A microdosimetry analysis of reversible electroporation in scattered, overlapping, and cancerous cervical cells

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Abstract
We present a numerical method for studying reversible electroporation on normal and cancerous cervical cells. This microdosimetry analysis builds on a unique approach for extracting contours of free and overlapping cervical cells in the cluster from the Extended Depth of Field (EDF) images. The algorithm used for extracting the contours is a joint optimization of multiple-level set function along with the Gaussian mixture model and Maximally Stable Extremal Regions. These contours are then exported to a multi-physics domain solver, where a variable frequency pulsed electric field is applied. The trans-Membrane voltage (TMV) developed across the cell membrane is computed using the Maxwell equation coupled with a statistical approach, employing the asymptotic Smoluchowski equation. The numerical model was validated by successful replication of existing experimental configurations that employed low-frequency uni-polar pulses on the overlapping cells to obtain reversible electroporation, wherein, several overlapping clumps of cervical cells were targeted. For high-frequency calculation, a combination of normal and cancerous cells is introduced to the computational domain. The cells are assumed to be dispersive and the Debye dispersion equation is used for further calculations. We also present the resulting strength-duration relationship for achieving the threshold value of electroporation between the normal and cancerous cervical cells due to their size and conductivity differences. The dye uptake modulation during the high-frequency electric field electroporation is further advocated by a mathematical model.

Introduction
Reversible Electroporation (RE) is where the exposures to electrical pulses cause pore formation but these pores reseal after some time. This method is used for drug delivery and DNA transfer [1–6] among others. Microdosimetry is used for studying the interaction of electrical energy with biological matter. It has been frequently used for electroporation studies since the real process of electroporation is still being investigated, as evidenced by several numerical studies. While in vitro electroporation studies employ both experiment-driven and model-driven approaches, the latter provides benefits in interpreting the biological outcomes since the biological responses of the cells are very complex, and current microdosimetry models are still not able to capture this complexity completely for them to be acceptably predictive. [7, 8]. Most of the numerical studies have been done using cell contours with parametric definitions, giving spatial distribution of the membrane potential [9–15]. The morphology of single and packed cells have been extracted from neuroblastoma cells, which exhibited a decrease in TMV for packed cells when compared to the isolated cells [16]. Confocal microscopy has been used to reconstruct the 3D cell [17] and RE has been achieved both numerically and experimentally. The 3D construction is also used to create the topology of the endoplasmic reticulum in the work presented [8] to develop an electroporation model. While studying electroporation the effect of cluster cells cannot be ignored. Studies have shown that it is hard to electroporate a cluster of cells due to the presence of multiple cell boundaries [18].

Cervical cancer is the fourth most lethal form of cancer worldwide. In 2018, 570 000 women were diagnosed with cancer leading to 311 000 deaths due to this
malignant disease, according to World Health Organisation (WHO) data [19]. The liquid-based in vitro cytology test or Pap smear test is the most common screening test, employed for cervical cancer. The staining agents used, cover the cytoplasmic area but factors such as inadequate spread of dye, inadequate sample acquisition and/or handling of samples, etc lead to error rates as high as 40% in the Pap smear test [20, 21]. The Cervical Intraepithelial Neoplasia (CIN) is the abnormal growth of the cells that are seen in the cervical tissue, which undergoes three phases of change categorized as CIN1, CIN2, and CIN3 [22, 23]. The cervical cancer cells show an increase in conductivity by a factor of 1.8 (maximum) [24, 25]. While employing electroporation, a significant quantum of works explores electroporation by the application of a pulsed electric field with the time period 25 μs-100 μs and pulse amplitude from 500 V/cm to 4.5 KV/cm with repeated pulses applied at a frequency of 1 Hz [26, 27]. These experiments were carried out in vitro with a mono-layer cell line. Twenty-five Gaussian pulses were applied for 70ns and 25KV/cm electric field strength to electroporation in cervical cancer cells [27]. In our earlier work, a realistic numerical model of single 2D cervical cells has been proposed in which nanosecond electrical pulses produced by a Marx generator were applied [28].

The aim of the work is to build a microdosimetry model for reversible electroporation in cervical cells for that can be used for future experimental studies. This method aims at reducing the hit-and-trial method of electroporation using a concrete strategy before experimentation. The electric field duration and strength could be beforehand analyzed using this model thus stopping the wastage of the cervical cell. It also uses the dye delivery diffusion equation to find out the quantity of dye that can be used and find out which cell rapidly intakes the dyes. A complete picture of reversible electroporation can be simulated and understood before going for experimentation. Thus a model-driven approach could be done. We have also analyzed various configurations of cervical cells using overlapping and scattered cells. The earlier studies have not worked with these configurations thus our model stands unique as it covers all the aspects that can be seen under a microscope during electroporation. Our work has also included cervical cancer cells of various stages through which a detailed analysis can be done upon the behaviour of electroporation with change in morphology of the cells during cancer. The pulse strength, duration, and pulse count applied are evaluated on the basis of high cell viability and permeability. The real cervical cell contours were extracted from EDF images which were prepared in the Australian Centre for Visual Technologies, University of Adelaide [29]. The cancerous cells were downloaded from a different database [30]. The algorithm is implemented to get the contour of various overlapping cells, isolated cells and a combination of both to yield a heterogeneous cluster of cancerous and normal cells. This topology is imported to the multi-physics area for further simulations. The threshold electric field for high-frequency uni-polar pulses is used in diffusion analysis for exploring the electrically mediated uptake of dyes or drugs into the cervical cell. A faster development of the required TMV and pore density is observed in the cancerous cervical cells. The proposed setup shows considerable promise for the validation and development of model-driven microdosimetry. The modulated uptake of dyes into cells could be further used for observing changes in the morphology of the cells.

