Light-cell interactions in depth-resolved optogenetics

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Abstract: Light as a tool in medical therapy and biological research has been studied extensively and its application is subject to continuous improvement. However, safe and efficient application of light-based methods in photomedicine or optogenetics requires knowledge about the optical properties of the target tissue as well as the response characteristics of the stimulated cells. Here, we used tissue phantoms and a heart-like light-sensitive cell line to investigate optogenetic stimulation through tissue layers. The input power necessary for successful stimulation could be described as a function of phantom thickness. A model of light transmission through the tissue phantoms gives insights into the expected stimulation efficiency. Cell-type specific effects are identified that result in deviations of the stimulation threshold from the modelled predictions. This study provides insights into the complex interplay between light, tissue and cells during deep-tissue optogenetics. It can serve as an orientation for safe implementation of light-based methods in vivo.

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1. Introduction

Light has become a versatile tool in biological research and medical and cosmetic applications. Methods like photodynamic therapy (PDT) [1], low-level laser therapy (LLLT) [2], laser hair removal [3] and the research field of optogenetics [4] rely on specific light-tissue-interactions for targeted treatment. One advantage of using light for manipulation of biological systems is the high spatio-temporal precision of its application to the target area. Especially for optogenetic control of nerve or muscle cells, the increased precision of the light-based method compared to a traditional electrical approach has opened up new possibilities for basic research [4–6]. A number of devices for the application of this method with in vivo-models has been developed [7–9]. These devices are designed to provide controlled light stimuli with the required input power. Yet, the most efficient combination of beam shape and power is difficult to estimate and has to be determined empirically. Many theoretical and experimental approaches exist to describe light distribution in biological tissue [10–14]. Simulations can be built on extensive studies of the scattering characteristics of different tissue types [15,16] to provide an informed estimate. While these methods are useful to track the average scattering path and correspondingly the intensity of the traversing light throughout the tissue, they do not necessarily allow for conclusions about
successful cell stimulation. Whether or not a cell is activated by a light stimulus depends on the incident light intensity as well as cellular characteristics, interactions between the tissue components and the tissue’s scattering behavior. Simulations and experiments show that the intensity can increase close to the tissue surface due to backward scattered photons and refraction at tissue interfaces [14,17]. Several studies have found that interference between photons can change the light intensity within a tissue as well [18–20]. However, it is not known whether interference and multiple scattering events in deeper tissue layers also affect the cellular reaction to a light stimulus. Experimental studies are further complicated by the limited reproducibility of the optical properties of natural tissues. All phenomena and interactions that are strong enough to activate a light sensitive cell need to be taken into account for optimal cell stimulation. If the input power is too high, uncontrolled activation and phototoxicity may occur. Insufficient intensity on the other hand will not yield the desired outcome. Therefore, an experimental assessment of the light-cell-interaction in deeper tissue layers is critical to optimize optogenetic applications.

In this study, we give insights into depth-resolved optogenetics. Tissue phantoms were used to reproducibly mimic the optical properties of native tissues. A light sensitive HL-1 cardiomyocyte cell line was used to investigate the effect of increasing the thickness of the artificial tissue. Two different modes of illumination were combined with two read-out modalities to investigate the importance of the input beam characteristics and avoid measurement biases. Light transmission in the forward direction was modelled based on Kubelka-Munk theory. The effect of a varying illumination area was investigated without tissue phantoms. The input power necessary for cell stimulation was evaluated for each setting, and was shown to not only depend on the tissue phantoms, but on cell-type specific characteristics as well. We show that optogenetic stimulation beneath the tissue surface is possible with low input power and that a complex interplay of light-tissue, light-cell and cell-cell effects plays an important role for estimating the input power required to stimulate heart-like cells.

2. Materials and methods

2.1. Cell culture

HL-1 cells [21] transfected with Channelrhodopsin-2 H134R and eYFP (HL-1-ChR2) have been used for optical stimulation before [22]. The cells were cultured according to a standard protocol for HL-1 cells provided by Merck Millipore [23]. Culture ingredients were supplied by Merck as well (Merck Millipore, Germany). Cells were split 1:3 at confluency, every 4-5 days, and kept in an incubator at 37°C and 5 % CO2. For stimulation experiments, 105 cells (passage number 28-33) were seeded on tissue phantoms or microelectrode (MEA) chips (60MEA200/30iR-Ti-gr, Multi Channel Systems MCS GmbH, Germany) coated with fibronectin/gelatin (5 µg/mL fibronectin + 0.02 % gelatin) and cultured in the incubator for three days.

