Super-Quiet Microfluorometry: Examples of Tumor Cell Metabolic Dynamics

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Microscope photometry is a widely used tool that provides quantitative information regarding a sample. To improve the precision of these measurements, we have reduced multiple sources of noise. A Hamamatsu super-quiet mercury-xenon or xenon bulb is used in conjunction with a highly stabilized Newport power supply and photofeedback detector installed on the microscope to minimize lamp noise. Furthermore, a cooled Hamamatsu photon-counting photomultiplier tube and photon counting apparatus are used for detection. In kinetic studies using fluorescent latex beads, the output noise of the fluorescence microphotometer was less than twice the fluorescent sample's expected quantum noise. To illustrate the application of this methodology, changes in tumor cell NAD(P)H autofluorescence in response to metabolic perturbations are provided.

Keywords precision photometry, super-quiet illumination, fluorescence, tumor cells

1. Introduction

Photometry using photoelectric detectors such as photomultiplier tubes (PMTs) has been used in the physical and biological sciences for ~100 years [e.g., 1]. In cell biology, these tools are generally used to provide rapid and quantitative measurements of the fluorescence intensity of fixed cells or changes in the fluorescence intensity of living cells in real time [2]. During the past fifteen years, the commercial introductions of digital photon counting systems and low noise PMTs have dramatically reduced detector noise [e.g., 3]. However, the primary source of noise in these experiments is illumination noise. Arc lamps, light emitting diodes (LED), and lasers produce, to varying degrees, illumination noise in both intensity and spatial distribution. LEDs and lasers have an additional disadvantage in that only one or a few wavelengths are available. Mercury arc lamps are preferable, as many dyes have been developed to take advantage of their emission wavelengths. In some experiments, illumination noise can be minimized by using emission ratioing protocols [4]. During emission ratioing studies, fluctuations in excitation light cause fluctuations in the emission light that, when ratioed, cancel out. However, emission ratioing is not applicable in many experimental settings. One example is excitation ratioing. In this case lamp intensity fluctuations occur at the same time-scale as real time data acquisition thus making such experiments noisy or impossible. In addition, endogenous fluorochromes such as NAD(P)H are not appropriate for emission ratioing. In this paper we present a versatile methodology using arc lamps to provide precise measurements of fluorescence intensities in real time using microscope photometry.

2. Instrumentation and Methods for Low Noise Microfluorometry

Figure 1 shows a schematic illustration of a super-quiet microscope fluorometry system. The system utilizes a Zeiss Axiovert inverted fluorescence microscope. The illumination and detection systems shown will be described in more detail.

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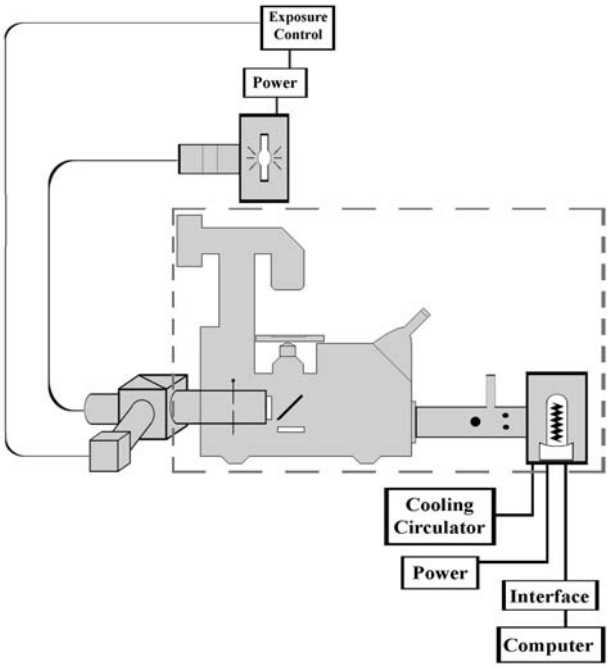

Fig. 1. Microfluorometry system. A schematic representation of a microfluorometry system designed to reduce major sources of noise is shown. The microscope, photometer and PMT detector are housed inside an aluminum enclosure (grey dashed line). The lamp power supply is connected to an exposure controller that regulates the lamp's output intensity. The lamp housing contains a super-quiet mercury-xenon light source, and connects via a liquid light guide to a Rapp Flash-Cube attachment at the rear of the microscope containing a 85:15 beam splitting mirror. 15% of the light is directed onto a thermoelectrically cooled detector that provides feedback to the exposure controller, thereby regulating the input intensity at the microscope. The remaining 85% of the lamp's intensity continues through the microscope where the excitation wavelength is selected by a filter cube. The low-noise PMT is connected to a cooling circulator, power supply and computer interface. All components resting on the optical table are securely attached to the vibration isolation air table.