Methods and materials

Cervical cells contour extraction

We try to replicate the earlier work done in which the joint optimization level set method is used to extract the contour of overlapping cervical cells [31]. The method proposed employs three methods to extract the full contour of the cells using EDF images. The first step is to employ Gaussian mixture model (GMM) for generating clump mask(s) followed by identifying the Maximally Stable Extremal Region (MSER) for generating nuclei mask and finally performing a joint level set optimization for detecting the boundaries of overlapping cells. The process start with pre-processing the EDF image figure 1(a), where these images are converted to grayscale followed by application of an anisotropic filter [32] to denoise the image. A subsequent application of the image quick-shift algorithm [33] generates superpixels using the vlquickseg() function from the VLFeat toolbox [34]. This is then followed by the application of an edge detector for the morphological gradient of the generated superpixel image which is then binarized at a threshold. The number of connected components in the binary image are identified using the bwlabel() (inbuilt function in Matlab). To build the GMMs the ratio of likelihood between the two classes of each pixel is used for classification. The re-estimation process of the likelihood map is repeated until a stable GMM is developed. The mleGmm() function is used to apply GMM on the cell clusters present in the Statistical Pattern Recognition toolbox [35] in MATLAB. This function gives us the mean, covariance, and prior of the image. The foreground and background GMM models are created leading to a convex mask which will be used further. Figure 1(b) shows the derived outer boundary of the cluster through this process.

After the isolation of the outer contour of the cell clump(s), the task of identifying the nuclei within the clump starts by the application of the Maximally Stable Extremal Region (MSER) algorithm [36] which yields a starting point of a nuclei mask which is then cleaned of all fragment using Distance Regularized Level Set Evolution (DRSLE) [37]. The cleared image is
binarized followed by the formation of blob sets of nuclei. To refine the nuclei mask additional properties such as the area ratio of the clump and the nuclei and the intensity of the obtained MSER blob. The MSER blob is filtered iteratively using these conditions till a stable nuclei mask is created. Figure 1(c) shows the isolated nuclear regions within the cellular clump after implementing the MSER algorithm in image 1(a).

For identifying the overlapping cells, it is important that the initial guess is proper. An initial segmentation process begins with building a shape prior which is further used for level set optimization. In order to build shape prior to each point on the clump boundary, it is associated with its nearest nucleus so that the cell ownership could be claimed. The interpolation function is used to draw the initial segmentation (TriScatteredInterp) in Matlab. In the overlapping region, this function draws the cell boundaries of each cell by following the extreme clump points. The geometric center of each cell is calculated. This boundary is then used for level-set optimization. For each cell in the clump following constraints were used:

\[
h_i(x) = \begin{cases} 
1, & \text{if } x \text{ outside clump } C \\
\frac{-2}{1 + \exp(-\beta t(x))}, & \text{if } x \text{ inside cell } i \\
0, & \text{otherwise}
\end{cases}
\]

where C represents set of clumps |C| denoted by \(h_i\). The function \(t(.)\) denotes the distance between the point x on the 2D image domain and the geometric center of the initial segmentation, \(\beta\) represents the free parameter whose value is taken after a lot of repeated trials. The shape prior constant is then defined as \(h_e(x) = \max(h_i(x))\) where \(h_i \in C\). If the clump mask created in the above process contains one nucleus, it may be safely asserted that the mask is not overlapping hence, it represents a single cell contour and they are not involved in level set evolution. however, if more than one nucleus is present, then the clump has an overlapping contour. The cell boundaries near the geometric centre of nuclei are assumed to be part of that cell. The cell boundaries are associated with a nucleus so that cell ownership can be established. The extreme point of the cell boundaries is taken and used for extrapolating the cell boundary of the overlapping region. Now the centroid of the clump containing the extrapolated boundaries is calculated using a connecting component function that contains all the information including the centroid of the cell. Applying joint level set optimization on the initial guessed boundary leads to the boundary of the overlapping areas. The joint level set optimization is used in this paper in which an initial segmentation in the form of shape prior is used. \(\phi(x, y, t) : \Omega \rightarrow \mathbb{R}\) denotes a 2D time-dependent level set function (LSF) with N cells that have been detected. LSF is denoted by \(\{\phi_i\}_{i=1}^{N}\) and the minimized energy function by equation

\[
\epsilon(\{\phi_i\}_{i=1}^{N}) = \sum_{i=1}^{N}(\epsilon_u(\phi_i) + \sum_{j=1}^{N} \sum_{i \neq j}(\epsilon_b(\phi_i, \phi_j)))
\]

where \(\epsilon_u\) is the unary energy function for each LSF, \(\epsilon_b\) represents the binary function over LSF and N(i) is the represented by level set function \(\phi_i\) such that it intersects zero level set function of \(\phi_j\). The unary function \(\epsilon_u\) is defined as:

\[
\epsilon_u(\phi_i) = \mu R(\phi_i) + \kappa D(\phi_i)
\]

where \(\mu > 0\), \(\kappa \in \mathbb{R}\), the first regularization term:

\[
R(\phi_i) = \int_{\Omega} p(|\nabla \phi|) \, dx
\]

where \(p: [0, \infty] \rightarrow \mathbb{R}\) and \(|\nabla \phi| = 1\) is the regularized term that maintains this property.
Table 1. Cervical cell dimension table.

