Electrochemiluminescence as a tool for microscopy at the nanoscale

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ABSTRACT

The particular form of electrochemiluminescence (ECL) used for analytical assays relies upon the discovery that tris(2,2′-bipyridyl)ruthenium(II) ($Ru(bpy)_3^{2+}$) emits a 620 nm photon when adjacent to an electrode held at about one volt relative to Ag/AgCl. This reaction occurs within nanometers of the electrode. The enormous economic investment in nanoscale lithography tools is leading to tools capable of routinely producing 32 nm features by 2009. We propose that these two technologies could be combined to produce a nanoscale microscopy system. We constructed a macroscopic test-bed and performed tests on it to explore the feasibility of such a system. We tested an ECL solution containing 1 mM $Ru(bpy)_3^{2+}$ 0.2 mM ammonium oxalate monohydrate in a 0.1 M ammonium acetate buffer at pH 5.0. Using this solution, we found that the ECL light was most intense at an applied voltage of 1.6 Volts, that the effect had excellent reproducibility and that the time to reach maximum intensity was several seconds after applying a voltage.

Keywords: Electrochemiluminescence, microscopy, electro-generated chemiluminescence

1. INTRODUCTION

Excited electrons can decay to normal energy states by the emission of an optical photon. When electrons are excited by an electrochemical reaction, this process is known as electrochemiluminescence (ECL) or electro-generated chemiluminescence. ECL is widely used for measuring clinical analytes.

The lithography process used in semiconductor fabrication has seen continuous improvements over the past 30 years. Year 2002 production capabilities were down to 130 nm line widths for critical features. There are significant resources being poured into new technologies for production-scale sub-100 nm lithography by the semiconductor industry. The technology road map published by Intel projects 65 nm features in production by 2005 and 32 nm features by 2009.[1]

We propose that these two robust commercial technologies could be combined to yield a nano-scale imaging system. In order to better understand the engineering issues that need to be solved to develop such a system, we will build a microfluidic device with a linear array of nanoscale electrodes. This device will be designed to measure one-dimensional motion of $Ru(bpy)_3^{2+}$tagged nano-particles on the scale of 50 nm.

The resolution of such a device is not limited by the optical wavelength, but rather by the size of the electrode exciting the $Ru(bpy)_3^{2+}$. The minimum electrode size is determined by the lithography system. This scheme transforms the limiting resolution of the imaging system from a fundamental one (the wavelength of light) to a technological one (minimum feature size of a lithography system). Furthermore, the technological limit has an enormous ongoing research and development effort in reducing it.

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1.1. Electrochemiluminescence

Electrochemiluminescence (ECL) first became of interest for analytical assays with the discovery in the 1970's that tris(2,2′-bipyridyl)ruthenium(II) ($Ru(bpy)_3^{2+}$) emitted a photon near 620 nm when excited by an electrode held at about +1.3 V (relative to Ag/AgCl).[2,3] Furthermore, it was found that this reaction had a fairly high quantum efficiency (2%) in water. Previous systems that were known to exhibit ECL were only useful in non-aqueous solutions, like acetonitrile.

The technology has been developed into a robust analytical chemistry technique by commercial vendors for use in clinical chemistry analyzers.[4] The number of electrochemiluminescent reagents is also expanding rapidly. For instance, a commercial vendor, Roche, (formerly IGEN of Gaithersburg, MD), currently sells reagents for linking the $Ru(bpy)_3^{2+}$ complex to oligonucleotides, thiols, carbohydrates and carboxyls.

Table 1. The $Ru(bpy)_3^{2+}$reaction mechanism.[2,5] Note that the molecular tag, $Ru(bpy)_3^{2+}$, is regenerated after the emission of each photon, enabling multiple cycles and many photons for each tag.

$$\begin{aligned} Ru(bpy)_3^{2+} - e^- &\rightarrow Ru(bpy)_3^{3+} \\ Ru(bpy)_3^{2+} + e^- &\rightarrow Ru(bpy)_3^{+} \\ Ru(bpy)_3^{+} + Ru(bpy)_3^{3+} &\rightarrow Ru(bpy)_3^{2+*} + Ru(bpy)_3^{2+} \\ Ru(bpy)_3^{2+*} &\rightarrow Ru(bpy)_3^{2+} + h\nu \end{aligned}$$

