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Semiconductor ultra-violet light-emitting diodes for flash photolysis

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Abstract

'Caged' compounds are biological molecules that are rendered inactive by a protecting (cage) group. Photocleaving of chemical bonds associated with the cage species with intense UV light results in the release of the active molecules. This technique, called flash photolysis, allows for real-time study of interacting biological molecules and typically involves the use of high intensity lasers or flash lamps to deliver the UV pulse to the biological specimen [Callaway EM, Katz LC. Photostimulation using caged glutamate reveals functional circuitry in living brain slices. Proc Natl Acad Sci USA 1993;90(16):7661–5; Parpura V, Haydon PG. "Uncaging" using optical fibers to deliver UV light directly to the sample. Croat Med J 1999;40(3):340–5; Denk W. Pulsing mercury arc lamps for uncaging and fast imaging. J Neurosci Methods 1997;72(1):39–42]. Here, we introduce compact, custom-designed semiconductor UV light-emitting diodes (LEDs) as a viable and efficient source for performing flash photolysis studies, focusing specifically on the application of these devices for uncaging neurotransmitters locally onto neurons cultured on artificial substrates. The illumination design feature incorporated in these devices allows for direct placement of the UV source in immediate proximity with the neuron of interest and provides a means for optical triggering of activity in the neuronal culture.

Keywords: Ultra-violet light-emitting diodes; LEDs; Flash photolysis; UV uncaging; Caged glutamate; Cultured hippocampal neurons

1. Introduction

Flash photolysis techniques have been successfully used for manipulating biochemical events across neuronal preparations, thereby allowing the complex neural signal integration process to be studied in detail. This technique involves the release of compounds that are of physiological interest, like calcium, neurotransmitters, fluochromes, peptides and enzymes, by photo cleaving the caged precursors (Lester and Nerbonne, 1982; Kaplan and Somlyo, 1989; Niu and Hess, 1993). The light source used in these experiments must deliver a high intensity ultra-violet (UV) irradiation (300–380 nm) in order to ensure adequate photoconversion of the inactive species to the active form. Arc lamps and bench top lasers, currently used in such photolysis studies, are either coupled directly to microscopes or coupled via fibers to deliver UV pulses to the biological samples (Callaway and Katz, 1993; Denk, 1997; Parpura and Haydon, 1999). These sources tend to be bulky and expensive, and require additional focusing elements to create a spot size in the micrometer range. By contrast, the newly developed semiconductor UV light-emitting diodes (LEDs) provide viable alternatives that are compact and inexpensive, yet high intensity sources when configured appropriately. High efficiency longer wavelength blue (460 nm) and green (530 nm) Indium Gallium Nitride (InGaN)-based LEDs have been recently successfully used in biological applications including voltage-sensitive dye imaging (Venkataramani et al., 2005). Recent developments in the material and device science of the more challenging AlGaN and AlGaInN-based LEDs emitting in the 280 and 340 nm wavelength ranges, with relatively high output power and operating speeds, has seen these devices being tested for a number of applications ranging from bioaerosol detection to water purification (Davitt et al., 2005a; Peng et al., 2004). In this paper we demonstrate the use of custom-designed and fabricated compact UV light-emitting diodes (LEDs), based on the quaternary AlGaInN semiconductors, as a flexible means to perform flash photolysis of caged neurotransmitters. We have designed and

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fabricated small area planar UV LEDs (<100 μm diameter) in which individual LED elements can be aligned to illuminate a specific hippocampal neural cell in close physical proximity, here in vitro. Careful positioning of the UV element to illuminate specific regions in the cultured hippocampal cell in proximity allows for precise control over the release of neurotransmitter.

2. Materials and methods

2.1. Cell culture

Cultures of rat hippocampal neurons were made as described previously (Brewer et al., 1993). Briefly, the hippocampus was removed from embryonic day 18 (E18) rat embryos, trypsinized (0.25%), and dissociated by titration. Cells were plated onto poly-L-lysine coated sapphire substrates at a density of 5000–10,000 cells/cm^2. After 3 h, the DMEM/10%FBS was removed and replaced with 37 °C Neurobasal (Gibco, 21103049) media (containing Neurobasal, B27, pen-step, and Glutamax). Cultures were subsequently fed every 4 days until use.