2.2. Tissue phantoms

Tissue phantoms were designed to mimic optical properties of natural tissues as well as provide a robust and reproducible system for cell stimulation experiments. Phantoms were manufactured from PDMS (Sylgard 184, Dow Corning, USA) with defined thicknesses of 0.5, 1, 1.5, 2, 2.5 or 3 mm and a diameter of 19 mm. Titanium dioxide particles (<5 µm, rutile, Sigma Aldrich, USA) were mixed by hand with the silicone at a concentration of 1.5 mg/mL. TiO2 particles are frequently used as scatterers in tissue phantoms and other contexts [24]. The mixture was degassed in a desiccator, poured into the respective mold, and hardened at 60°C for three hours.

Optical characterization of the tissue phantoms was performed with an UV/Vis-spectrophotometer with an integrating sphere (beam aperture 2x10 mm, Lambda 1050, Perkin Elmer, USA). Three phantoms with thickness \( z = 2 \text{mm} \) were measured at 400-500 nm and averaged. Total forward scattering, total side scattering, remaining scattering of the system and total absorption were...
determined in different configurations (Fig. 1). The obtained measurements were then divided by the input power to derive relative absorption ($\tau_{\text{abs}}$), transmission ($\tau_{\text{st}}$) and side scatter ($\tau_{\text{ss}}$), as well as $\tau_{\text{sr}}$ for the remaining scatter. The standard deviation of the three samples was $<7.5\%$ over the whole range for the scattering parameters $\tau_{\text{st}}$ and $\tau_{\text{ss}}$, and $<3\%$ for $\tau_{\text{abs}}$.

According to ISO 19962:2019, total forward scattering at 450 nm was calculated based on the measured parameters:

$$S_{\text{for}} = \frac{\tau_{\text{ss}} - \tau_{\text{st}} \cdot \tau_{\text{rs}}}{1 - \tau_{\text{rs}}}$$  \hspace{1cm} (1)

$S_{\text{sca}}$ was calculated to account for the proportion of total incident light that was not scattered in forward direction:

$$S_{\text{sca}} = \frac{S_{\text{for}}}{\tau_{\text{st}}}$$  \hspace{1cm} (2)

For an estimate of the phantoms’ absorption ($\mu_a$) and reduced scattering coefficients ($\mu'_s$), the Beer-Lambert law was applied:

$$\mu_a = -\frac{\ln(\tau_{\text{abs}})}{d} = 0.123 \text{ mm}^{-1}$$  \hspace{1cm} (3)

$$\mu'_s = -\frac{\ln(1 - S_{\text{sca}})}{d} = 1.146 \text{ mm}^{-1}$$  \hspace{1cm} (4)

where $d$ is the phantom’s thickness. These values are in accordance with those determined for native tissues such as muscle or liver tissue [15].

2.3. Experimental setups and stimulation experiments

An optical setup was designed that allowed for simultaneous blue-light stimulation with a 450 nm laser diode, fluorescence imaging and electrophysiological recordings with a MEA-system. Experiments were carried out with two different modes of illumination and two different read out methods. This allowed for an evaluation of the effect of illumination mode and quality and a comparison of fluorescence and electrophysiology data.
In the first configuration, light from the laser diode was coupled to a 200 µm glass fiber (NA = 0.22; LifePhotonic, Germany) (Fig. 2(a)) which illuminated the sample space from below. The diameter of the illumination cone was approx. 210 µm at the well bottom. HL-1 cells were grown on coated PDMS phantoms. Fluorescence imaging was used to visualize calcium fluctuations in the cells with a red calcium-sensitive dye (Cal-630 AM, 5 µM, Biomol GmbH, Germany) and a sCMOS-camera (exposure time 300 ms, Andor Zyla 5.5; Andor Technology Ltd, UK). The Cal-630 dye was chosen in combination with an orange LED (590 nm) for excitation which does not affect ChR-2 activity [25]. The camera’s exposure time was adjusted so that the baseline fluorescence was still visible but maximum fluorescence upon stimulation did not result in overexposure.