1.2. Nanometer Scale Lithography

There is tremendous economic interest in reducing the minimum feature size in lithography due to the multi-billion dollar market for electronic chips. Current (year 2002) production capabilities are down to 130 nm line widths for critical features, exceeding the resolution typically obtainable from high numerical aperture fluorescent microscopes. There are significant resources being poured into new technologies for sub-100 nm lithography by the semiconductor industry for use on a production scale.[1] Improvements to these numbers are expected to continue their rapid progression, enhancing the value of this proposed electrochemiluminescence microscopy. The technology roadmap published by Intel[1] projects 65 nm features in production by 2005 and 32 nm features by 2009. A prototype extreme ultra-violet (EUV) ($\lambda = 13$ nm) lithography system already exists that can pattern 70 nm features.[1]

1.3. Long Term Goal

Our long term goal is to develop an "electrochemiluminescent (ECL) microscope" with nanometer resolution. Electrochemiluminescence is a relatively new technology that has been developed for the *in vitro* diagnostics (blood testing) industry. The principle is simple: an electron transfer excites a particular molecule into a state that decays *via* emission of a (620 nm) photon. The electron transfer occurs by way of quantum tunneling from an electrode, and is therefore extremely unlikely to occur over a distance longer than a few nanometers. This localized excitation allows one to know precisely (within a few nanometers) where the molecule is, provided that the source of excitation itself (the electrode) is that small. The goal, therefore, requires the development of techniques for nanometer scale lithography of electronic circuits and the adaptation of commercially available electro chemi-luminescent probes to single molecule detection. This is followed by the construction of a data acquisition and control system that combines single molecule detection (*via* 620 nm photons) with excitation information to build up a nanometer scale image. The complete system would work as shown in Figure 1.

The proposed ECL imaging system will require sensitive optical detection, a data acquisition and control system and software to analyze the data of the $Ru(bpy)_3^{2+}$ tagged sample. The greatest hurdle to achieving single molecule detection (not single photon detection) is usually contamination by background signals. An important advantage of the ECL microscope is the lack of a background signal compared to fluorescence microscopy. A number of sources contribute to the background intensity of fluorescence measurements including autofluorescence and scattering of the excitation light. There are also substantial optical losses of the fluorescent signal caused by the optical filters that are needed to remove the excitation light. Since ECL is excited by an electric signal, there

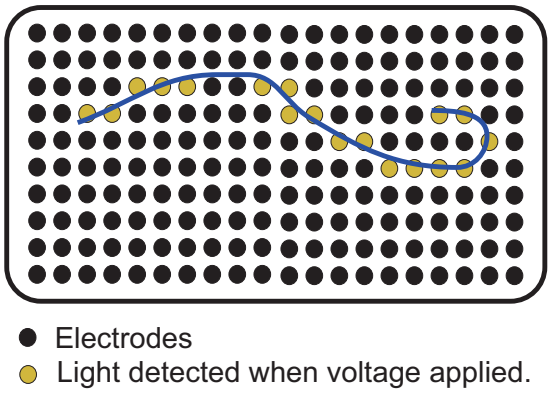

Figure 1. A schematic example of the long term goal of this project. A chip with multiple electrodes is fabricated. Molecules labeled with $Ru(bpy)_3^{2+}$ are deposited onto the chip. A small voltage is applied to each electrode sequentially. Electrodes which are close (within a few nanometers) will excite the $Ru(bpy)_3^{2+}$ and lead to the emission of a photon. The photons are collected and detected through optical lenses to a sensitive detector. An image is synthesized by correlating the photon detection with the applied voltage. The resolution of this image is not limited by the wavelength of light, rather it is limited by the minimum size of the electrode.

is no need for filters in front of the detector. Compared to fluorescence microscopy systems, we would expect to have a higher collection efficiency and a much lower background.