| Celltypes               | Cell X     | Cell Y     | Nucleus X | Nucleus Y |
|-------------------------|------------|------------|-----------|-----------|
| Superficial             | 66 ± 9.2 μm| 66 ± 9.2 μm| 8.4 ± 1.1 μm| 8.4 ± 1.1 μm|
| Intermediate            | 52 ± 10 μm | 52 ± 10 μm | 8.6 ± 1.2 μm| 8.6 ± 1.2 μm|
| Parabasal               | 31 ± 6 μm  | 31 ± 6 μm  | 8.3 ± 1.0 μm| 8.3 ± 1.0 μm|
| Basal                   | 16 ± 3.2 μm| 16 ± 3.2 μm| 9.0 ± 1.8 μm| 9.0 ± 1.8 μm|
| CIN1 (superficial)      | 46 ± 10 μm | 46 ± 10 μm | 8.0 ± 2.2 μm| 8.0 ± 2.2 μm|
| CIN2 (superficial)      | 38 ± 7.1 μm| 38 ± 7.1 μm| 8.4 ± 2.2 μm| 8.4 ± 2.2 μm|
| CIN3 (superficial)      | 20 ± 5.5 μm| 20 ± 5.5 μm| 13 ± 2.2 μm | 13 ± 2.2 μm |

Numerical model of the cervical cells

The cervical cells are first exposed to uni-polar rectangular pulses of low-frequency pulse of time duration 100 μs and electric field strength of 1 kV/cm. Different Cellular clusters and isolated cell configurations are subjected to electric stress, as shown in figures 2–4. The computational domain of 500μm×500 μm is used, and copper electrodes (500 μm×10 μm) of resistance 0.0038 Ω are attached to the top and bottom of the rectangular domain. The arrow indicates the orientation of the applied electric field. The points noted in each figure are selected from the contour for analysis to highlight the effect of the applied electric field on overlapping cells and free cells (as applicable). The temperature analysis is done to verify that no thermal ablation is seen at the applied pulse indicating RE. The TMV and pore density are plotted at these points to evaluate whether the membrane(s) have reached the threshold pore density of 10^{14} m^{-2} and TMV 1–1.5 V—in concurrence with the reported works [38–40]. Experimental data [26] has been used to evaluate the pulse strength and duration to establish concurrence with our proposed numerical model. In the experiment, the cells were exposed to pulses of range 500 V/cm to 4500 V/cm with a number of pulses ranging from 1 to 60 at a frequency of 1 Hz. The paper reveals that the required strength of the electric field is around 1 kV/cm where the viability is 97.625 % for 100 μs pulse. This is the point of threshold where the cervical cells, when exposed to the electric field, have higher vitality and reversible electroporation is reported at this electric field strength and duration [26]. Hence we start with the numerical modelling of the cervical cells after deciding on the electric field required for reversible electroporation.

The cell membrane is made up of a bi-lipid layer, which when exposed to an electric field causes the dipoles to get pushed away—leading to an “opening” -pore. The pores formed are generally of radii r_p = 0.8 nm [9] and their creation causes an increase in the conductivity of the cell membrane. These pores formed are represented by a statistical Smoluchowski equation given by:

\[ D(\phi_i) = \int_{\Omega} h_C(x) g(\phi_i) (|\nabla \phi_i|) \, dx \]

where \( h_C(x) \) is the shape prior constraint as stated above and here \( g = \frac{1}{1 + |\nabla \phi|} \) where I represents the image domain. \( D(\phi_i) \) is derived from the Geodesic active contour length term provided.

\[ e(\phi_i, \phi_j) = \chi \int_{\Omega} h_C(x) g H(-\phi_i H(-\phi_i) \, dx \]

For finding minimum energy of the function \( (\phi_i)_{N=1} \) a gradient flow equation of the the each LSF \( \phi(x, y, t) \) is given as:

\[ \frac{\partial \phi_i}{\partial t} = -\frac{\partial (\phi_i)_{N=1}}{\partial t} \]

where \( \frac{\partial (\phi_i)_{N=1}}{\partial t} \) represents the Gateaux derivative the function. This derivative PDE is solved using finite difference scheme with time stepping given to solve the curve formation. Based on equation (1) [31] A joint level set optimization is employed here, in which the shape prior is used as shown in figure 1(d). We refer the reader to the original work [31] for complete details and the source code(s). After applying these steps, the contour is obtained—as shown in figure 1(e) which is then re-scaled to the size provided in table 1 [22, 23].

