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Determination of macromolecular exchange and PO_2 in the microcirculation: a simple system for *in vivo* fluorescence and phosphorescence videomicroscopy

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Abstract

We have developed a system with two epi-illumination sources, a DC-regulated lamp for transillumination and mechanical switches for rapid shift of illumination and detection of defined areas (250-750 μm^2) by fluorescence and phosphorescence videomicroscopy. The system permits investigation of standard microvascular parameters, vascular permeability as well as intra- and extravascular PO_2 by phosphorescence quenching of Pd-meso-tetra (4-carboxyphenyl) porphine (PORPH). A Pechan prism was used to position a defined region over the photomultiplier and TV camera. In order to validate the system for *in vivo* use, *in vitro* tests were performed with probes at concentrations that can be found in microvascular studies. Extensive *in vitro* evaluations were performed by filling glass capillaries with solutions of various concentrations of FITC-dextran (diluted in blood and in saline) mixed with different amounts of PORPH. Fluorescence intensity and phosphorescence decay were determined for each mixture. FITC-dextran solutions without PORPH and PORPH solutions without FITC-dextran were used as references. Phosphorescence decay curves were relatively unaffected by the presence of FITC-dextran at all concentrations tested (0.1 µg/ml to 5 mg/ml). Likewise, fluorescence determinations were performed in the presence of PORPH (0.05 to 0.5 mg/ml). The system was successfully used to study macromolecular extravasation and PO_2 in the rat mesentery circulation under controlled conditions and during ischemia-reperfusion.

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- Phorphyrin

Intravital microscopy has become a standard technique for investigating microvascular phenomena. In the past, most studies measured only variables such as internal diameter and red blood cell velocity. More recently, investigators have been using fluorescence intravital microscopy for studying microvascular permeability to small and large molecules (1-3), leukocyte-endothelium interaction (4,5), tissue mitochondrial bioenergetics (6), and oxidative stress (7). Fluorescein isothiocyanate (FITC) is probably the fluorescent dye most frequently used to label dextran or albumin. Recently, phosphorescence has also been used in microcirculation studies. Noninvasive measurement of PO_2 can be obtained using phosphorescence signals from a metalloporphyrin probe

(8). Using a new investigating tool, Torres Filho et al. (9,10) measured intravascular and extravascular PO_2 in small tissue areas. This PO_2 measurement system has been implemented also in other laboratories (6,11).

Due in part to the specific nature of light sources and collection devices, it is difficult to perform physiological studies using the same set-up for bright-field microscopy as well as fluorescence and phosphorescence. While there have been previous reports of systems capable of measuring time-resolved luminescence (12,13), there are no reports of such a system using FITC and metalloporphyrins in the microcirculation. Therefore, the aim of the present study was to implement a simple system that allows a rapid shift for illumination and capture of well-defined areas using fluorescence and phosphorescence microscopy as well as transillumination. The system allows quantitative on-line evaluation of intra- and extravascular PO_2, macromolecular extravasation as well as blood vessel diameter and length. In order to allow *in vivo* evaluations, *in vitro* and *in vivo* tests were performed with fluorescence and phosphorescence probes in concentration ranges that can be found in the microvascular studies. We also investigated whether fluorescence determinations were affected by the presence of the phosphorescence probe and whether phosphorescence evaluations were affected by the presence of FITC.

Figure 1 schematically illustrates the system used to measure fluorescence and phosphorescence in small regions. All studies were performed using a Leitz Orthoplan microscope equipped with an epi-illumination system (Ploemopak; Leitz, Wetzlar, Germany), a tungsten-halogen source for transillumination and a bright-field substage condenser. The epi-illumination light sources were a high-pressure mercury vapor lamp (HBO50, Aus Jena, Jena, Germany) and a xenon strobe arc (EG&G Electro Optics, Salem, MA, USA). A mechanical switch allowed rapid shifts of epi-illumination sources. The epi-illumination head included three rapidly interchangeable cubes: one for observations during transillumination and two additional cubes with appropriate dichroic mirrors as well as excitation and emission filters for fluorescence and phosphorescence microscopy (see below).

A specially designed optical adapter allowed the image magnified by the objective to be projected either to a charge coupled device (CCD) camera (model STPM314, Sony) or to a photomultiplier (model 77344, Oriel, Stratford, CT, USA). The camera had a sensitivity of 0.5 lux (F1.2) A Pechan prism image rotation unit (Hoptik, Tucson, AZ, USA) was inserted into the optical path, allowing better positioning of the region of interest. Observations were made using water immersion objectives (Zeiss 20X, NA = 0.50; Zeiss 40X, NA = 0.75; Leitz 55X, NA = 0.84) yielding final magnifications of 1230-3080 times on the screen of the video monitor (model WV5400BN, National, Tokyo, Japan). The horizontal resolution of the monitor was 700 lines at center. These magnifications were equivalent to 2-5 raster lines per μm.