The optical collection system of the proposed system is shown in Figure 2. It will consist of a high numerical aperture microscope objective coupled to an avalanche photodiode. A data acquisition computer system will combine this information with a control system that sequentially applies a voltage to different electrodes. The computer will acquire a measurement of $Ru(bpy)_3^{2+}$ as a function of position, and a synthetic image can be generated from this information. The resolution of the synthetic image is not limited by the optical wavelength, but rather by the size of the electrode exciting the $Ru(bpy)_3^{2+}$. The minimum electrode size is determined by the lithography system. This scheme transforms the limiting resolution of the imaging system from a fundamental one (the wavelength of light) to a technological one (minimum feature size of a lithography system). Furthermore, the technological limit has an enormous ongoing research and development effort in reducing it.

1.4. Related work by others

Several studies to date have shown the feasibility of using electrochemiluminescence from microfabricated electrodes for analytical measurements. The pioneering study by Smith's lab[6] appeared in 1996, where the authors constructed a cell that contained a gold cathode and an indium tin oxide anode. Indium tin oxide is a transparent conductor. This cell allowed the observation of induced luminescence by an external photodiode, through the anode. They could measure the concentration of free $Ru(bpy)_3^{2+}$as low as 1 nM in this cell.

Later work by Smith's lab[7] demonstrated an assay to quantitate polymerase chain reaction (PCR) product DNA, by incorporating a $Ru(bpy)_3^{2+}$tag onto the primers. This work also used an integrated PIN diode onto the micromachined flow cell.

Smith's lab was also the first to show that electrochemiluminescence could be observed in water at carbon microelectrodes without the presence of a reducing agent. This effect was unexpected and is still not completely understood. This underscores the importance of experimental work at the micro level.

Integrated electrodes and detection of chemiluminescence was also the goal of the work of Fiaccabrino *et. al.*[8] They showed that their system could detect ruthenium in the range of $0.5 - 50\,\mu$M.

Manz's lab has published two interesting papers[9, 10] on the use of electrochemiluminescence in microfabricated total analysis systems. The first[9] was a proof of principle showing that they could detect as few as 3×10^4 molecules in a probe volume of 100 nl. The second[10] demonstrated a unique floating electrode design inside of a large electric field used for the electrophoretic separation of two forms of the ruthenium complex.

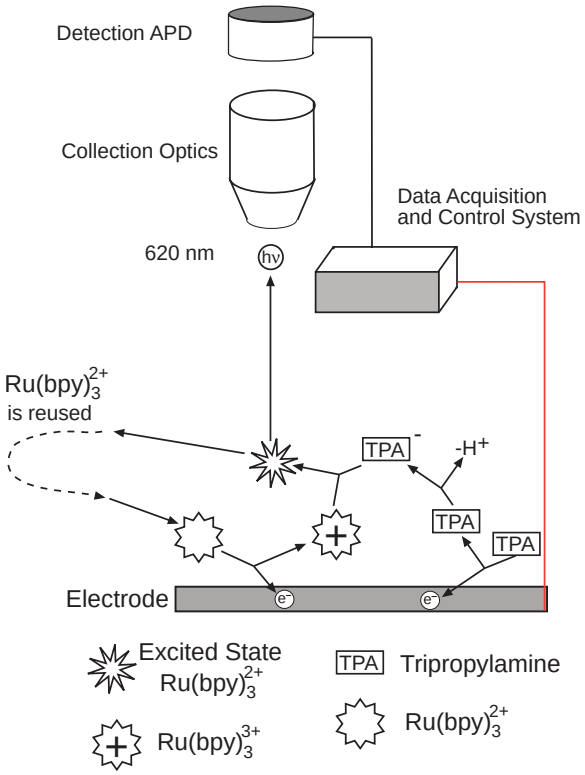

Figure 2. An illustration of the optical set-up to be used for the proposed experiments. The electrochemiluminescence (ECL) chemical reaction as used with commercially available reagents[4] is shown with the proposed data acquisition and control system. The reaction takes place in an aqueous solution within a few nanometers of an electrode. Photons emitted during the reaction will be collected by a high numerical aperture objective and focused onto an sensitive photo-detector (an avalanche photo-diode). A computer simultaneously will observe the detector output and apply a voltage to different electrodes in a linear array. By knowing which electrode has voltage applied to it and the resultant photo signal, the computer can rapidly synthesize a one-dimensional distribution of the $Ru(bpy)_3^{2+}$ tags.

