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14. ABSTRACT Radiofrequency (RF) radiation is in the frequency range in which the outer membrane of mammalian cells is no longer a barrier to electric field penetration, allowing access of the RF to subcellular structures. The biological effect of RF radiation, in particular high power, wideband radiation or ultrashort electrical pulses, is the topic of an AFOSR-administered MURI research program with the PI of this proposal serving as PI of the MURI. Experimental studies in which human cells were exposed to pulsed electric fields of up to 300 kV/cm amplitude, with durations as short as 10 ns, have confirmed that these pulses have strong effects on subcellular structures. In order to identify all of the intracellular targets of these short-pulse electric fields, we must conduct live cell imaging with very high temporal resolution. An instrument has been developed for exactly this type of live-cell imaging. This new instrument scans 1000 microbeams across the cell simultaneously using a spinning disk and can generate images at a rate of 350-1000 frames per second. Reality limits the speed images can be captured to the readout speed from the camera to the computer, but image acquisition at over 120 frames per second is still possible for a 300X250 pixel image. Using organelle-specific fluorescent dyes, we are able to image the live cell response of specific organelles, as well as calcium concentration and membrane potential at a resolution of 8 milliseconds avoiding the phototoxicity problems of conventional laser-scanning confocal microscopy. First experiments on the effects of pulsed electric fields on the generation of reactive oxygen species and calcium release have demonstrated the importance of this new, high resolution of this diagnostic system.

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Abstract

Radiofrequency (RF) radiation is in the frequency range in which the outer membrane of mammalian cells is no longer a barrier to electric field penetration, allowing access of the RF to subcellular structures. The biological effect of RF radiation, in particular high power, wideband radiation or ultrashort electrical pulses, is the topic of an AFOSR-administered MURI research program with the PI of this proposal serving as PI of the MURI. Experimental studies, in which human cells were exposed to pulsed electric fields of up to 300 kV/cm amplitude, with durations as short as 10 ns, have confirmed that these pulses have strong effects on subcellular structures. Initial results on the breaching of intracellular granule membranes without permanent damage to the cell membrane were published in 2001 and 25 more papers exploring this phenomenon have been published since then. In order to identify all of the intracellular targets of these short-pulsed electric fields we must now conduct live cell imaging with a very high temporal resolution. A DURIP grant has allowed us to acquire an instrument which has recently been developed for exactly this application of rapid live-cell imaging. It is the PerkinElmer UltraView RS Spinning Disk Confocal Imaging System. Instead of scanning a single laser beam across the cell, this new instrument scans 1000 microbeams simultaneously using a spinning disk and can generate images at a rate of 350-1000 frames per second. In practice, we are limited in the speed images can be captured by the readout speed from the camera to the computer. However, image acquisition at over 120 frames per second is still possible for a 300X250 pixel image. Using organelle-specific fluorescent dyes we will now be able to image the live cell response of mitochondria, endoplasmic reticulum, nucleus, calcium concentration and membrane potential with a resolution of 8 milliseconds while avoiding the phototoxicity problems of conventional laser-scanning confocal microscopy. Two examples for the use of this system in bioelectrics are discussed in the following. One is the detection of reactive oxygen species in melanoma cells caused by nanosecond pulses; the second is the temporary calcium response of Jurkat cells to nanosecond pulses. The latter is the topic of a thesis, which was successfully defended on June 27, 2006.

The Concept of an UltraView Spinning Disk Confocal Imaging System

The technique of using a spinning disk impregnated with holes to transfer an image as an electrical signal was discovered in 1884 by Paul Nipkow. The term Nipkow disk refers to a type of scanning disk with multiple, symmetrically placed spirals of pinhole apertures through which illumination light is passed and split into multiple 'minibeams'. When spun, the light scans the sample in a raster pattern. Light emitted from the sample can be detected to form an image of the sample.

It was not until 1968 that Nipkow disk technology was integrated into a microscope by Petran and co-workers in Prague and New Haven. Early Nipkow disk-based confocal systems suffered from the reduction in illumination that occurs by placing an opaque disk containing pinholes in front of the light source. In order to make the images confocal, the pinholes on a Nipkow disk need to be placed up to 10 diameters apart. This, in turn, means that they only transmit 1% of the light shining on them onto the sample, which was not ideal for many biological applications.