2.2. Substrate preparation

Sapphire substrates (Meller Optics) used to culture hippocampal neurons were treated in concentrated nitric acid overnight, washed in distilled water, and then stored in 100% ethanol until further use. These were then fire polished and placed in 24-well culture plates. Poly-L-lysine (P2636, Sigma, St. Louis, MO) dissolved in borate buffer at a concentration of 0.3 mg/mL was added to the coverslips and allowed to adsorb overnight. The coverslips were washed in distilled water and DMEM (Dulbecco's Modified Eagle Medium, Gibco, 11995065, Carlsbad, CA) with 10% FBS (Fetal Bovine Serum, Atlanta Biologicals) was added to the substrates, which were stored in the incubator (5% CO_2, 37 °C) for 2 h before plating the neurons.

2.3. Caged glutamate and picospritzer system

In this study, DMNB-caged glutamate (L-glutamic acid, α-(4,5-dimethoxy-2-nitrobenzyl)) ester, and hydrochloride (α-(DMNB-caged) L-glutamic acid) was used. A stock solution of this compound was prepared at a concentration of 5 mM in aqueous buffer and kept in the dark at all times. Pipettes pulled from borosilicate glass using a Sutter Instrument puller were filled with the caged glutamate solution (this will henceforth be referred to as the puffer pipette). The puffer pipette was connected to a picospritzer unit, which applied controlled pressure pulses releasing precise amounts of solution into the medium.

2.4. Electrophysiology

Whole-cell patch clamp recordings were performed using an Axoclamp2B amplifier. The recording solution contained 145 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 1 mM Mg Cl_2, 10 mM Glucose, 10 mM HEPES. The osmolality was adjusted to 315 mmol/kg. The intracellular pipette solution contained 9 mM NaCl, 136.5 mM K-gluconate, 17.5 mM KCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM EGTA. The osmolality was adjusted to 310 mmol/kg. pH of both the solutions was adjusted to 7.25 using KOH. All chemicals were obtained from Sigma–Aldrich, USA. The cultures were studied at room temperature (27 °C) after 1 week in vitro. The patch pipettes made of borosilicate glass and had a resistance of 3–4 MΩ when filled with the pipette solution.

2.5. UV LED fabrication procedure

The 340 nm wavelength LEDs were fabricated from MOCVD-grown quaternary AlGaInN quantum-well p-n junction heterostructures (Ren, 2005), according to a material design typical for these compact solid state light emitters. Epitaxial growth of the active material was on sapphire substrates, which are transparent at this wavelength and hence light emission was extracted from the polished backside through the 500 μm thick substrate. Mesa-type devices were etched using chlorine-based RIE, and a high-temperature annealed multilayer Ti/Pd/Al/Ti/Au metallization served as the contact to n-type AlGaN (Peng et al., 2004). An opaque Ni/Au p-electrode 50 μm in diameter defined the optical aperture, as illustrated in the inset to Fig. 1(a). In contrast to longer wavelength blue and green InGaN LEDs, 340 nm devices have lower n-type conductivity and require n and p contacts which are relatively closer for high

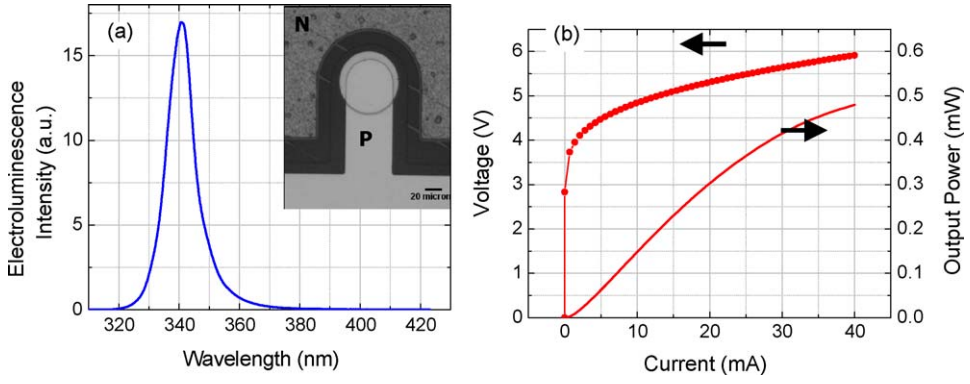

Fig. 1. (a) Electroluminescence spectrum and (b) typical dc light-output (measured through the transparent sapphire substrate using a UV-enhanced silicon photodiode (Hamamatsu) placed in close proximity the backside of the device) and current–voltage characteristics for a 50 μm diameter 340 nm LED. *Inset*: Photographic image of single device.