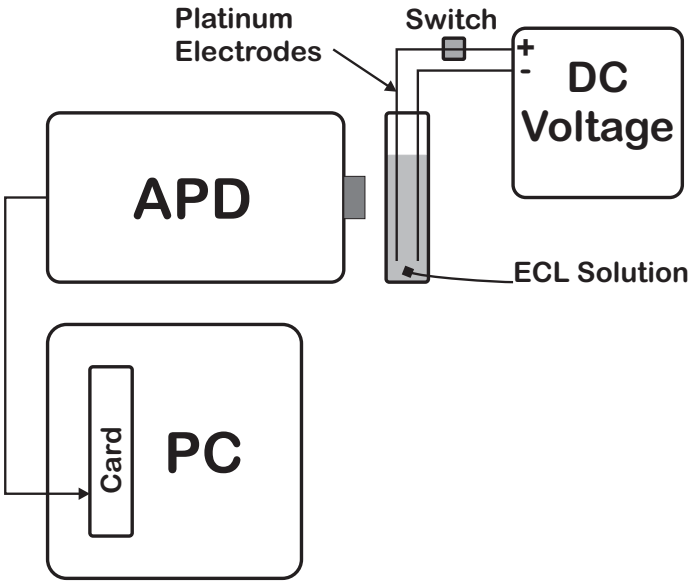

Figure 3. A schematic diagram of the experimental system used here. A variable (0-12 V) power supply was attached to two platinum electrodes through a switch. The electrodes were immersed in the ECL solution (1 mM $Ru(bpy)_3^{2+}$ 0.2 mM ammonium oxalate in a 0.1 M ammonium acetate buffer at pH 5.0). The emitted ECL light was observed with an avalanche photodiode module (APD), SPCM-AQR-14 from Perkin Elmer. The signal, consisting of digital pulses representing individual photons, is sent to a board (National Instruments) mounted inside of a standard PC. The board, along with custom software, records the number of counts within a certain time period.

Arora's 1997 paper from Manz's lab[9] gives strong support to the feasibility of this proposal. To quote:

> The lowest measurable concentration (S/N=3) is 5×10^{-13} M, which compares favorably with the best-reported literature value.[5] This concentration corresponds to only 3×10^4 molecules in the probe volume of 100 nl. It should be realized that since ECL occurs only for molecules in the immediate vicinity of the working electrode, the actual number of molecules contributing to the measured signal could be considerably less.

The areal size of the electrodes in this work[9] are 1 mm^2 and they are spaced by 100 μm. (The probe volume is 1 mm × 1 mm × 0.1 mm or 100 nl.) If one takes "the immediate vicinity of the working electrode" to be 10 nm, one arrives at only (10 nm/100 μm) about 10^{-4} or about 3 molecules contributing to the signal detected in this work.[9] To further support this, other work[11] states that "generally, an electrochemical reaction occurs within a limited reaction layer, the so-called electrical double layer and/or the electrical diffuse layer at several nm distances from the surface of the working electrode." Recall that a single tag of $Ru(bpy)_3^{2+}$ is recycled during the electrochemiluminescence reaction and gives rise to multiple photons.

1.5. Experimental Plan

Experiments performed here are meant to establish basic parameters of the electrochemiluminescence process. Specifically, we aim to answer three questions: (1) What is the optimal voltage to excite the electrochemiluminescence? (2) How reproducible is the electrochemiluminescence intensity? and (3) How fast does the electrochemiluminescence intensity reach its peak when a voltage is applied?

2. METHODS

We constructed a testbed to measure basic parameters of the ECL process, see 3. The testbed consisted of a cuvette with two platinum electrodes. The electrodes were made of 36 gauge platinum wire (Fisher). Each

