come to light when in silico predictions are compared to in vitro and in vivo experiments.”. This highlights the reason why in silico models are necessary and how they complement in vivo/in vitro models. In other words, simulations using in silico models show us how much of the real process we can account for with what is known. By comparing simulation results with real world measurements we can uncover the existence of other phenomena taking place.

* Figure 2 is very informative even though its description is quite lengthy, maybe a different format would be more useful?

The legend of Figure 2 has been modified to improve readability and its length was decreased by 100 words. The figure itself was also reworked to better reflect the legend: two additional panels have been added to cover the Equation of continuity and the Charge conservation law.

The text introducing the figure was also modified to clarify why this figure is relevant in the context of our model:

“To combine all components, all interface signals must be compatible. The common denominators in those units are charge and current density – Figure 2. The current protocol was used to study how scaffold dependent parameters can be used to modulate these two signals, independent of the EF. Results show that. Results show that variations to those metrics can be produced by modifying the scaffold and highlight the necessity of accounting for the ECM electrical properties when pursuing an understanding of the ES process in vivo.”

became

“Charge and current density – Figure 2 – can act as interface signals between models of the ES device and the biological sample, or between different components of the ES device. The proposed FEM based protocol uses the equations described in Figure 2 and was used to study how scaffold dependent parameters can be used to modulate those two signals, independent of the EF generated by a direct coupling setup. Results stress that it is necessary to account for scaffold or ECM electrical properties when investigating how ES impacts target cells.”

* Formatting in table 1, I believe there are some subscripts that are missing V_0 instead of V_o . It might also be useful to have a separate column for units as well as using the proper symbols I stated of theta.

Table 1 is formatted as it is within the software – units between square parentheses after the parameter expression, and no option to use symbols or subscripts as or within parameter names.

* Figure 6 and 7 are introduced in line 637, but I have not seen figures 3-5 introduced beforehand which are key to understand the different variants to the model and parameters used. I also think that the steps for compose could be introduced in a form of a schematic instead of the pages of different steps.

The reference to figures 6 and 7 at line 637 (end of first paragraph in representative results section) was removed. That paragraph is meant to restate the purpose of the proposed model, not to demonstrate its effectiveness, thus the reference to the figures was redundant and potentially confusing to the reader.

The protocol can indeed be summarized in a flow chart. However, as JOVE is a methods-based journal, the focus of the paper is the protocol and all steps (e.g., button clicks, software commands, any user inputs, etc.) must be included.

4) The authors are missing important refs in their introductory work. They are strongly encouraged to refer to a wider range of publications to reflect on the advances of the field accurately. These two refs are very relevant and out to be cited:

Lynch KJ, Skalli O, Sabri F. Growing neural PC-12 cell on crosslinked silica aerogels increases neurite extension in the presence of an electric field. *Journal of functional biomaterials*. 2018 Jun;9(2):30.

This paper reports the effect of electrical stimulation (ES) with a capacitive coupling setup on neural cells seeded on top of the collagen coat of either an aerogel scaffold (PCSA) or tissue culture polystyrene (TCPS). We added this reference in the introduction under in vitro studies of ES impact on cellular development.

Hadley J, Hirschman J, Morshed BI, Sabri F. RF coupling of interdigitated electrode array on aerogels for in vivo nerve guidance applications. MRS Advances. 2019 Apr;4(21):1237-44.

This paper presents an interesting device able to wirelessly induce current in a gold secondary coil with interdigitated electrodes. The secondary coil can be placed inside the body, to be in direct contact with damaged neural cells, while the primary coil and the power source can be kept outside – thus eliminates the need to implant additional components. This coil is coated on a porous biostable aerogel surface because increased rate of axon growth was reported on such materials and they are suitable materials for nerve conduits. The secondary coil has two interdigitated electrodes connected to its ends – if a current is induced within the coil, there is a potential difference between the electrodes.

It is mentioned that the damaged nerve is potentially fixed between those electrodes, on the aerogel substrate, although this topic has not been investigated or thoroughly discussed within this paper. That is understandable, as the purpose of the article is to show that a wireless energy transfer to the inside of the body is feasible, and not to investigate how that energy is further used by cells.