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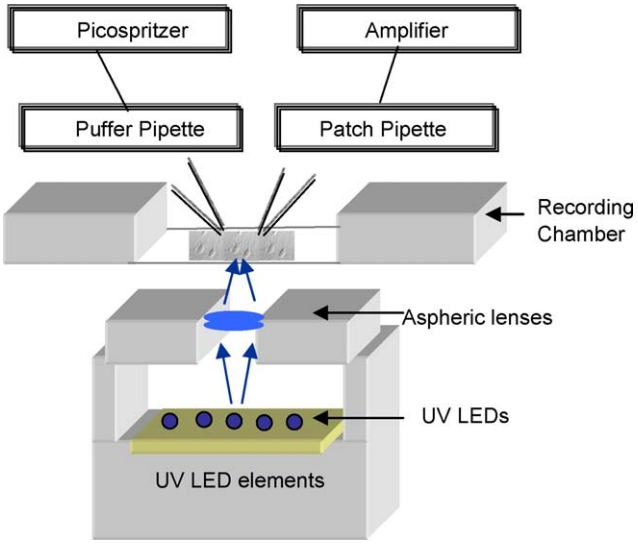

Fig. 2. Schematic showing the set-up: individual LED elements are focused onto neural cells using a pair of aspheric lenses; the entire assembly is mounted on a custom built holder that can be positioned under the neural cell of interest.

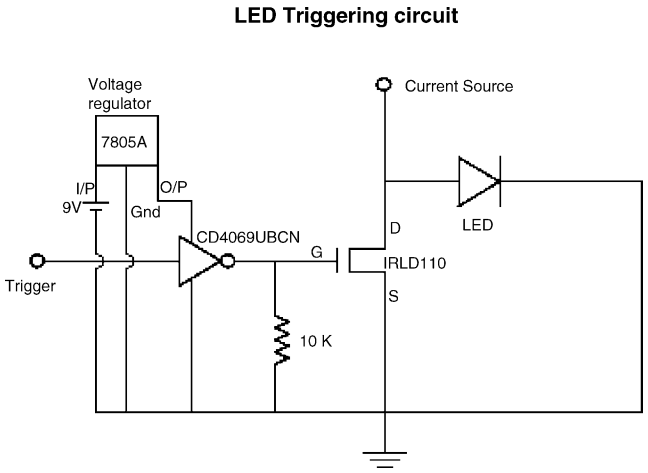

Fig. 3. Circuit used for triggering individual UV LED elements.

electrical-to-optical conversion efficiency. In our devices, the lateral n to p distance was more than 20 μm, eliminating so-called current crowding effects, which can reduce the efficiency of larger area devices, particularly in sub-300 nm wavelength devices. The device emission spectra and a typical light output power and current–voltage characteristics of fully fabricated 50 μm diameter (optical aperture), 340 nm emitting LEDs are shown in Fig. 1(b).

2.6. Optical system configuration

Ultra-violet light-emitting diodes fabricated by the method described above were mounted on a copper ring for thermal management and inserted into a 1" mirror mount for easy handling. As mentioned above, the UV light is extracted from the sapphire side (backside emission). This offers the additional advantage of removing the electrical wiring from the optical path thereby allowing the LEDs to be placed in close proximity to the cultured neuron substrate. A pair of fused silica aspheric lenses with appropriate focal lengths (Thorlabs) was used to focus the UV light onto the sample and the entire assembly was mounted on a custom built holder and placed below the chamber containing the cultured cells; the schematic of the set-up is shown in Fig. 2. With this arrangement, it was possible to achieve a tight focus of 50 μm spot from a 50 μm device as illustrated in Fig. 5(a).

3. Results and discussion

A simple circuit (shown in Fig. 3) was used to drive the light-emitting diode elements. For the flash photolysis experiments performed, the LED element was operated at modest current injection levels (30–55 mA) which from Fig. 1 translates to a power of 0.4 mW at the LED substrate. The light output power shown in Fig. 1(b) was measured through the transparent sapphire substrate using a UV-enhanced silicon photodiode (Hamamatsu) placed in close proximity the backside of the device. During this measurement, there was no additional heat sinking and dc current injection with simultaneous voltage and photocurrent measurement was accomplished with a semiconductor parameter analyzer (HP4145). This method, which does not use any integrating sphere geometry, collects only the fraction of light emitted through the substrate and more closely reflects the useful amount of light output power. The timing of the LED pulse was controlled using a delay generator (Stanford Research SR 535 delay generator).