Fig. 2. Setup for cell stimulation experiments. (a): Setup for fiber-based stimulation and fluorescence readout. Light from the laser diode is coupled into a glass fiber (F) and directed to the phantom from below. Cells are grown on top of the phantom. The inset shows the configuration of cell stimulation. An LED and a camera are used for fluorescence-based calcium imaging. (b): The same setup modified for MEA measurements. Cells are located directly on the MEA chip (M), and covered with a silicone phantom (inset). Light from the laser diode (450 nm) is regulated by the λ/2 plate and a polarizing beam splitter (PBS) and then directed through the objective (O) onto the chip. At the same time, an LED (590 nm) can be used to excite fluorescence, imaged with a camera (C). A power meter (PM) is used to control the laser power for cell stimulation. A shutter (not shown) was placed between the PBS and the following mirror.

For MEA-experiments, the beam of the laser diode was directed onto the sample from above through the objective (40x, water immersion, NA = 0.8, f = 4.125mm; Zeiss, Germany) (Fig. 2(b)). The beam was nearly collimated behind the objective, with a diameter of approx. 150 µm. HL-1 cells were seeded on the MEA-chips. The phantoms were cut into rectangles and placed on top of the cell layer. The tissue phantoms on the MEA chips were carefully exchanged in the course of the experiments, avoiding cell detachment. Consequently, the same cells were used with different phantoms in the MEA experiments, while each phantom was seeded individually for the fluorescence experiments.
For cell stimulation, the shutter frequency was set higher than the cells’ spontaneous contraction frequency. Opening time of the shutter was fixed at 50 ms. The power was controlled with a power meter positioned perpendicular to the polarizing beam splitter. For each phantom thickness, laser power at the power meter was decreased in steps of 0.2 mW (corresponding to an increase of approx. 0.05 mW behind the objective) with a λ/2-plate until pacing was observed by at least ten consecutive pulses, which was defined as reliable activation. The respective power value behind the objective was recorded for each experiment. To avoid distortion of the observations by photobleaching in the fluorescence setup, a new area on the phantom was illuminated each time the laser power was increased.

2.4. Beam expansion experiments

The effect of the beam diameter on stimulation thresholds was investigated by removing a collimating lens from the MEA-setup \( f = 125 \text{mm} \), resulting in a focused beam with high divergence and small Rayleigh range behind the objective. The threshold power was then determined at different locations in the beam path using MEA-recordings (see Fig. 3(a)). To relate the threshold values to the illuminated area, the beam diameter at each measurement location was estimated from the Gaussian beam propagation (see Figure 3(b)). The beam waist \( w_0' \) behind the objective was calculated from

\[
 w_0' = w_{0R} = w_0 \frac{f}{\sqrt{(s-f)^2 + z_R^2}} \tag{5}
\]

where the laser’s beam waist \( w_0 \) was estimated from \( w_0 = \frac{\lambda}{2\theta} \) with \( \theta = 0.5 \text{mrad} \), \( z_R \) is the Rayleigh range, \( f = 4.125 \text{mm} \) is the objective’s focal length and \( s = 1400 \text{mm} \) is the distance from the laser to the objective. The beam radius \( w(z)' \) at any distance \( z \) from the laser focus was calculated by

\[
 w(z)' = w_0' \sqrt{1 + \left(\frac{z}{z_R}\right)^2} \tag{6}
\]

The resulting values for beam radius and cross sectional area should be regarded as estimates as they are based on the assumption of an ideal Gaussian beam. However, they provided a reasonable basis for relative comparisons of threshold intensities during cell experiments.

![Fig. 3.](image)

2.5. Data analysis

Fluorescence data was analyzed in ImageJ [26] and quantified with a custom macro. Three active cells within the illuminated area were chosen by hand, and a reference area without cells was
marked as background (Fig. 4(a)). The average grey value was determined for each cell and is given as the relative fluorescence intensity by

$$\Delta F = \frac{((F_i - B_i) - (F_0 - B_0))}{(F_0 - B_0)}$$

where \(F_i\) and \(B_i\) are the mean grey values of the \(i^{th}\) image of the cell (\(F\)) or the background (\(B\)), and \(F_0\) and \(B_0\) are the mean grey values of the first recorded image. The low-intensity laser pulse was not visible in these experiments. The results were plotted against recording time to illustrate the cellular reaction to laser stimulation (Fig. 4(e)). The peak-to-peak time interval was compared to the stimulation interval set by the shutter. A reference experiment was conducted with increased power to visualize the laser pulse and confirm the temporal connection of illumination and cellular reaction. MEA data was analyzed with the Multi Channel Analyzer software (Multi Channel Systems MCS GmbH). The time interval between action potentials