Although conventional mercury arc lamp illumination is appropriate for most fluorescence microscopy applications, it has a substantial amount noise in both short time-scale fluctuations and drift over time. One alternative is conventional xenon bulbs, which produce slightly less noise but are dimmer than mercury bulbs. However, both of these bulbs are inadequate for precision photometry. Recently, "super-quiet" xenon and mercury-xenon lamps have been introduced by Hamamatsu (Bridgewater, NJ). Super-quiet lamps have highly durable barium-impregnanted cathodes that eliminate arc point shift and fluctuations [5]. Cathode erosion is also reduced, which substantially increases bulb lifetime.

Although Hamamatsu's super-quiet bulb can substantially reduce illumination noise of the bulb, it is essential to use a highly stabilized power supply to maintain constant illumination intensity. For this purpose, we employ a Newport Corp. (Irvine, CA) model 69907 arc lamp power supply, which exhibits changes of <0.02% for large line voltage swings. The power supply is connected to a research lamp housing. As the housing is fan cooled, it is located on a shelf away from the optical table. Filters to reduce the delivery IR and UV light to the microscope can be inserted into the condenser/fiber bundle focusing assembly. Light is delivered to the microscope via a liquid light guide. The liquid light guide is attached to the microscope via a custom Flash-Cube (Rapp OptoElectronic, Hamburg, Germany). The Flash-Cube delivers 85% of the light to the microscope and 15% to a thermoelectrically-cooled photodetector. A filter cube within the microscope selects the appropriate excitation and emission wavelengths. The signal from the photodetector is routed to an Oriel light intensity controller (model

68950, Newport). The exposure control unit monitors the light intensity at the microscope and rapidly regulates the power supply to remove fluctuations and drift from the lamp's output.

To minimize systematic sources of noise, steps were taken to reduce light contamination and vibration [e.g., 6]. Light contamination was minimized by performing experiments in a darkroom and by enclosing the photomultiplier tube (PMT) housing, photometer and the microscope inside an aluminum enclosure. The front of the enclosure has a black felt drape to enable easy access to the system components, while maintaining a light-isolated environment. As vibration can introduce noise, the microscope, photometer, PMT housing, aluminum enclosure and feedback photodetector are all firmly attached to the optical table at one or more points. Care is taken to ensure that the microscope system does not touch the enclosure at any point, to minimize the opportunity for the transfer of vibrations. Components such as power supplies, water circulators, computers, camera controllers and other system elements are placed on free-standing shelves. As a further precaution, care is taken to ensure that cords, hoses and light guides that enter the enclosure do not touch the table, enclosure or instrument except for their attachment point on the system. Finally, to minimize the entry of irrelevant light into the microscope, the desired area is placed in the center of the field then the diaphragm is stopped down. Furthermore, the region of emission can be further selected using the X and Y slits in the photometer.

Emission light is quantified using a Photon Technology International (PTI; Birmingham, NJ)) D-104 microscope photometer attached to a refrigerated PMT housing (model PC177CE, Products for Research, Danvers, MA). For detection, a Hamamatsu R1527P (photon counting) PMT is employed. The measured dark count rate for this detector apparatus is ~5 cps. As the photon counting mode exhibits less noise than current measurement and biological samples are generally dim [3], a PTI photon counting apparatus is employed. This is important because conventional uncooled photodetectors would produce detectable levels of noise when coupled with super-quiet illumination systems. Furthermore, photon counting data are generally acquired at 3 Hz, which is much longer than the sampling rate of the digital exposure controller.

3. Experimental Analysis of Noise Levels using Conventional Mercury and Super-Quiet Mercury-Xenon Illumination Sources

To examine the experimental performance of the super-quiet microfluorometry system, studies were performed on 6 micron diameter blue fluorescent latex beads (Polysciences, Inc., Warrington, PA). The size and color of the beads mimic the properties of the tumor cells described below. The beads were mounted in ProLong-Gold media (P36934; Invitrogen-Molecular Probes, Eugene, OR), which hardens (thereby preventing the beads from moving or vibrating during a recording) and inhibits photobleaching. Figure 2 shows recordings of the same field of mounted beads using this microscope and detector apparatus. One recording was collected during illumination with a conventional mercury lamp and power supply (Fig. 2, trace 1) whereas the second was illuminated using a super-quiet mercury-xenon lamp and a stabilized power supply with photofeedback regulation (Fig. 2, trace 2). Statistical analyses of the data show that the signal's measured standard deviation for the super-quiet illumination system is roughly a tenth of that observed for the conventional illumination system and less than twice the expected shot noise (Table 1).