In order to test the viability of these devices to efficiently "uncage" molecules, we used a caged version of fluorescein molecule, caged-FITC (CMNB-caged Fluorescein, Molecular Probes). A drop of solution containing 2 mM of caged-FITC dissolved in water was added on a microscope slide, and a cover slip was placed on top. Fluorescent images of the sample surface before and after UV irradiation were captured. Fig. 4 shows the fluorescent intensity as a function of distance. The red trace

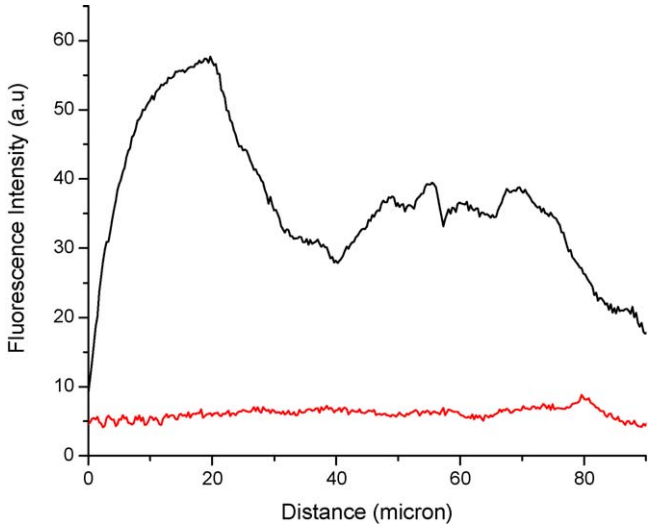

Fig. 4. Fluorescent intensity before (red trace) and after (black trace) illuminating caged-FITC sample with UV light from a 50 μm LED device. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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represents the resting fluorescent intensity of the sample region before UV irradiation, and the black trace shows the intensity from the same region after illuminating with UV light from a 50 μm LED element for 1 s. The varying intensity peaks in the black trace correspond to irregularities in the substrate. Integrating the area under these curves, it can be seen that there is 2.8-fold increase in fluorescence intensity; implying that the light from the UV LED is efficiently uncaging the chemical bonds and releasing fluorescein, which is causing the fluorescence intensity to increase.

The capability of UV LEDs to uncage neurotransmitter at/near single hippocampal cultured cells was tested on cultured neural cells ($n = 5$) and responses from two example cells are shown. Fig. 5(a) displays an image of a single 9 div hippocampal neuron indicating the positions of both the patch and the puffer pipettes. The entire LED assembly with the corresponding focusing optics was mounted on a 3D stage that allowed for careful and precise positioning of the LED element below the region of interest. Whole-cell recordings were made on a selected neuron; action potentials were triggered by injecting current through the patch pipette at the beginning of the experiment, but thereafter this pipette was used only to record the response of the cell (no current injected). The timing and delay of the picospritzer and LED circuit was carefully and independently controlled using two channels of a delay generator (SR535 delay generator). The LED was typically operated between 30 and 55 mA drive current for 250–350 ms. During these experiments, the picospritzer was typically regulated at a pressure between 6 and 20 psi for 20–200 ms. In the examples shown in Fig. 5(d) and (e), the device was operated at 55 mA for 350 and 250 ms, and the picosprizer at 6 and 20 psi for 200 and 50 ms, respectively.

Control experiments were performed wherein the response of the neuron to only the LED being ON and only the puffer being ON was recorded. These are shown in Fig. 5(b) and (c). The lack of any neural response at these conditions indicates that neither the LED nor the caged glutamate trigger any neural response from the cells. When both the puffer and the LED were turned ON (ON times indicated by the red and blue insets), it was possible to record action potential from the neuron, as shown from the recordings from two cells in Fig. 5(d) and (e). The ability to trigger action potentials in the neuron indicates that the UV LED effectively uncaged the glutamate that was puffed in the vicinity thereby exciting the neuron. In sum, we have demonstrated the application of these compact ultra-violet light-emitting diodes as efficient and effective sources for flash photolysis experiments. It is also clear from Fig. 5(d) and (e) that