Our introduction references the following:

- in vivo and in vitro studies of ES impact on cellular/tissue development
- clinical trial results of ES using devices available for clinical use
- fibrous scaffold studies that report the structure of scaffolds, their effects on cells in the presence of electric fields
- studies on the structure and properties of extracellular matrix

Although the design of electrical stimulation devices is a related subject to our paper, we focus on the set of devices that make use of fibrous scaffolds and have the electrodes in direct contact with the medium the cells are in. That is why the broader range of existent ES devices is but briefly introduced in our paper by general features, not referencing any specific devices or aiming to make comparisons between different designs. For that, the reader can access referenced reviews with a wider grasp of the topic^{3,4}.

As we did not reference any other specific ES devices and the suggested paper does not fit any of the other topics mentioned above, we cannot reference Hadley J, Hirschman J, Morshed BI, Sabri F. RF coupling of interdigitated electrode array on aerogels for in vivo nerve guidance applications. MRS Advances. 2019 Apr;4(21):1237-44. in our introduction. However, we will keep this publication in mind for our future work, and we did modify the following passage to avoid giving the reader the impression that the power source must always be implanted along with the electrodes:

“Devices used in vivo for longer treatments need to be wearable, thus the electrodes and most times the energy source are either implanted or attached to the skin as wound dressings or electroactive patches. The generated voltage gradient displaces charged particles in the treatment area.”

Reviewer #4:

The paper reports on finite element modelling to describe the cellular microenvironment and the changes generated by exposure to electric fields. In particular, it is shown the electric field coupling with geometric structure to determine charge distribution. Further, time dependent inputs on

charge movement is shown. Two case studies are presented.

This work is in an area of increasing activity and interest and the development of models related to cellular electric microenvironment is relevant.

The description of the protocol seem correct and the results consistent. With respect to the possible impact of the result, it would be interesting to see discussion/comment on the following issues, in order to work to be more significant for the scientific community in the field:

-many works on electric field microenvironment is based on dipolar variations (such as piezoelectricity in bone tissue engineering). See, for example the works of C. Ribeiro et al or S. Ribeiro et al describing both materials and bioreactors. This electrical microenvironment has been proven to be relevant in cell differentiation, growth and maturation. Can the authors account for those electrical microenvironment? How can be achieved?

This model does not account for piezoelectric effects. The aspect of piezoelectricity should be covered in models that couple mechanical and electromagnetic interactions. Ours focuses solely on electric interactions (the electric field changes slow enough so that the induced magnetic field is negligible).

-the most relevant effect on cell fate, related to scaffolds is mechanotransduction. There are relevant models for that. It seems more relevant and correct to properly couple mechanotransduction models with the electrical ones, for a proper description of the microenvironment. Can the authors comment/address this issues?

While we agree that mechanical forces are also an important set of physical cues that the microenvironment provides the cell (as can be observed in figure 1), electromagnetic interactions are of a different nature (as they only occur between charged particles). We believe that each model of a stimulation mechanism has to first be experimentally validated separately, before coupling with others of different nature and/or with models of cellular response to stimulation. Otherwise, there is no way to quantify the contribution of each.

To better expose this point we changed the following paragraph in the discussion section:

“Other physical phenomena that could be coupled in later versions of the model are fluid flow (used in bioreactors and inherent in vivo), non-uniform ohmic heating and observed resulting morphological deformations of composites with aligned fibers⁵. Signals such as mechanical stress and transferred heat can then be added to the cellular input set.”

to:

“As the end goal of tissue engineering is to create bioreactors that not only mimic one or two aspects of in vivo environments, but replicate and control all cellular developmental cues⁶, electromagnetic and mechanical in silico models as well as models of heat transfer between bioreactor components will need to be combined. In a subsequent modelling phase, coupling phenomena between those interactions such as ohmic heating, electrolytic fluid flow, morphological scaffold deformations in response to electrical stimulation⁵ and piezoelectricity⁷ can also be added. However, models should be merged only after each one has been experimentally validated. This

way, we can gain a better understanding of each component's influence in the cellular microenvironment, and how stimuli can be optimized. “

Thus, event when the work is potentially interesting, those relevant issues must be improved.

We hope you find our revised manuscript improved.

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