Algorithm 1. Algo of Contour extraction

Input: Cytoplasm Specimen
Output: Individual cytoplasm, nucleus and overlapping contour
1: Stage 1: Scene Segmentation
2: Compute super-pixel map from I using Quick Shift
3: Compute gradient map from super pixels
4: Compute convex hull from gradient map to initialize clump and background GMM models
5: while Clump and background GMM models not stable
6: Re-estimate clump and Background
7: GMM Models
8: Post processing to eliminate noise in the clumps and clumps with preset size less than the threshold
9: Detect and segment nuclei using blobs representing MSER
10: Compute initial segmentation for the cells \( i \in \{1, 2, \ldots, N \} \) and shape priors, \( h_C(\Omega) \).
11: Stage 2: Joint Level Set for overlapping cell segmentation
12: \( (\phi_i)_{N=1} = \arg\min_{(\phi_i)_{N=1}} e(\phi_i) \) \( e(\phi_i) \)
13: Re-compute \( h_C(\Omega) \) using \( (\phi_i)_{N=1} \) as new initial segmentation for each N cells and iterate 10-11 until reaching a local minimum.
Cell contours are zero level sets of \( \phi_i^k \) for \( i \in \{1, 2, \ldots, N \} \)
\[
\frac{\partial N}{\partial t} = \alpha e^{\left(\frac{w}{z}\right)} \left[ 1 - \frac{N}{N_0} e^{\left(-\eta \left(\frac{w}{z}\right)\right)} \right]
\]  
(4)

where \(N_0\) is the initial pore density on the cell membrane \((3.3 \times 10^6 \text{ m}^{-2})\). The \(N\) represents temporal pore density, \(tmv\) is the trans-membrane potential developed at that instant of time. The \(V_{ep}\) represents the characteristic electroporation voltage with \(\alpha\) and \(q\) representing electroporation constant. The conductivity of the cell membrane increases due to the formation of pores and it represented by an average value:

\[
\sigma(x, y, t) = \sigma_0 + \pi \sigma_p r_p^2 N(x, y, t) K
\]  
(5)

where \(\sigma_0\) is the static conductivity of the lipid bi-layer. The factor \(K\) is calculated as given by the following equation.

\[
K = \frac{e^{\psi_0} - 1}{w_0 e^{\psi_0} - \eta \psi_0} \frac{w_0 e^{\psi_0} + \eta \psi_0}{w_0 - \eta \psi_0}
\]  
(6)

where \(w_0\) is the energy barrier, \(\eta\) is the relative entrance length and \(\psi_m = \frac{\alpha}{K} V_m\) is the compensated transmembrane voltage. By solving the Laplace equation the electric potential is calculated in the simulation domain.

\[
\nabla \cdot \nabla \left(\sigma + \varepsilon_0 \frac{\partial \phi}{\partial t}\right) - \frac{\partial \nabla \cdot P}{\partial t} = 0
\]  
(7)

\[
E = -\nabla \phi
\]  
(8)

where \(\phi\) represents potential developed in the computational domain. Equations (4), (5), (7) and (8) are used in the calculation of developed potential. The Transmembrane potential is calculated using equation (9).

\[
TMV = \phi_{\text{outer}}(x, y, t) - \phi_{\text{inner}}(x, y, t)
\]  
(9)

A thin layer is used as boundary impedance for easy computation applied on the plasma membrane given by equation (14) available in COMSOL 5.5a electric current module. The membrane thickness is taken to be 5 nm.
The temporal development of the pore radius is given by equations (11)–(12).

\[
\frac{\text{d}r}{\text{d}t} = \frac{D}{K_T} \left( \frac{V_m^2 F_{\max}}{1 + r_0/(r + \tau)} + 4\beta \left( \frac{L_0}{r} \right)^{\frac{3}{2}} + 2\pi \gamma + 2\pi N\delta_{\text{eff}} \right)
\]

where \( V_m \) is the transmembrane potential developed during electroporation. The number of hydrophilic pores developed \( (r > r^*) \) are from \( j = 1, 2, 3, \ldots k_p \), K represents Boltzmann constant, \( \delta_{\text{eff}} \) is calculated as:

\[
\delta_{\text{eff}} = 2\delta^* - \left( 2\delta^* - \delta_0 \right) \left( 1 - \frac{\lambda_1}{\lambda_2} \right)^2
\]

where \( A \) is the area of the plasma membrane and total perforation area is given by \( A_p \) which is calculated as [9]:

\[
A_p = \oint_S S^2 N(t) \tau_{\text{eff}}^2 \text{d}S
\]

where \( S \) is the plasma membrane surface. The Multi-relaxation Debye-based relationship is used to model the cervical cell dielectric properties. The cytoplasm and nucleus of the cervical cells in this study use a dispersive medium whose dielectric properties have been modelled using a second-order equation [10].

\[
\epsilon(\omega) = \epsilon_\infty + \frac{\Delta \epsilon_1 + \Delta \epsilon_2}{1 - \omega^2 \tau_1 \tau_2 + j\omega \tau_1 \Delta \epsilon_1 + j\omega \tau_2 \Delta \epsilon_2}
\]

where \( \epsilon_\infty \) denotes the high frequency permittivity, \( \tau_1 \) & \( \tau_2 \) are the relaxation times and \( \Delta \epsilon_1, \Delta \epsilon_2 \) are the relaxation amplitudes. The polarization vector \( P \) can be expressed in the second-order differential equation which is known as Debye Dispersion model. In this equation, the polarization \( P \) is expressed as a time-varying model of a homogeneous medium in terms of a time-varying electric field given by [41]:

\[
\text{Figure 3. (a) EDF image of the cervical cells (b) TMV contour map at 50 } \mu \text{s (c) Pore Density plot in Log scale at 50 } \mu \text{s (d) Temperature (K) map at 100 } \mu \text{s (e) Temporal development of TMV at P1-P4 (f) Temporal development of pore density in Log-scale at P1-P4 (g) Temporal development of pore radius at P1-P4.}
\]
Temperature change during electroporation

The changes in the temperature during electroporation is measured using the Bio-Penes equation [42]-[43] which is defined as:

$$\nabla \cdot (k \nabla T) + \sigma |\nabla \psi|^2 + q'' - W_b c_bT = \rho c_p \frac{\partial T}{\partial t}$$  \hspace{1cm} (17)

where $T$ is the temperature (initially taken as 310.15 K), $W_b c_b$ is heat generated in the medium, $q''$ is the heat generated due to metabolic component (taken as zero here), $K$ is the thermal conductivity, $c_p$ is the heat capacity, $\psi$ is the potential developed, and $d$ is the conductivity of the cell membrane, cytoplasm, and nucleus. This equation has been used to observe the temperature developed in the cells during electroporation to analyze whether the thermal stresses developed exceed the safety threshold or not.