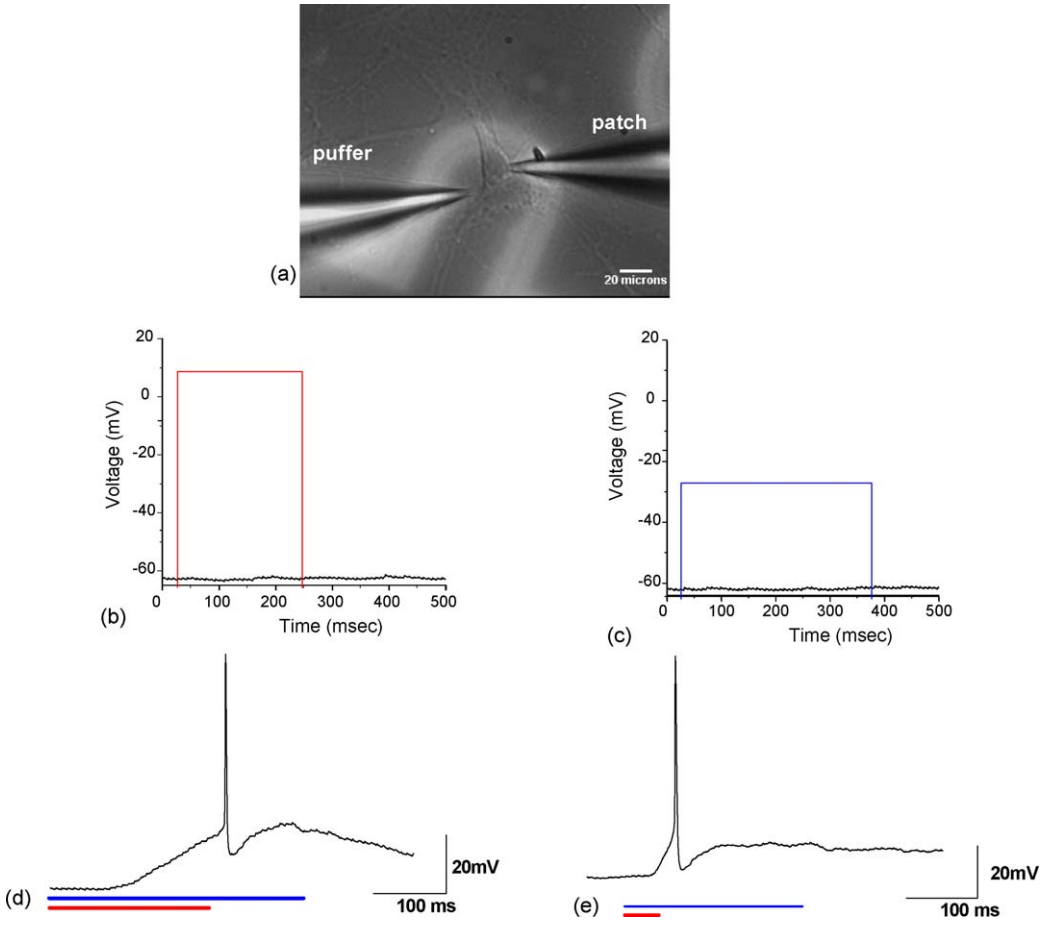

Fig. 5. (a) Image of the neuron with corresponding puffer (left) and patch (right) pipettes, (b) Control experiment with the puffer: red trace indicating the duration of puffer pulse, black trace shows the cell's response, (c) control experiment with the LED; blue trace indicating the duration for which LED is ON, black trace shows the cell's response, and (d and e) response recorded from two different cells when both the puffer (red) and LED (blue) were ON. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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by carefully controlling the picospritzer conditions, it is possible to achieve shorter latencies between beginning of depolarization and the firing of action potential. While, in cells recorded using longer puffer ON times at lower pressures, this latency was in the order of 200 ms, whereas with conditions of shorter puffer ON times and higher pressure, the latency was much shorter, in the order of 50 ms. While, this also depends on the position of the puffer with respect to the cells' processes, this has not been evaluated in detail here.

4. Conclusion

Flash photolysis experiments have required expensive and complicated instrumentation. There has been a lack of an affordable and stable light source that would be bright enough to cause efficient uncaging. Light-emitting diodes as excitation sources cover the whole visible range and offer excellent stability, power efficiency and economy. These have been used extensively in steady state lighting and in fluorescent spectroscopy. With recent advances in material growth and device processing technology, light-emitting diodes have become available with emission in blue and ultra-violet spectral regions. In this paper, we have described and demonstrated the applicability of using compact, planar ultra-violet LEDs as effective local sources for flash photolysis. The LED-based flash photolysis system detailed in this paper is intended to describe a compact and inexpensive approach for uncaging experiments. These LEDs can be fabricated into planar arrays, where independent electrical control of each LED with microsecond precision can be achieved (Davitt et al., 2005b) making them particularly suitable for examining network properties by stimulating the neurons at different locations. The ability to select individual elements and turn them ON, gives a unique advantage of selecting different stimulating location. These devices can also be operated at very high speeds, typically in <μs range. It is also possible to fabricate these devices in the nanometre range using e-beam lithography techniques (He et al., 2004). Though, currently these sub-nanometer devices cannot be directly applied for flash photolysis studies (due to low powers that are currently extracted from these devices), with significant improvements in the material growth, devices with much higher power can be obtained using these small-scale structures, which will then open up immense applications for these devices.

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