The phosphorescence decay technique permits noninvasive measurements of PO_2. The technique has been described in detail previously (9,10). Briefly, palladium (Pd)-meso-tetra (4-carboxyphenyl) porphine (Porphyrin Products, Inc., Logan, UT, USA) previously bound to albumin was used as a probe. Phosphorescence was excited with a xenon strobe arc flashed at 10 Hz. The filters used for excitation of the porphyrin and phosphorescence emission were carefully cut to fit the cube inserted into the Ploemopak system. These filters were selected to match the excitation and emission peaks of porphyrin, i.e., 416 and 687 nm, respectively (8). The excitation light passed through a narrow band pass interference filter (peak at 420 nm) and an adjustable rectangular slit to illuminate an area of 5-15 x 50 μm (relative to the actual microscopic field). A focussing

lens in the optical path allowed the slit to be placed on a conjugate plane with the image. The phosphorescence emission from the epi-illuminated area passed through a light filter with cut off at 630 nm before being captured by a photomultiplier (model 77344, Oriel), with a peak radiant sensitivity (approximately 70 mA/W) in the 600-800 nm range. The signals from the photomultiplier were sent to an oscilloscope (model 54603B, Hewlett-Packard). The oscilloscope averaged 10-40 curves, yielding a single smoothed curve after 1-4 s that was then digitized and stored for later analysis. Each smoothed phosphorescence decay curve was computer processed for the calculation of PO_2.

The PO_2 of a given area was calculated by mathematically fitting the decay of phosphorescence to a single exponential using a conventional least squares method. The time constant τ of the fitted exponential curve was used to calculate PO_2 according to the Stern-Volmer equation:

$$\tau_0/\tau = 1 + k_q \times \tau_0 \times PO_2$$

where τ_0 and τ are the phosphorescence lifetimes in the absence of O_2 and in the area