Mathematical modelling of dye uptake into cervical cells during RE

The cell membrane separates the extracellular and intracellular fluids and with the help of electrically mediated poration, dyes can be transported. Towards this, we consider Propidium Iodide (PI) dye for our computational work. The dye used for The concentration of dye throughout extracellular space is given by:

$$\frac{\partial C_E}{\partial t} = (\nabla \cdot (D_{eff} \nabla C_E)) - \left(1 - \frac{\epsilon}{\epsilon_c}\right) \left(\frac{\pi R_p^2 N_p}{V_c}\right) \times P \left[\exp\left(-t/\tau\right)\right] S F (C_E - C_{RE}) + \left(1 - SF\right) (C_E - C_{RE})$$  \hspace{1cm} (18)

where $C_E$ represents the concentration of dye or drug in the extracellular fluid which is taken as 0.01 mole/m$^3$. The $D_{eff} = 10^{-4}$mm$^2$sec$^{-1}$ is the
effective diffusion of the material in the extracellular fluid is proposed. Since RE numerical study observed the Survival Factor (SF) of the cell during electroporation is kept 1 the IRE part is cancelled out. The porosity of membrane \( \varepsilon \) is 0.18 and the resealing time constant of pores \( \tau = 5 \mu s \). The pore radius \( R_p = 0.8 \text{ nm} \) and \( P \) is the permeability of the drug and dye kept as \( 5 \times 10^{-4} \text{ mm/sec} \). \( V_o \) represents the volume of the cube in which the cell is kept which kept.

\[
\frac{\partial C_{RE}}{\partial t} = \left( \frac{\pi R^2 N_P}{V_o} \right) P [\exp(-t/\tau) - SF(C_E - C_{RE})]
\]

(19)

where \( C_{RE} \) represents the concentration of dye or drug in the \textit{intracellular} region of the cell. The initial conditions of \( C_E = 0.01 \text{ mole/m}^3 \) and \( C_{RE} = 0 \). The no flux boundary conditions are given as \( \frac{\partial C_{RE}}{\partial r} = 0 \) and \( \frac{\partial C_s}{\partial r} = 0 \). We studied the mathematical model proposed earlier [44] for dye delivery into the cells. The method used simple circle geometry of radius 30\( \mu \)m and used a 1 kV/cm electric field for 100 ms rectangular pulse with 1ms rise and fall time as proposed in the literature. We then used our single-cell cervical model to further simulate and check the results. Propidium Iodide (PI) dye has been considered in our simulation method.

The calculations are performed in COMSOL Multiphysics 5.5 a using the electric currents module (solution of the Laplace equations), the Weak form boundary PDE module (pore density calculation), the PDE interfaces module (polarization calculation and concentration of dye calculation) and the Bio-heat transfer module (thermal changes). The free triangular mesh strategy has been used with the Paradiso solver to solve the equations.