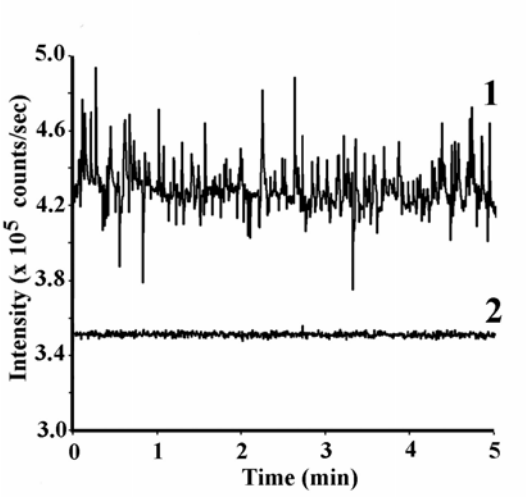

Fig. 2. Conventional Mercury-Based Illumination vs. Super-Quiet Mercury-Xenon Illumination. Two recordings of the same blue fluorescent beads were collected using different illumination techniques; the results were plotted as fluorescence intensity (counts/sec) vs. time (min). Trace 1 shows a recording collected using a conventional mercury illumination system whereas trace 2 is a recording of the same field using super-quiet mercury-xenon illumination. Corresponding error calculations are given in Table 1.

Table 1. Quantitative Fluorescence Measurements. Quantitative analysis of noise produced by the illumination systems. The mean intensities and standard deviations are shown for data of Figure 2. Although the expected shot noise for these two illumination systems are very similar under these conditions, the measured standard deviation is ten times greater for the conventional mercury illumination system.

	Conventional Mercury Illumination	**Super-Quiet Mercury-Xenon Illumination**
Mean Intensity (cps)	**4.36×10^5**	**3.47×10^5**
Standard Deviation (cps)	**1.06×10^4**	**1.07×10^3**
Expected Quantum Noise (cps)	**6.60×10^2**	**5.89×10^2**

Microfluorometry experiments were also conducted on living cells. For living cells, it is best to use adherent cells or non-adherent cells that are firmly attached to the substrate using attachment factors such as those in cell-tak (Becton-Dickinson, Franklin Lakes, NJ). Experiments using adherent HT1080 tumor cells are shown in Figure 3. These cells were obtained from the ATCC (Manassas, VA). The kinetic data provided by conventional and super-quiet illumination are shown in Fig. 3, panel A. These recordings show NAD(P)H autofluorescence intensity as a function of time for HT-1080 tumor cells. Cells were grown on cover-glass bottom culture dishes then the culture medium was replaced with HBSS (Invitrogen, Carlsbad, CA). Cells were studied at 37°C on a heated stage using a 32x/0.4NA objective. This objective was chosen because large changes in cell shape would not affect the measured fluorescence intensity [e.g., 7]. The results show that the super-quiet illumination system performs substantially better in cell studies than the conventional bulb and power supply system.

4. Detection of Metabolic Perturbations Using Super-Quiet Hg-Xe Illumination

To determine whether super-quiet microfluorometry can detect small perturbations in NAD(P)H autofluorescence, tumor cells were incubated in the absence or presence of metabolically-active

compounds. In Fig. 3B, trace 1, a control recording of a field of untreated cells is shown. In Fig. 3B, trace 2, 2-deoxy-D-glucose was added to HT1080 cells at a final concentration of 20 mM (arrow), resulting in an increase of NAD(P)H intensity. Fig. 3B, trace 3, shows the effect of adding NaCN to a final concentration of 4 mM (arrow). In this case, NaCN causes the NAD(P)H intensity to slowly decline. This behavior of HT1080 cells may be due to the fact that tumor cells have aberrant metabolic energy production [e.g., 8]. Although these changes are well-resolved with the super-quiet microfluorometry system, they would be difficult to detect with a conventional system.