In the UltraView RS, PerkinElmer has overcome this problem by using a scanhead which includes a collector disk containing microlenses placed in front of the Nipkow disk. The microlenses--one for each pinhole—ensure that most of the light illuminating the disk is focused onto the pinholes. Transmission efficiency is increased from 1% to nearly 70% percent of the light falling on the disks, allowing the sample to be illuminated with a sufficient quantity of light. As a result of this advance, the spinning disk confocal is now the technology of choice for real-time imaging of living cells.

In the UltraView RS, 20,000 pinholes are arranged in an elliptical pattern across the disk. Since 1000 of these are illuminated at any one time, the sample is excited by 1000 minibeams. Microlenses in the upper disk, aligned exactly with the pinhole apertures of the lower disk, focus collimated light from the laser on to corresponding pinholes in the lower disk

In the UltraView RS, the excitation light is focused onto the sample through the objective of the microscope. Thus, instead of one beam being shone point-by point across the sample as done in a conventional LSCM, the whole field is illuminated at any one time by over a thousand minibeams.

Fluorescent light from the sample returns along the same path through the objective lens and pinholes, which exclude light from above and below the plane-of-focus, generating a confocal image. Fluorescence emission light is split from any reflected excitation light by a dichroic mirror through a relay lens to the imaging point in a camera. The upper disk containing microlenses is mechanically connected to the lower disk containing pinholes, and both are rotated, thus raster-scanning the sample. As there is multipoint excitation, light is emitted from the whole sample at the same time, so instead of using a photomultiplier tube, detection can occur via a CCD camera, making the image immediately visible.

The disk is designed to scan 12 frames in one rotation, and as it rotates at 1800 rpm, the disk generates images at 360-1000 frames per second (observable at the eyepiece of the scanner) and recorded by a video camera. In practice, we are limited in the speed images can be captured by the readout speed from the camera to the computer. However, image acquisition at over 120 frames per second is still possible for a 300X250 pixel image.

The major benefit of UltraView RS's Nipkow dual-disk based scanning is that true, real-time confocal imaging of living samples is now possible. This is delivered by the multipoint-excitation, multipoint-emission characteristics of UltraView RS, which confers three main advantages over traditional beam scanning LSCMs for live cell imaging:

Higher Acquisition Speed

The use of microlens-enhanced Nipkow disk technology with CCD-based image capture allows the recording of very fast intracellular events, for instance the activation and propagation of calcium release events underlying heart muscle cell contraction. Acquisition speeds can be increased by looking at smaller areas of the image ("subarraying") or by combining pixels together (binning) to reduce the volume of information flow from the camera, but the latter reduces lateral resolution. The combination of high speed of acquisition and high spatial resolution allows genuine 4-D imaging.

Reduced Photobleaching

As discussed above, traditional beam scanning LSCMs subject each point in the sample to high doses of radiation, which can lead to photobleaching. The multibeam scanning mechanism of Nipkow disk based confocal microscopes means that each point in the sample is subjected to a much lower dose of radiation compared to LSCMs. This low intensity illumination makes two-photon processes, which would cause excitation of the molecule into a triplet state highly unlikely. Reduced photobleaching is important when performing live cell work to image proteins tagged with GFP family members.

Reduced Phototoxicity

Again, due to the reduced laser dose given to the sample, phototoxic events caused by release of free radicals during laser-induced breakdown of fluorophores are prevented. Reduced phototoxicity observed with UltraView RS enables experiments to continue for longer periods of time without anomalous results caused by the activation of cellular stress pathways.

Purchased Equipment:

PerkinElmer UltraView RS Spinning Disk Confocal Imaging System Including: confocal scanning module, Argon/Krypton laser controlled by an AOTF, cooled CCD camera, piezo objective driver, UltraView RS imaging Suite software to drive the system and Volocity II software for data analysis	$189,900
Funds provided through DURIP:	$ 147,400
Matching funds provided by Old Dominion University:	$42,500

Results Obtained with the Spinning Disk Confocal Imaging System

The Perkin-Elmer Ultraview ERS Rapid Confocal Imager was installed at the Center on July 11, 2005. Perkin-Elmer provided two days of training to faculty and students at the Center. During the past year, the spinning disk confocal has been used by five graduate students and four full-time researchers at the Center.