**Fig. 4.** Analysis of stimulation data. (a): Cells were chosen by hand and their gray values were evaluated over time. Scale bar 100 \(\mu\)m. (b)-(d): Evaluation of the marked area in (a). (b) and (c) were taken 300 ms apart, showing a fluorescence increase in the regions of interest that had previously been illuminated. The image shown in (d) was created to increase contrast by subtracting image (b) from (c). Scale bars 50 \(\mu\)m. (e): Time course of the cells’ relative fluorescence intensity. Blue arrows mark the laser pulse, which was applied manually. The baseline decreases due to photobleaching of the dye. (f): Plot of an excerpt of a MEA-recording from an electrode showing the Becquerel-effect. (g): Excerpt of a MEA-recording from a different electrode showing field potentials. The amplitudes of the field potentials are substantially smaller than those caused by the Becquerel-effect. The time interval between the peaks could easily be determined within the corresponding software.
could be identified directly from the recorded data. The temporal correlation was confirmed by evaluating electrodes that were directly hit by the laser pulse, as these electrodes showed the Becquerel effect—a voltage evoked by a photoelectrochemical effect, leading to measuring artifacts [27,28]. The respective amplitude was clearly distinguishable from the smaller field potentials (Figs. 4(f) and 4(g)).

2.6. Fitting and modelling

Quantitative fits were calculated to predict the laser power necessary for cell activation depending on phantom thickness, based on fluorescence data and MEA data, respectively. Calculations were performed with Origin 2019. To estimate the proportion of collimated ($T_c$) and diffuse transmission ($T_d$) behind the phantom as well as the diffuse reflection ($R_d$) within the phantom, the Kubelka-Munk terms from [29] were rearranged to calculate $T_c$, $T_d$ and $R_d$:

$$T_c = e^{-(\mu_a + \mu_s) \cdot d}$$  \hspace{1cm} (8)
$$T_d = \sqrt{1 + R_d^2 - 2aR_d}$$  \hspace{1cm} (9)
$$R_d = \frac{a - b - a \cdot e^{2Sbd}}{e^{2Sbd} - (a - b)^2} - \frac{e^{2Sbd} - 1}{e^{2Sbd} - (a - b)^2}$$  \hspace{1cm} (10)

with

$$S = \frac{3\mu_s \cdot (1 - g) - \mu_a}{4}$$  \hspace{1cm} (11)
$$a = \frac{2\mu_a}{S}$$  \hspace{1cm} (12)
$$b = \sqrt{a^2 - 1}$$  \hspace{1cm} (13)

The absorption coefficient $\mu_a$ and the reduced scattering coefficient $\mu'_s$ from the phantom characterization (section 2.2) were used for the calculations. The anisotropy factor $g$ was estimated at $g = 0.94$, based on measurements from [30], resulting in a scattering coefficient of $\mu_s = \frac{\mu'_s}{(1-g)} = 19.1 \text{mm}^{-1}$.

3. Results

3.1. Correlation of calcium imaging signal and electrophysiology

To ensure that both readout modes reflect the same cellular reaction, an experiment with simultaneous calcium imaging and MEA recording was conducted using the setup in Fig. 2(b) without tissue phantom. Figure 5 shows the temporal correlation of both the $\Delta F/F_{\text{peak}}$ and the field potential with the laser stimulus. Therefore, both calcium fluctuations and membrane potential are indicators of the cells’ reaction to light and it is sufficient to observe either one. In all following experiments, only one readout method was used. Photobleaching of the dye resulted in a decline of $\Delta F/F$ over time.

3.2. Optogenetic stimulation through phantoms—calcium imaging

To evaluate fiber-based cell stimulation, experiments were conducted with the calcium imaging setup shown in Fig. 2(a) using tissue phantoms with a thickness of 0.5, 1, 1.5, 2, 2.5 and 3 mm and without tissue phantom. Three experiments per phantom thickness were performed. Cell growth on the phantoms was normal compared to growth in a T25 cell culture flask. The power thresholds for reliable pacing are plotted against phantom thickness in Fig. 6(a). Although the variability of the absolute values between the experiments was high, the general trend was reproducible. This was especially true for the initial dip of the curve.
3.3. Optogenetic stimulation through phantoms–field potential measurements

Field potential measurements with MEAs (see Fig. 2(b)) were conducted using tissue phantoms with a thickness of 0.5, 1, 1.5 and 2 mm and without tissue phantom. Cells were grown on the MEA chips to high confluency in the chip’s center to ensure an effective multi-electrode readout. Five experiments were performed with each phantom. One series of experiments yielded extraordinarily small values and was excluded from further analysis, resulting in four measurements per phantom thickness for analysis.