**Results**

Figures 2–4, plot (a) shows the cluster image with the selected points P1-P5,P4, P7. Plots (b) and (c) show the TMV (V) and the pore density respectively (computed at 50\( \mu \)s). The temperature map is plotted at 100 \( \mu \) s in plot (c) showing a profile that spans 310 K to 318K—which is not sufficient for cells to undergo ablation. Plots (e) and (f) show the temporal development of the TMV and the pore density. The TMV in figures 2(e), 3(e) and 4(e) show variations. The figures 2(e) and 3(e) represent clumps of cervical cells and various points are analysed. They show that the clumps are difficult to electroporate as many points have not reached the TMV above 1V but by observing figure 4(e) it is observed that TMV above 1V has been easily achieved for maximum point as they have isolated cells more than clumps. Similarly, if we analyze figures 2(f) and 3(f) and 4(f) pore density in log scale has been achieved above 14 in figure 4(f) but for 2(f) and 3(f), most points have not reached pore density in log scale above 14. Thus based on the results an idea can be easily achieved that clump cells need more electric field than scattered cells to achieve reversible electroporation. Figures 2(g), 3(g), and 4(g) represent the temporal development of pore radius (nm) at various points in the figures. With the proposed model validated, the plots in figures 2–4 present the results of the overlapping and isolated cells which have been reversibly electroporated. These plots also include the thermal profile, which shows that the temperature rise observed in the cells is not enough to cause ablation. The overlapping zones exhibit marginally lower levels of electroporation as cells behave as a single unit (a single large cell). A pulse strength of 10 kV/cm was also applied to the model for 40 ns in order to reversibly electroporate the cell to modulate the dye uptake by the cells. This is driven by the assumption that the dispersive model (using the Debye second-order expression) would have a lower threshold of the electric field as polarization is observed at these high-frequency levels—which is a reflection of dispersion in an electrical medium. Thereafter, a high-frequency pulse is applied to a mixture of cancerous and normal cervical cells. Figure 5(a) shows a mixture of superficial normal and cancerous cervical cells. The stages of cancer progression in the superficial cervical cells—CIN1, CIN2, and CIN3 are shown in the figure. These cells are introduced in a similar computational domain of 500 \( \mu \)m \( \times \) 500 \( \mu \)m. The electric field of 10 kV/cm for 40 ns is applied on the copper electrodes (described earlier). Figures 5(b)–(c) shows the TMV and pore density map of the membrane, captured at 20 ns. Figure 6(a) shows the TMV plot of four cells from P1-P4 where P1 is the point at the superficial cell, P2 is the point at first stage cancer cell stage CIN1, P3 is the point at second cancer cell stage CIN2 and P4 is the point at third stage cancer cell CIN3. We note that the rate of TMV evolution is faster in CIN3 (CIN3 > CIN2 > CIN1 > normal cells). Figure 6(b) shows that the pore radius evolution is faster in CIN3 and a similar order is observed. The pore density evolution shown in figure 6(c) exhibits a similar pattern. The rise in conductivity of the cell membrane is shown in figure 6(d). We also explored dye diffusion under electric stress through this high-frequency pulse application. The extracellular fluid was set up with a dye concentration of 0.01 mole/m\(^3\). The uptake of the dye in the cells at points P1-P4 are shown in figure 6(e), which highlights that CIN3 cells, due to ease of electroporation and with larger pore density, exhibit faster uptake compared to other cells. Figure 6(f) shows no considerable rise in the temperature of cells, but a relatively larger rise is observed in CIN3 cells. The polarization plot in figure 6(g) shows that the CIN3 cells undergo higher polarization at a faster rate. With the cellular clusters simulated containing normal and cancerous cervical cells, we note that cancerous cells exhibit a faster development of TMV and threshold pore density owing to an increase in conductivity and decrease in size with increasing
levels of neoplasia. The intake of drugs or dyes in cancerous cells is predicted to be higher than the normal cells due to higher pore density, as shown in figure 6(e). Figure 7 simulates the effect of electrically modulated dye uptake in the heterogeneous cluster. These colours represent an RGB map that is a function of the dye concentration in the cervical cells at 10 μs.

The concentration map shows that the dye intake by CIN3 cells of $2 \times 10^{-3}$ mole/m$^3$ and normal cell 0.0003 mole/m$^3$. Figure 8(a) compares the transmembrane potential generated by the single rectangular voltage pulse supplied as shown in figure 8(c). The TMV in the real cell model decays slower compared to the symmetric model (circle [10], Supergielis [13], and double shell model [33]) which almost showed a similar response to the pulse field. The pore density was also less compared to the other models shown in figure 8(b). Figure 8(d) shows the temporal development of the pore radius during reversible electroporation of the above-stated models. The results show that our model produced a greater development in pore radius compared to other models. Figure 8(e) shows
the polarization of the different models. The realistic cell shows more polarization compared to the symmetric cells. Aggregated, these simulation results seem to agree well with the existing models. Figure 9 shows the output results of the circle model and cervical cell model dye influx after the application of the electric pulse field. The intracellular and extracellular concentration is plotted with time for both models. Initially, the dye concentration is taken to be 0.01 mol/m$^3$. Both regions indicate a concentration of 0.002 mol/m$^3$ at 30 ms.

**Discussion**

This work begins by extracting the scattered, overlapping, and isolated contours from the EDF images of the cervical cells with image processing tools such as GMM, MSER, and joint-level set optimization. In our earlier work, we worked on single cervical cells and successfully extracted the contour [28]. The cells extracted for electroporation study have been reported in which scattered, isolated, and sparse neuroblastoma cells have been extracted successfully and electroporation multi-physics has been analyzed.
Our method extracts the contour of overlapping cells cervical cells of highly clustered formation. The usefulness of the study of clusters has been reported in many earlier works. The symmetric cell models produce a symmetric trans-membrane potential (TMV) distribution which is not the case in biological cells. In our study, we are able to study low-frequency electric fields and use a dispersive (change in permittivity and conductance with time) cell model to the clustered cervical cells to study the high-frequency electric field response. We also compared our work with the 2D circle model proposed in which a dispersive medium was proposed under the nanosecond electric field [10]. Then we simulated a parametric equation (Supergelis formula) to replicate the cervical cells and applied a rectangular pulse voltage for electroporation study [13]. We also used the double shell model as proposed earlier study and exposed it to the same rectangular pulse field [33]. We took one cell from the EDF image in figure 4(a) to compare it with these cell models. The method uses a multi-physics application to import the geometry of the cells and apply an electric field with high frequency on the cells for reversible electroporation. In a clustered orientation as shown in figure 4(a), it can be observed that the cells have not all cells have been properly electroporated. The cervical normal and cancerous cells close to the electrode have been easily electroporated. The mathematical model for dye uptake by the cervical cells is evaluated due to reversible electroporation in the cytoplasmic area. We studied the mathematical model proposed earlier [44] for dye delivery into the cells. We then used our single-cell cervical model to further simulate and compare the results plotted in figure 9. We then further used these equations and simulated multicellular cells using a nanosecond Gaussian pulse electric field as proposed above. Figure 6(e) shows the concentration of various cells for nanosecond electric field and used dispersive cells. The cancerous cells experience faster TMV development, higher pore density, and consequently, a faster uptake than the normal cells.