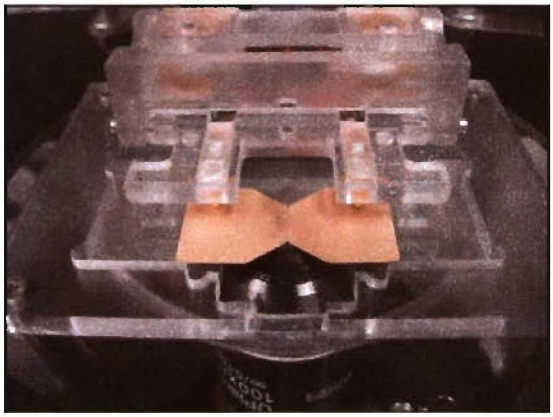

Figure 1 Chamber for viewing cells on inverted microscope during nsPEF application. Glass coverslip with gold-plated electrodes 100 μm apart supports the cells for optimal optical clarity.

We have developed a chamber that allows us to apply nanosecond pulsed electric fields (nsPEF) to cells while viewing them on the confocal and acquiring images as fast as every 15 milliseconds (fig. 1).

Coverslip nsPEF chambers: In order to obtain high resolution fluorescent images of cells on the spinning disk confocal microscope while applying nsPEF, we have developed a chamber in which the electrodes are vacuum-deposited directly onto coverslip glass (fig. 1). This vacuum deposition eliminates any gap between the metal and the glass as always occurs when a metal electrode is attached to the coverslip with adhesive. After depositing chromium onto the glass, we use standard photoresist photolithography techniques to generate the two-plate pattern and then electroplate copper followed by nickel and finally gold to form the final inert parallel plate electrode with a 100 μm gap into which the cells are placed.

Using this chamber, we have studied the cellular responses to nsPEF. These include changes in intracellular Ca^{2+} and stimulation of reactive oxygen species generation.

1. Imaging ROS generation

(This project is part of a study on the effect of nanosecond pulses on melanoma cells)

We have used the Image-iT live green reactive oxygen species detection kit from Molecular Probes to monitor ROS production in B16 cells with and without nsPEF application. Carboxy-H_2DCFDA is loaded into the cells for 30 min at 37 °C and cellular esterases cleave off the methyl ester group to leave behind a charged and impermeant carboxy-DCFH. This is non-fluorescent, but upon oxidation, forms carboxy-DCF which is very fluorescent. Our first experiments with this look very promising (figure 2). They show a punctuate ROS production near the plasma membrane within a few minutes after exposing cells to 5 pulses of 100 ns and 100 kV/cm. Within 10 min, the fluorescence increases in the cell cytoplasm and the nucleus.

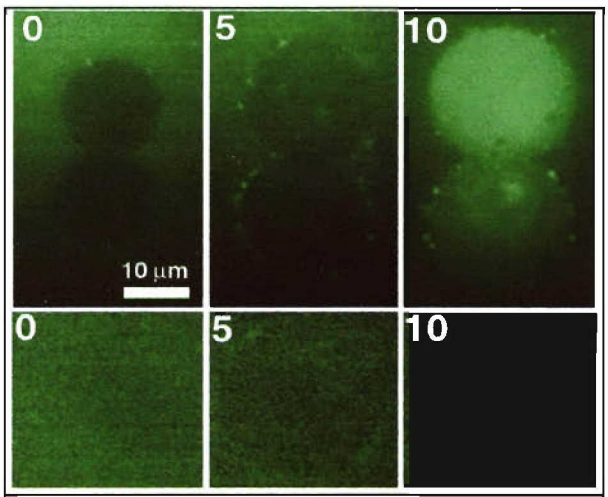

Fig. 2. Carboxy-DCFH fluorescence increase with time after nsPEF treatment. Top frames: two B16 cells that have been treated with 10 pulses 100 ns long and 100 kV/cm in amplitude. Numbers indicate minutes post-treatment. Bottom frames: control B16 cell that has not been treated with nsPEF.