Figure 6(b) shows the power thresholds for reliable pacing depending on phantom thickness. When a power threshold was reached, the rhythm set by the light pulse was visible on all recording electrodes, not only the ones that were directly illuminated. Measurements from the individual experiments showed large variations. However, the overall trend within each series was reproducible and comparable to the data obtained from fluorescence experiments. It has to be noted that for MEA-measurements, all data from one series (color coded in Fig. 6) was obtained with the same cells. The general trend, including the initial decrease of the threshold, is visible in each of the series although the absolute values differ largely. The values from individual experiments always appeared in the same order for each phantom thickness, emphasizing the reproducibility of the trend in spite of variations of absolute values. It is therefore more informative to compare the relative change of the stimulation threshold within the experimental series. Figure 6(c) shows the mean and individual power thresholds for each phantom thickness relative to the respective first measurement, which was set equal to 1. Standard deviations show that the development of the stimulation threshold with tissue depth varied considerably less than the absolute measurement values.

3.4. Model of transmission and power threshold

The proportion of light that should be expected to be transmitted or reflected was calculated as a function of phantom thickness based on the Kubelka-Munk theory and the optical characteristics.
Fig. 6. (a): Power thresholds for fiber-based cell stimulation and fluorescence readout plotted against phantom thickness. Mean values and standard deviations are shown. Although there is some variation in the absolute values, the overall course was highly reproducible. The initial decrease of the power threshold when a thin phantom was used was observed in each experiment. (b): Power thresholds for direct cell stimulation with MEA-readout plotted against phantom thickness. Data from the different experimental series vary considerably. (c): Power thresholds for cell stimulation in MEA-setup normalized to the respective measurement without tissue phantom. Black circles represent mean values with standard deviation. Smaller deviations as compared to (b) indicate that the dependence of the stimulation threshold on phantom thickness is less variable than the absolute values. Connecting lines in (c) are drawn for orientation and do not represent continuity of the x-variable.

of the phantoms produced during this study (see section 2.6). Notably, the calculated collimated transmission \(T_c\), i.e. the proportion of light that is expected to be transmitted by a phantom without undergoing a single scattering event, declined rapidly and was negligible for phantoms thicker than 0.25 mm. The diffuse transmission \(T_d\) showed a slower exponential decay, while the proportion of diffuse reflected light \(R_d\) increased with phantom thickness (Fig. 7).

The average threshold values obtained from both the calcium imaging and MEA-measurements were fitted to an exponential function to facilitate quantitative estimates concerning the development of stimulation thresholds with tissue depth. Fitted equations (dashed lines) and coefficients of determination are given in Fig. 8. The model shown in Fig. 7 was used to estimate the expected course of the stimulation power threshold for the calcium imaging experiments. Only the diffuse transmission \(T_d\) appeared to be relevant for stimulation of cells behind phantoms thicker than 0.25 mm. Therefore, its decay should give an estimate of how much the input power would have to be increased with increasing phantom thickness to achieve sufficient intensity after the phantom
Fig. 7. Collimated transmission \( (T_c) \), diffuse transmission \( (T_d) \) and diffuse reflection \( (R_d) \) calculated from Kubelka-Munk terms and the optical characteristics of the tissue phantoms. Absorption and direct reflection are not depicted.

for successful stimulation. This expected increase of the power threshold is shown in Fig. 8(a) as \( \frac{1}{T_d} \) (grey dotted line). The initial dip observed in the experimental data was not present in this model, and the difference between calculated and observed threshold values increased with phantom thickness.

Fig. 8. Quantification of stimulation power thresholds as a function of phantom thickness. (a): Data from calcium imaging. The dotted line represents \( \frac{1}{T_d} \) (see Fig. 7), multiplied by the observed power threshold without phantom. (b): MEA data. Fitted equations are shown as dashed lines.