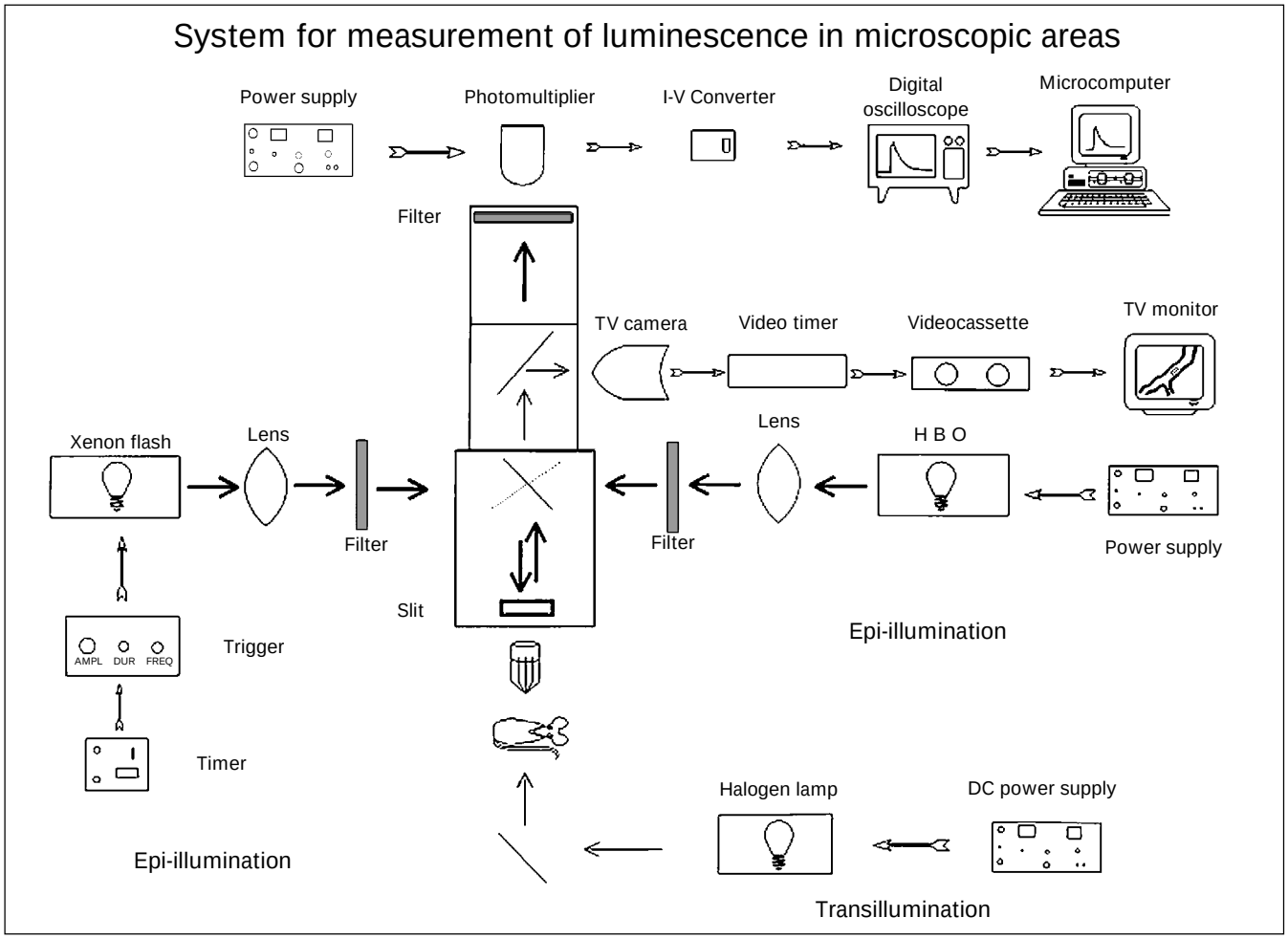

Figure 1 - Schematic diagram of the system used to measure fluorescence and phosphorescence *in vitro* and *in vivo*. The timer permits activation of a xenon flash during specific time intervals. Collector lenses placed after epi-illumination light sources allow maximum efficiency. The image of the slit can be focussed (using epi-illumination) on the same plane as the preparation and observed together with the transilluminated area. The position and size of the slit are verified through a calibrated microscale of the microscope ocular (not shown). Images captured by the TV camera are used to obtain morphometric data and off-line red blood cell velocity. The filter placed before the photomultiplier was changed according to the type of radiation under study.

being analyzed, respectively, and k_q is the quenching constant. Appropriate values for τ_0 and k_q (8,9) were used according to the probe and to the temperature of the area under study.

Photometric evaluation of the fluorescence emitted by extravasated FITC allows estimation of microvascular permeability. To elicit fluorescent signals, the area was illuminated with a 50-W mercury lamp. Photobleaching was avoided by keeping the light exposure time to 1-3 s. The filter system for fluorescence evaluation included a band pass filter (420-490 nm) and an emission filter with cut off at 515 nm. The excitation light passed through the same adjustable rectangular slit used for the phosphorescence studies. The fluorescence emission from the epi-illuminated area was also captured by the photomultiplier. The signals were sent to an oscilloscope, digitized and stored for later computer analysis. Since fluorescent light intensity is proportional to FITC-dextran concentration, the integrated optical density of the illuminated area after background subtraction would be proportional to the total amount of tracer molecules within the area. Background levels were obtained by measuring the light intensity in a given epi-illuminated area before the addition of the fluorescent compound.

In vitro studies were performed using blood collected from two male Wistar rats anesthetized with sodium pentobarbital and kept in heparinized blood collection tubes. Solutions with different concentrations (0.1 µg/ml to 5 mg/ml) of FITC-dextran (MW 150,000; Sigma Chemical Co., St. Louis, MO, USA), diluted in whole blood and in saline and mixed with different amounts of porphyrin were prepared. Final concentrations of porphyrin were 0.05 to 0.5 mg/ml. Glass capillaries (inner and outer diameters = 0.5 and 0.9 mm, respectively) were filled with these solutions, sealed and placed in the microscope system for the determination of fluorescence and phosphorescence. FITC-dextran solutions without porphyrin and porphyrin solutions without FITC-dextran were used as references. Background measurements were also obtained with glass capillaries filled only with blood or saline. Two capillaries were prepared for each solution and all capillaries were evaluated twice in random order.

In vivo studies were performed on 4 male Wistar rats (150-200 g) acclimated to an animal care facility and maintained on standard rat chow and water *ad libitum*. After 12 h of fasting, the animals were anesthetized with sodium pentobarbital (40 mg/kg, *ip*) and the trachea was cannulated to insure a patent airway. The left and right femoral veins were cannulated using polyethylene catheters (PE 50). The superior mesenteric artery was isolated and a snare was placed around it using a polyethylene tube. The rat mesentery was prepared for intravital microscopy as described in detail earlier (14). Briefly, after abdominal midline incision, the mesentery was carefully spread over a Lucite pedicle of a specially designed acrylic board and covered with thin plastic film to avoid drying and exposure to atmospheric gases. The Lucite pedestal consisted of a chamber through which thermostatically controlled warm water was circulated continuously to maintain the temperature at 37°C. Observations were performed in animals spontaneously breathing room air.