2. Imaging Ca^{++} Release

(This project is part of a MS thesis by Shaka S. Scarlett; preparations for a manuscript are underway)

Nanosecond pulsed electric fields (nsPEF) of megavolt-per-meter field strengths can interact with subcellular structures [1] and consequently trigger a variety of biological events. The exposure can induce apoptosis, as well as an array of differential "sub-lethal effects." One of the most sensitive and immediate responses is the increase in intracellular calcium concentration in pulsed cells [2, 3, 4]. We investigated the response of Jurkat cells (a leukemia cell line) to nsPEFs of 60 ns duration and field strengths from 25 to 100 kV/cm. The development of the induced calcium response in individual cells was studied by **spinning-disc confocal microscopy** with the calcium indicator Fluo-4. The fast imaging system allowed us to record the development of calcium levels with a temporal resolution down to a few milliseconds. Cells exposed to an electric field of 100 kV/cm in the presence of extracellular calcium exhibit an increase of fluorescence within one second, followed by a slow rise, which requires an average of 22 seconds to reach peak intensity. During the initial fast increase, the fluorescence signal rises to 80% of its peak intensity value. This initial fast increase could not be resolved by diagnostics employed thus far. As shown in Figure 3, the response is much faster than the naturally occurring calcium signaling events. However, the amplitude of the pulse-induced signals and natural responses are comparable. The secondary slow rise is absent in cells pulsed without extracellular calcium. The results suggest that the initial rise in calcium is caused by the release of calcium from internal stores. The subsequent much slower increase can be explained by extracellular uptake, triggered by the intracellular calcium release. Spatially resolved measurements of pulse-induced calcium release showed that the calcium release begins at the endoplasmic reticulum at the anode- and cathode-facing poles of the cell and propagates throughout the rest of the cell, with the peak intensity eventually spreading to the nucleus (Fig. 4). Since the pole areas are the locations of highest electric field strength, the observed temporal and spatial development indicates that calcium release is a subcellular effect possibly caused by electroporation of the ER membrane. In physiological calcium response, the calcium signal tends to increase evenly throughout the cell over a longer period of time.

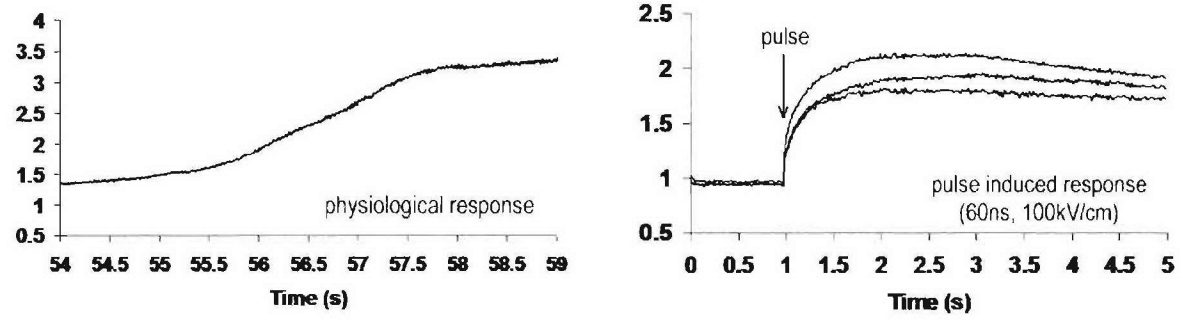

Fig. 3. Fluorescence response derived from images taken every 18 ms with an exposure time of 5 ms. For a physiological response (left), the increase in calcium levels extends over several seconds, whereas pulsed electric fields induce a fast response in less than 1 second.

(a) (b) (c) (d)

Figure 4. Spatial distribution of calcium increase. Upper row: Fluo-3 fluorescence; lower row: same in false colors to indicate the spatial distribution of calcium in the cells at various times. Cathode and anode are facing the top and bottom side of the cell, respectively. Two cells (a) before the pulse, (b) less than 18ms after 100kV/cm pulse, and (c) 18ms after show initial increase in calcium around the nucleus at the anode and cathode facing sides of the cell. (d) At approximately one second after the pulse calcium response the fluorescence is most intense at the nucleus. Images were taken with an exposure time of 5 ms per frame every 18 ms.

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