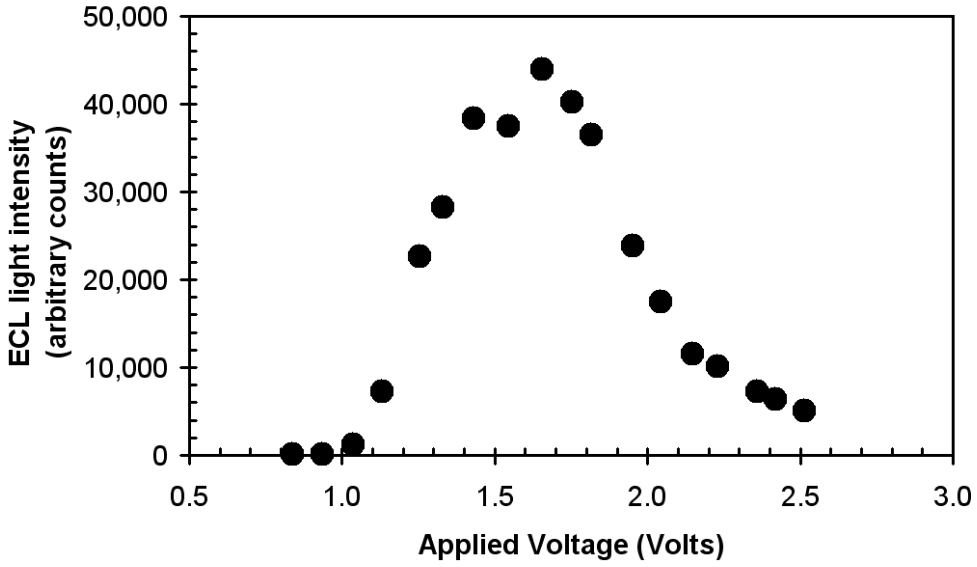

Figure 4. The ECL light intensity as a function of applied voltage.

electrode was soldered to a copper wire. One copper wire was connected to a switch, the other directly to the ground terminal of the power supply. The positive terminal of the power supply (BK Precision, model 1670A) was connected to the other end of the switch. The voltage was monitored with a Fluke digital multimeter.

The optical signal was monitored with a avalanche photodiode module, (SPCM-AQR-14 from Perkin Elmer). This module combines an avalanche photodiode (APD) with an active diameter of 175 μm, a two-stage thermoelectric cooler, amplifier, discriminator, and TTL output driver. It has a dark-count rate of about 75 counts/second. The module has a TTL output where each pulse represents a distinct photon detected. The counting rate is linear to within 10% at a count rate of one million counts per second.

The output signals were fed to a counting board (PCI-6601 from National Instruments) through the BNC-2121 breakout box (National Instruments). The counting board records the number of counts between gates, the rising edge of two pulses. The gates were chosen to be either one second apart or one millisecond apart depending on the study being performed. In either case, custom software recorded the number of pulses per time period for later display and analysis.

The entire testbed was built within a darkened room and the APD and cuvette were covered in black rubberized fabric (BK5, Thor Labs) to eliminate ambient light.

The electrochemiluminescence solution contained 1 mM $Ru(bpy)_3^{2+}$ (Tris 2,2,-bipyridyl ruthenium(II) chloride hexahydrate, CAS number 50525-27-4, molecular weight 748.6) and 0.2 mM ammonium oxalate monohydrate (CAS number 6009-70-7, molecular weight 142.1) in a 0.1M ammonium acetate buffer at pH 5.0. The chemicals were obtained from Sigma.

3. RESULTS

3.1. Determination of the optimal voltage

In Figure 4, we plot the measured ECL light intensity as a function of the voltage applied to the solution. We find that the optimal voltage is close to 2.0 volts.

3.2. ECL light intensity as a function of time

In Figure 5 we plot the ECL light intensity as a function of time before and after a voltage is applied. We find that although it takes about five seconds for the maximum intensity to be reached, a detectable change occurs within 100 milliseconds.