The board was attached to the microscope stage and the mesenteric tissue image was projected onto the TV camera connected to a videotape recorder (model SLV88HFBR, Sony), a video timer (model VTG33, For A) and a video monitor. A schematic drawing of the microvascular anatomy in the area under observation was made to facilitate later analysis. Each field was recorded under transmitted light for at least 1 min before or after each PO_2 determination. The temperature of the area being studied was periodically measured using a thermocouple placed over the pedestal (YSI Co., Inc., Yellow Springs, OH,

USA). Microvessel inner diameters were measured off-line from videotape recordings with a calibrated digital caliper (Mitutoyo).

In order to evaluate the influence of FITC on PO_2 measurements, each animal received a slow (3-5 min) intravenous injection of Pd-meso-tetra (4-carboxyphenyl) porphine previously bound to albumin. The dose used was 15 mg/kg body weight at a concentration of 18 mg/ml. A series of intravascular PO_2 determinations were performed and the animals then received FITC-dextran (50 mg/kg body weight) intravenously at a concentration of 50 mg/ml. After 5 min, PO_2 determinations were repeated at the same locations. In order to evaluate the influence of porphyrin on fluorescence measurements, each rat received an intravenous injection of FITC-dextran (50 mg/kg body weight) at a concentration of 50 mg/ml. After a period of at least 20 min, a series of fluorescence determinations were performed in intravascular and perivascular areas before, during and after ischemia. Changes in microvascular permeability were provoked by subjecting the mesentery to an ischemic period of 20 min followed by reperfusion. Ischemia was induced by occluding the previously isolated superior mesenteric artery. The animals then received the phosphorescence probe (15 mg/kg body weight at a concentration of 18 mg/ml). After a period of 5 min, fluorescence determinations were repeated at the same locations.

The switch for illumination and emission between fluorescence and phosphorescence set-ups was mechanically simple, took about 10-15 s, but precluded simultaneous measurements. The *in vitro* calibration showed that the system was linear for fluorescence measurements and FITC-dextran concentrations ranging from 0.1 µg/ml to 5 mg/ml yielded average light intensities (which were measured as voltages) from 10^1 to 10^4 mV. The analysis of 80 capillaries filled with test solutions (320 luminescence measurements) showed that phosphorescence decay measurements were unaffected by the presence of FITC-dextran at all concentrations tested (0.1 µg/ml to 5 mg/ml). Likewise, *in vitro* fluorescence determinations were unaffected by the presence of porphyrin at all concentrations tested (0.05 to 0.5 mg/ml).

Similar findings were obtained using the mesentery *in vivo*. Intravascular and extravascular measurements of fluorescence were unaffected by the injection of porphyrin (Figure 2, top panel). Ischemia-reperfusion caused the extravasation of FITC-dextran to perivascular spaces. This increase in permeability could be monitored by measuring the fluorescence in well-determined regions. Since optical characteristics of *in vivo* preparations are different from the *in vitro* set-up, absolute values of light intensity cannot be directly translated into absolute values of FITC-dextran levels *in vivo*. However, the linearity of the system is preserved and fluorescent light intensities measured *in vivo* can be readily used to express relative changes in intra- and extravascular FITC-dextran concentrations.

Intravascular and extravascular measurements of phosphorescence were unaffected by the injection of FITC-dextran. An example of an intravascular PO_2 measurement under control conditions is presented in Figure 2 (lower panel). In addition, we noted that in some poorly perfused areas, late injection of porphyrin would not allow measurements of interstitial PO_2 since the leakage of the probe under these conditions would be too small.

We have implemented a simple and versatile system for studying several variables at the microcirculatory level. The videomicroscope system included two different epi-illumination sources and a DC-regulated trans-illumination lamp. The system allows the investigation of morphological and micro-hemodynamic parameters, leukocyte-endothelium interaction, as well as noninvasive determination of PO_2.