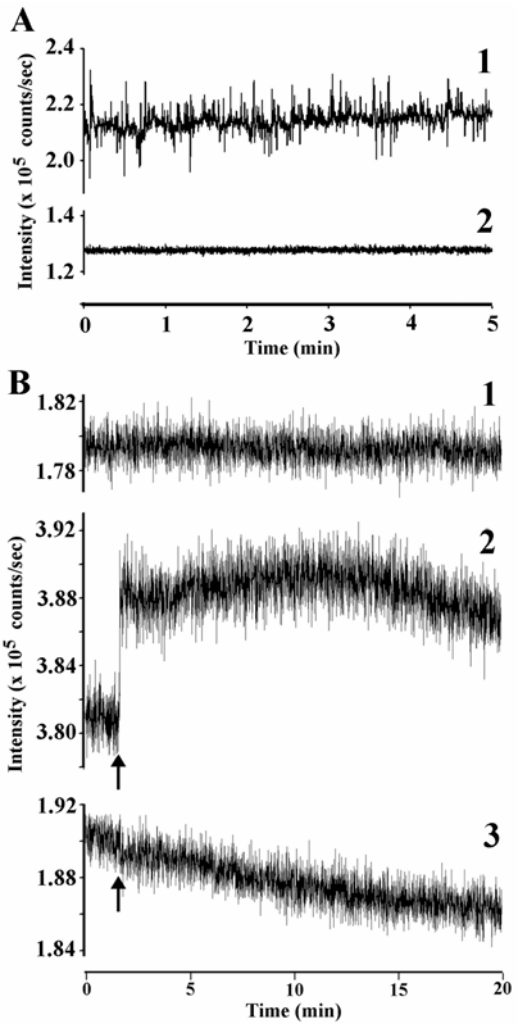

Fig. 3. NADPH autofluorescence in HT-1080 Tumor Cells. NAD(P)H auto-fluorescence recordings of HT-1080 tumor cells were plotted as intensity (counts/sec) vs. time (min). Panel A shows NAD(P)H autofluorescence in resting cells illuminated by a conventional Hg lamp (trace 1) and illuminated with a super-quiet mercury-xenon system (trace 2). The improved performance of the super-quiet illumination system in comparison to standard microscope illumination is apparent. Panel B shows NAD(P)H autofluorescence changes following addition of metabolically-active compounds. Trace 1 shows a 20 minute recording of an untreated cell. In Trace 2 the reagent 2-deoxy-D-glucose was added (arrow) to cells at a final concentration of 20mM. Trace 3 shows cells during NaCN added (arrow) to a final concentration of 4mM. It should be noted that the ordinate scales in panels A and B differ.

5. Discussion

In the present study we have described the apparatus and methods for the precise photometric characterization of samples, including living cells, using a fluorescence microscope. Although some component fabrication and modification were performed, most of the components were obtained from well-known optical and electro-optics manufacturers. The total noise associated with fluorescence emission is only slightly greater than the expected quantum noise (the square root of the number of counts). Consequently, microphotometry experiments, especially non-ratiometric experiments, can be performed with much greater precision. As illustrated in Figure 3, this increase in precision allows the detection of small signals that would be otherwise buried in the noise of conventional systems.

Many additional applications of super-quiet microfluorometry are envisioned. Several of these applications may be:

- Improved precision in the numerous excitation ratioing experiments, such as the calcium dye fura-2.
- Detection of small cellular responses to drugs. On a larger scale, super-quiet microphotometry could be useful in drug screening applications.
- Examination of noise phenomena that may underlie cell signals, such as oscillations. In this vein, "excess" calcium noise has recently been described in calcium signaling [9].
- Rigorous computational modeling of experimental findings.
- The use of spectral analysis, wavelet analysis and other mathematical tools to extract information and biological mechanism from time series data. These tools have been used, for example, to study ion channel signaling [10].

These and other experiments requiring precise photometric measurements in real time are now possible.

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References

[1] Braddick, H. J. J. (1960) Photoelectric photometry. *Rep. Prog. Phys*. **23**, 154-175.
[2] Swatland, H. (1997) *Computer Operation for Microscope Photometry*. Boca Raton: CRC Press.
[3] Ellis, R., Wright, A. (1999) Optimal use of photomultipliers for chemiluminescence and bioluminescence applications. *Luminescence*, **13**, 11-18.
[4] Inoue, S., Spring, K.R. (1997) *Video Microscopy*, 2nd Ed. Plenum Press, NY.
[5] Hamamatsu Corp. (2005) Super-Quiet Xenon Lamp Super-Quiet Mercury-Xenon Lamp. *Product Guide.* Web site: http://sales.hamamatsu.com/assets/pdf/catsandguides/Xe-HgXe_TLSX1044E03.pdf
[6] Petty, H. R. (2007) Fluorescence microscopy: Established and emerging methods, experimental strategies, and applications in immunology. *Microsc. Res. Tech*., in press.
[7] Salzberg, B. M., Grinvald, A., Cohen, L. B., Davila, H. V., Ross, W. N. (1977) Optical recording of neuronal activity in an invertebrate central nervous system: Simultaneous monitoring of several neurons. *J. Neurophys.* **40**, 1281-1291.
[8] Fantin, V., St-Pierre, J., Leder, P. (2006) Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell*, **9**, 425-434.
[9] Melamed-Book, N., Kachalsky, S., Kaiserman, I., Rahamimoff, R. (1999) Neuronal calcium sparks and intracellular calcium "noise". *Proc. Natl. Acad. Sci*. **96**, 15217-15221.
[10] Payne, R. (1981) Suppression of noise in a photoreceptor by oxidative metabolism. *J. Comp. Physiol*. **142**, 181-188.