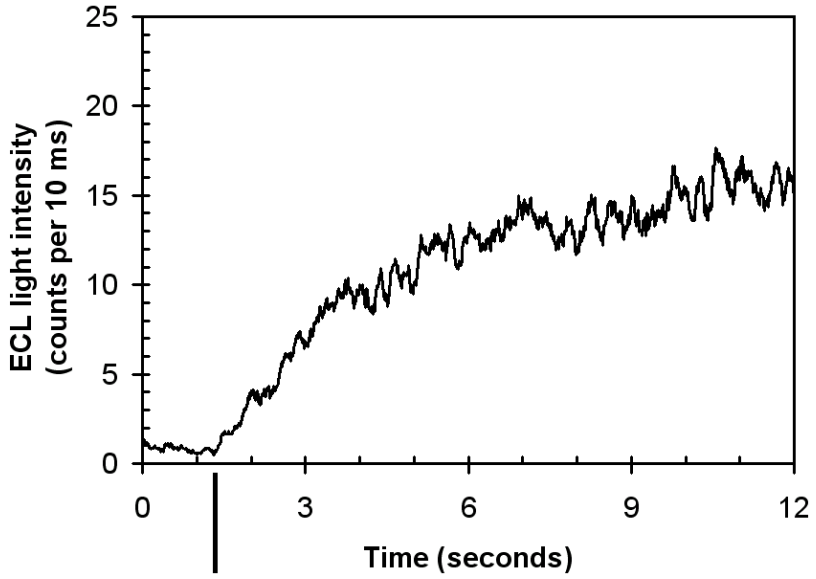

Figure 5. This measurement determines the time for the ECL light intensity to increase to its maximum after a voltage is applied to the solution. At about 1.4 seconds (indicated by the black bar on the x-axis) a voltage of 1.1 V is applied to the ECL solution.

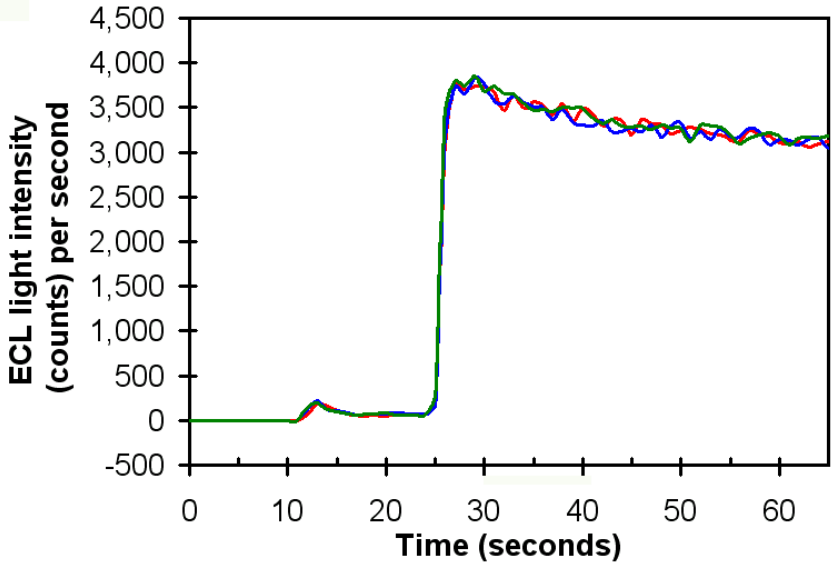

Figure 6. Three consecutive runs showed excellent repeatability. In each case, the ECL solution contained 1 mM Ru(bpy), 0.2 mM oxalate in a 0. M ammonium acetate buffer. The applied voltage was 1.1 V.

3.3. ECL repeatability

Finally, we measured the overall reproducibility of the ECL process. We performed three repeated measurements (see Figure 6) and found excellent overall reproducibility.

4. DISCUSSION

The ECL intensity showed excellent reproducibility, despite the sensitivity to the applied voltage. Reproducibility is a key feature of any robust instrumentation. Hence, this supports the feasability of the ECL microscopy concept.

The ECL intensity reaches a maximum at about 1.6 or 1.7 volts. However, water electrolysis occurs at about 1.3 volts. Hence, the optimal voltage for ECL microscopy may not be at the level of peak ECL intensity, but instead it may be just below the threshold for the electrolysis of water.

The most significant hurdle to the concept of ECL microscopy appears to be the slow response time of electrochemiluminescence to the applied voltage. ECL microscopy will require the sequential application of voltage to individual electrodes. Hence the image acquisition time will be directly proportional to the electrochemiluminescence reaction time. Here, we measured the electrochemiluminescence reaction time to be as long as ten seconds. However, we did note that a detectable change occurs within the first few tens of milliseconds. This reaction time could be a function of electrode and cell geometry or the specific composition of the ECL solution or both. Further experimentation is needed to determine the feasability of ECL microscopy.

ACKNOWLEDGMENTS

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