3.5. Beam diameter

The MEA setup was used to determine the input power depending on the beam diameter. The power threshold for cell activation was highest when the sample was positioned in the focal plane (Fig. 9(b)). With increasing beam diameter, the input power threshold initially decreased and subsequently increased at a distance of approx. 1 mm from the focal plane. The illuminated area \( A \) was estimated from the calculated beam diameters (see section 2.4, Fig. 9(a)) to determine the threshold intensity \( I = \frac{P}{A} \) (Fig. 9(c)). This analysis shows that the threshold intensity in the focal plane was more than five orders of magnitude higher than at larger distances. Cell stimulation
with a focused beam was therefore highly inefficient. It must be noted, however, that the absolute intensity values are based on the simplified assumption of an ideal Gaussian beam and should be evaluated with care. The intensity threshold decreased rapidly with increasing area, and appeared to remain constant at approx. 1.5-2 mm distance from the focus. This corresponds to an illuminated area of 0.2-0.4 mm$^2$ and diameter of 500-700 µm.

4. Discussion

The tissue phantoms used in this study were manufactured to mimic the optical properties of natural tissues. The reduced scattering coefficient was similar to that of heart or muscle tissue, while the absorption coefficient was modeled after a generic tissue with average contents of water, fat and blood [15]. These parameters were calculated according to the Beer-Lambert law. However, as this is a highly simplified method to determine these parameters for scattering phantoms [31,32], the values can only be regarded as estimates. Also, it has to be noted that while the absolute parameters are similiar, the strong backscatter of the TiO2 particles may not match the true scattering properties of native tissues. Importantly for this study, the phantoms were easy to fabricate and had highly reproducible optical properties. They could be coated and used for cell culture without further modifications. Particles leaking from the phantoms were not observed.

The data obtained from cell experiments presented large variations. Especially from the MEA measurements, where the same cells were used with different phantoms, it was clear that
these variations were mostly based on the different batches of cells used for each experimental series. Although all cells had similar passage numbers, some batches required consistently higher stimulation powers than others. Small variations in ambient variables such as temperature or humidity cannot be fully excluded either. The overall trend however was reproducible between different cell batches as well as different methods of stimulation and measurement (see Fig. 6).

Surprisingly, in each series of experiments, the power threshold was lower with a thin phantom (0.5-1 mm) than without a phantom. Such results could have been expected during the study of irradiated tissues, where the light intensity is known to increase due to photons scattered backwards from deeper layers [14,17]. In the present study however, the cells were situated behind the tissue phantoms as opposed to within them. Backscattered photons could therefore not be expected to alter the stimulation threshold. Kubelka-Munk calculations predicted that even after passing through the thinnest phantoms (0.5 mm), virtually all photons would have been scattered at least once and collimated transmission ($T_c$) would be neglectable (see Fig. 7). This would expand the incident laser beam and thus increase the number of illuminated cells. Due to an effect that is inherent to heart muscle cells, this increase was assumed to be the cause of the observed reduction of the pacing threshold by thin phantoms (see below). The stimulation threshold increased with thicker phantoms both in the experimental results and the model predictions (see Figure 8), although the predicted threshold was consistently higher than the observed values. The absolute values presented in the model are based on simplified measurements and assumptions about the phantoms’ anisotropy, but the actual values for real tissues can be expected to range within the same order of magnitude.