**Conclusion**

This agglomerative work relies on the extraction of two-dimensional contours of the overlapping and freestanding cervical cells with the help of an algorithm developed using the level set method. This was followed by a mathematical model to study RE—which was successfully validated against experimental data. The simulation study is done for cellular clumps of different orientations with a different number of cells and for isolated cells combined with overlapping cells. For every orientation, we observe that a threshold has been achieved for reversible electroporation proving the validity of the proposed model. The temperature analysis is done for all the models to highlight that the thermal envelope is not breached - which indicates a significantly low ablation likelihood. We subsequently apply a high-frequency pulse to the model of normal and cancerous cells. The cervical cells of various types including those at varying stages of neoplasia have been used. The simulations show that the normal cells exhibit a delayed and attenuated response compared to the rest. The use of dye delivery diffusion equation has also been used to show the molar uptake by the cells is modulated. The pore evaluation of normal and cancerous cells has been analyzed, showing faster evolution in cancerous and CIN cells as compared to normal. These results indicate a feasible mechanism that employs reversible electroporation for modulating dye uptake in cells. The experimental schematic employs a low-cost and simple nano-pulse generator, that has already been proposed [28]. We have been able to introduce a microdosimetry model in which a reversible electroporation study under a nanosecond pulse for the realistic contour of isolated, overlapping and scattered cervical cells was done and introduction of mathematical dye delivery equation into the simulation work. The paper tells about the importance of considering realistic situations in which the experiments are carried on. The realistic cell clusters along with a dispersive medium have been used to show the reality of the situation.
under a nanosecond pulse field are used to increase the accuracy of our model. The future work lies in proceeding with experimental work under the nanosecond pulse field and verifying our work. Another possible outcome of the experimental work using this model-driven approach could be to increase the screening accuracy of cervical cancer using reversible electroporation. The dyes could be transferred as shown in the microdosimetry work. The cancerous cells intake of dyes will be more than the normal cells hence under the microscope the morphological changes in the cells could be viewed. Thus in future, this work can be extended to cervical cancer screening.

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Data availability statement

http://mde-lab.aegean.gr/index.php/downloads

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Author declaration

MK developed the multi-physics model for single and overlapping cells using extracted contours, performed the multi-physics studies and developed the result figures under supervision of AM. AM did the major writing part and conceptualization part. All authors read and approved the final manuscript.

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References

[1] Kotnik T, Rems L, Tarek M and Miklavčič D 2019 Membrane electroporation and electroporationalization: Mechanisms and models Ann. Rev. Biophys. 663–91
[2] Miklavčič D and Puc M 2006 Electroporation, Wiley Encyclopedia of Biomedical Engineering (Wiley)
[3] Yarmush M L, Golberg A, Sersa G, Kotnik T and Miklavčič D 2014 Electroporation-based technologies for medicine: principles, applications and challenges Annu. Rev. Biomed. Eng. 16 295–320
[4] Kotnik T and Miklavčič D 2000 ‘Theoretical evaluation of the distributed power dissipation in biological cells exposed to electric fields Bioelectromagnetics 21 385–94
[5] Kotnik T and Miklavčič D 2006 Theoretical evaluation of voltage inducement on internal membranes of biological cells exposed to electric fields Biophys J. 90 480–91
[6] Kumar M and Mishra A 2023 Multiphysics analysis of reversible electroporation and electrodeformation of cervical cells using a nanosecond pulse generator IEEE Trans. Plasma Sci. 51 534–43
[7] De Angelis A, Denzi A, Merla C, André F M, Mir I M, Apollonio F and Liberti M 2020 Confocal microscopy improves 3d microdosimetry applied to nanoporation experiments targeting endoplasmic reticulum Front. Bioeng. Biotechnol. 8 552261
[8] Merla C, Paffi A, Apollonio F, Leveque P, d’Inzeo G and Liberti M 2011 Microdosimetry for nanosecond pulsed electric field applications: a parametric study for a single cell IEEE Trans. Biomed. Eng. 58 1294–302
[9] Krassovska W and Filev P D 2007 Modeling electroporation in a single cell Biophys J. 92 404–17
[10] Salimi E, Thomson D J and Bridges G E 2013 Membrane dielectric dispersion in nanosecond pulsed electroporation of biological cells IEEE Trans. Dielectr. Electr. Insul. 20 1256–65
[11] Puchar G, Miklavčič D and Kotnik T 2009 A time-dependent numerical model of transmembrane voltage inducement and electroporation of irregularly shaped cells IEEE Trans Biomed Eng. 56 1491–501
[12] Mescia L, Chiapperino M A, Bia P, Gielis J and Caratelli D 2018 Modeling of electroporation induced by pulsed electric fields in irregularly shaped cells IEEE Trans. Biomed. Eng. 65 414–23
[13] Chiapperino M A, Bia P, Caratelli D, Gielis J, Mescia L, Dermol-Černe J and Miklavčič D 2019 Nonlinear dispersive model of electroporation for irregular nucleated cells BioEM 40 131–42
[14] Smith K C, Gowrishankar T R, Esser A T, Stewart D A and Weaver J C 2006 The spatially distributed dynamic transmembrane voltage of cells and organelles due to 10-ns pulses: meshed transport networks IEEE Trans. Plasma Sci. 34 1394–404
[15] Joshi R P, Mishra A and Schoenbach K H 2008 Model assessment of cell membrane breakdown in clusters and tissues under high intensity electric pulsing IEEE Trans. Plasma Sci. 36 1680–8
[16] Denzi A, Camera F, Merla C, Benassi B, Consales C, Paffi A, Apollonio F and Liberti M 2016 A Microdosimetric study of electropulsation on multiple realistically shaped cells: effect of neighbours J. Membr. Biol. 249 691–701
[17] Chiapperino M A et al 2020 Experimental and numerical study of electroporation induced by long monopolar and short bipolar pulses on realistic 3D irregularly shaped cells IEEE Trans. Biomed. Eng. 67 2781–8
[18] Pavlin M, Pavačič N and Miklavčič D 2002 Dependence of induced transmembrane potential on cell density,
arrangement, and cell position inside a cell system IEEE Trans Biomed Eng. 49 605–12