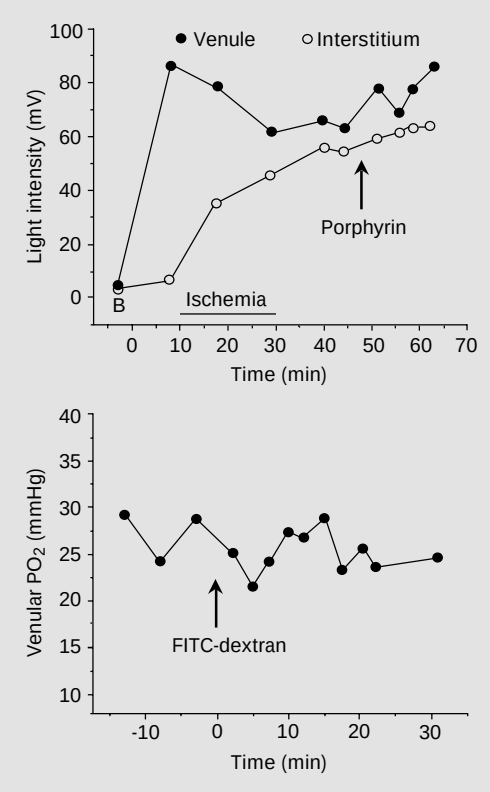

Figure 2 - Examples of luminescence measurements from two *in vivo* experiments in the rat mesentery preparation. *Top panel*, Typical curves of fluorescence measurements obtained before, during and after mesenteric ischemia in one experiment. B represents background measurements before FITC-dextran injection. The curve on top was obtained from measurements in a 40-µm venule. The bottom curve presents fluorescence from a tissue area as a result of increased levels of extravasated FITC-dextran. The arrow indicates the *iv* injection of Pd-meso-tetra (4-carboxyphenyl) porphine (15 mg/ml). *Bottom panel*, Typical curve showing PO_2 levels from a 45-µm venule before and after *iv* injection of FITC-dextran in one experiment.

There have been previous reports on systems capable of measuring fluorescence and phosphorescence (12,13) but only a few have been applied to microvascular preparations (6,15,16). These systems utilize more sophisticated approaches, ranging from acousto-optic tunable filters and phase-locked mechanical choppers to slow-scan CCD cameras and gated multichannel plate image intensifiers. A major advantage of the present system is that quantitative fluorescence and phosphorescence may be performed in the same precise microregion using a microscope normally found in most microcirculation research laboratories. Therefore, on-line determinations of PO_2 can be done in regions following changes in microvessel permeability. Assembly, calibration, data collection and analysis are rather straightforward, basically requiring the computation of signals produced by a photomultiplier inserted into the optical path.

Previous investigators have used rectangular fields to determine integral optical density (1). A similar procedure has been employed in our study, since only a particular region was excited by epi-illumination. This is advantageous also because the total tissue area exposed to strong illumination is minimized. In the case of PO_2 measurements, this procedure of limiting illumination has been successfully implemented earlier (11).

Once injected into the circulation, fluorescent and phosphorescence probes reach plasma and interstitial concentrations that have been previously estimated (17). In order to validate the system for *in vivo* studies, *in vitro* tests were performed with probes within the concentration ranges that can be found in blood and interstitium. We observed that phosphorescence decay curves were relatively unaffected by the presence of FITC-dextran at all tested concentrations (0.1 µg/ml to 5 mg/ml). Likewise, fluorescence determinations could be performed in the presence of porphyrin (0.05 to 0.5 mg/ml). This is not surprising since the decay time of the porphyrin phosphorescence is long enough to avoid the prompt fluorescence while maintaining sufficient data for PO_2 determination.

A complete evaluation of mesenteric microvessel permeability was beyond the scope of the present study. However, following appropriate calibrations and adequate fluorescence determinations at precise times and locations, we showed that the system can be readily used to estimate microvessel permeability. Macromolecular extravasation following ischemia-reperfusion, as used in the present study, has been used by several authors (18,19).

The system may be further improved in several ways. A three-wavelength labeling method could be used, for instance, by employing porphyrin for PO_2 determinations, rodamine labeling for white cell visualization, and fluorescein markers for blood plasma. Since a closed circuit TV system is available, video-image digital processing may

also be employed, as described previously (1,7,20). Once appropriate analysis is performed, a single illumination source could be used for all luminescence measurements and a multiple wavelength filter cube could be developed to detect both fluorescence and phosphorescence. These improvements would lead to a faster detection system since the switch between fluorescence and phosphorescence light sources and filter cubes would be eliminated.

In summary, a system is described which permits *in vitro* and *in vivo* quantitative fluorescence and phosphorescence in well-defined microregions. On-line determinations of PO_2 and macromolecular extravasation can be made after changes in microvessel permeability. Preliminary experiments using the rat mesentery confirmed this versatility.

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