The pacing threshold during optogenetic stimulation of whole hearts has been shown to decrease with larger illumination areas [33,34]. Therefore, stimulation experiments without phantoms with different beam diameters were carried out to investigate the influence of the illuminated area independently of scattering. The results clearly showed that small beam diameters (less than the size of a single cell in the focal spot) were considerably less efficient during cell stimulation (see Fig. 9(c)). With increasing irradiation area, the intensity threshold declined, and appeared to remain constant when the illuminated area was approx. 0.2-0.4 mm$^2$ (corresponding to 1.5-2 mm distance from the focus and a diameter of 0.5-0.7 mm). This spot was expected to irradiate approx. 300-600 cells based on the estimated cell size of 600-700 $\mu$m$^2$. From these results, it can be concluded that a larger number of synchronously illuminated HL-1 cells required less input power for effective stimulation. This observation is consistent with the lower stimulation thresholds obtained with the fiber setup, were the illuminated area was larger than in the MEA setup. However, the discrepancy between the diffusely transmitted light and the observed pacing thresholds, the former of which does not display the initial dip (see Fig. 8(a)), indicates that the area increase alone is not sufficient to explain the results. The phenomenon can be well explained by considering the electrical syncytium of cardiomyocytes and the HL-1 model used in this study. The cells are electrically coupled through gap junction to form an electrical syncytium [22]. This is corroborated by the field potential measurements with MEA which showed that once the threshold power for stimulation was reached, all cells on the MEA chip were synchronized to the new frequency. Thus, the threshold determined here has to be attributed to activation of the whole electrical syncytium of cardiomyocytes rather than to activation of one single cell. It has been shown before in simulations that in electrically coupled heart tissue, a minimum number of synchronously depolarized cardiomyocytes (termed the "source") is required to synchronize activity of adjacent non-depolarized cells (termed the "sink") with strong repolarization $K^+$ conductances at rest [35]. Otherwise, a source-sink mismatch occurs and the stimulated activity ceases to propagate. In the heart, this mechanism prevents spontaneous diastolic depolarization in a few cardiomyocytes from evoking a pro-arrhythmic extrabat. Previous models have shown that a large depolarizing current can partly compensate for a small source [35]. Analogously, in the present study, the ChR-2 induced depolarization occurring in the illuminated HL-1-cells ("source")
must be large enough to also depolarize adjacent cells ("sink"), which are not illuminated but electrically coupled to the source. Only a large enough source current can evoke synchronized activity. The focused laser beam stimulated only few cells, and the resulting ChR2-current was not large enough to depolarize the adjacent cells. When the beam was expanded, and thus the number of cells acting as "source" increased relative to the surrounding sink, less input power was necessary for initiation of the depolarization wave. Likewise, this effect was likely the cause for the lowered power threshold caused by thin tissue phantoms (see above). High scattering and low absorption expanded the beam traveling through the phantoms and thus increased the effectively illuminated area. This effect is also reflected in the observation that the MEA-setup, were the stimulating beam was smaller, the power thresholds were higher as well. Overall, the two setups did not show distinct differences, indicating that a scattering medium quickly compensates for any differences in beam quality, such as speckle patterns or divergence angle.

This study provides insights into the interplay of physical (beam expansion by scattering) and biological (electrophysiology of heart cells) effects during stimulation of optogenetic tissues. As the beam diameter behind the tissue phantoms could not be precisely determined, it remains unclear whether attenuation within the phantoms or compensation for the expanded beam played a larger role for the increasing power threshold values. It can be expected however that the increasing number of stimulated cells (i.e. the expanding source) would at least partially compensate for light attenuation within the tissue phantoms. This assumption is underlined by the initial dip of the threshold values with thin phantoms and the deviations of the experimental observations to from the Kubelka-Munk calculations.

The present study emphasizes the relevance of tissue optics not only considering light attenuation, but especially deflected photons. Subsequent studies should expand the model used here to investigate two important factors that are present in native tissues: 1) When a volume of optogenetic tissue is illuminated, the source expands in three dimensions. The effects of the new size of source and sink should be quantified; 2) Backscattering effects are expected to further reduce the input power necessary for stimulation in a specific plane, as the intensity is increased by backscattered photons and/or interference. The magnitude of these effects depends on tissue characteristics and should be quantified in conjunction with the afore mentioned cell-cell-interactions. Reproducible tissue phantoms would provide a reliable basis for such studies as well to minimize the variability of optical properties that is inherent to native tissues. Furthermore, studies using such a well-controlled setting can highlight cell-specific and differences in the stimulation characteristics of cells of different origins.

Developing a theory that accounts for both the attenuation and expansion of the incident light and the biological factors within the target tissue will be challenging. However, such a theory could provide a comprehensive model to choose the best illumination strategy for the optical stimulation of tissues.

5. Conclusion

Controlled optogenetic stimulation in native tissues depends on cell and tissue characteristics that are difficult to predict. The available literature provides valuable estimations of the intensity distribution within a tissue. Our study highlights that apart from light and intensity distribution, cell-specific effects must be considered to adjust the illumination strategy. Knowledge of a tissue’s optical characteristics, specifically the extend of side and backscattering, the established scattering theories as well as tissue-specific cell-cell-interactions is required to optimize an experimental setup for deep-tissue optogenetics.

This study provides an estimate of how cell activation develops with tissue depth during optogenetic stimulation. While the results cannot be directly transferred to a less controlled in vivo setting, it provides valuable insights into the complexity of the light-cell-interplay and
the resulting implications concerning the setup and interpretation of deep-tissue optogenetic experiments.

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**Disclosures**

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