[19] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebello M, Parkin D M, Forman D and Bray F 2015 Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012 Int. J. Cancer. 136 E359–86

[20] https://who.int/health-topics/cervical-cancer---tab1

[21] Das L, Das S and Chatterjee J 2015 Electrical bioimpedance analysis: a new method in cervical cancer screening. I. Med. Eng. 2015 636075

[22] Walker D C, Brown B H, Hose D R and Smallwood R H 2000 Modelling the electrical impedivity of normal and premalignant cervical tissue Electron. Lett. 36 1603–4

[23] Walker D C J, Brown B H, Smallwood R H, Hose D R and Jones D M 2002 Modelled current distribution in cervical squamous tissue Physiol Meas. 23 159–68

[24] Balidemaj E, de Boer P, van Lier A L, Remis R F, Stalpers L J, Westerveld G H, Nederveen A J, van den Berg C A and Crezee J 2016 In vivo electric conductivity of cervical cancer patients based on B maps at 3T Phys. Med. Biol. 61 1596–607

[25] Al Ahmad M, Al Natour Z, Mustafa F and Rizvi T A 2018 Electrical characterization of normal and cancer cells IEEE Access 6 25979–86

[26] Yang Y, Moser M A J, Zhang E, Zhang W and Zhang B 2018 Development of a statistical model for cervical cancer cell death with irreversible electroporation in vitro PLoS One 13 e0195561

[27] Mitsutake K, Satoh A, Mine S, Abe K, Katsuki S and Akiyama H 2012 Effect of pulsing sequence of nanosecond pulsed electric fields on viability of HeLa S3 cells IEEE Trans. Dielectr. Electr. Insul. 19 337–42

[28] Kumar M and Mishra A Reversible electroporation study of realistic normal and cancersous cervical cells model using, avalanche transistor-based nano pulse generator IOP, Biomedical Physics & Engineering Express http://iopscience.iop.org/article/10.1088/2057-1976/ac240b

[29] http://cs.adelaide.edu.au/~zhil/project/TIP_CellSegJointLS_2014/Dataset.zip

[30] http://mde-lab.aegean.gr/index.php/downloads

[31] Lu Z, Carneiro G and Bradley A P 2015 An improved joint optimization of multiple level set functions for the segmentation of overlapping cervical cells IEEE Trans. Image Process. 24 1261–72

[32] Perona P and MalikJ 1990 Scale–space and edge detection using anisotropic diffusion IEEE Trans. Pattern Anal. Mach. Intell. 12 629–39

[33] Guo F, Zhang L and Liu X 2020 Nonlinear dispersive cell model for microdosimetry of nanosecond pulsed electric fields Sci. Rep. 10 19456

[34] Vedaldi A and Fulkerson B 2008 An Open and Portable Library of Computer Vision Algorithms (http://vlfeat.org/)

[35] Hlaváč M 2002 V: Ten Lectures on Statistical and Structural Pattern Recognition (Kluwer Academic Publishers) (https://doi.org/10.1007/978-94-017-3217-8)

[36] Matas J, Chum O, Urban M and Pajdla T 2002 Robust wide baseline stereo from maximally stable extremal regions Proc. BMVC. Cardiff. U.K. 384–96

[37] Li C, Xu C, Gui C and Fox M D 2010 Distance Regularized Level Set Evolution and Its Application to Image Segmentation IEEE Trans. Image Process. 19 3243–54

[38] Retelj L, Pucihar G and Miklavic D 2013 Electroporation of intracellular liposomes using nanosecond electric pulses—a theoretical study IEEE Trans. Biomed. Eng. 60 2624–35

[39] Pakhomov A G, Kolb J F, White J A, Joshi R P, Xiao S and Schoenbach K H 2007 Long-lasting plasma membrane permeabilization in mammalian cells by nanosecond pulsed electric field (nsPEF) Bioelectromagnetics 28 655–63

[40] Joshi R P, Hu Q, Aly R, Schoenbach K H and Hjalmarson H P 2001 Self-consistent simulations of electroporation dynamics in biological cells subjected to ultrashort electrical pulses Phys. Rev. E 64 011913

[41] Garcia S G, Rubio R G, Bretones A R and Martin R G 2003 Extension of the ADI-FDTD method to Debye media, Antennas and Propagation IEEE Transactions 51 3183–5

[42] Khorasani A et al 2020 The effect of conductivity changes on temperature rise during irreversible electroporation Frontiers in Biomedical Technologies 3 178–85

[43] Davalos R V, Mir L M and Rubinsky B 2005 Tissue ablation with irreversible electroporation Ann. Biomed. Eng. 33 223–31

[44] Mondal N, Chakravarty K and Dalal L C 2018 Mathematical modelling of drug delivery in tissue cells using electroporation Appl. Conf. Proc. 1973 030017