



## INTERNALIZATION OF NEAR-INFRARED FLUORESCENT DYES WITHIN ISOLATED MACROPHAGE POPULATIONS

C. T. REESE<sup>1</sup>, C. NTAM<sup>1</sup>, T. V. MARTIN<sup>1</sup>, S. CARRINGTON<sup>2</sup>, J. LEOTAUB<sup>2</sup>, L. COX<sup>2</sup>, R. J. WILLIAMS<sup>2</sup> AND D. A. HILL<sup>1</sup>✉

Departments of Biology<sup>1</sup> and Chemistry<sup>2</sup>, School of Computer, Mathematical and Natural Sciences, Morgan State University, Baltimore, Maryland 21251

<sup>1</sup>✉ Dwayne Hill, Morgan State University, The School of Computer, Mathematical and Natural Sciences, Department of Biology, 1700 E. Cold Spring Lane, Baltimore, MD 21251. Phone 443 885 1865 Fax: (443) 885-8285 email: hilllab@yahoo.com

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**Abstract-** The development and application of microsensor technology has enhanced the ability of scientists to further understand various biological activities, such as changes in the intracellular environment after injury or toxic exposure. NIR microsensor technology may be useful in detecting the cellular injuries or adverse changes during the early onset period, allowing for the administration of therapies to initiate recovery. The development and use of Infrared (IR) and near infrared (NIR) dyes as biological micro-sensors due to their advanced spectral characteristics may be helpful. Three of the more useful NIR dye characteristics include the ability to minimize background interference by extraneous biological matrices, the ability to exhibit optimal molar absorptivity and quantum yields, and the ability to maintain normal cellular activity. Thus, the current study was designed to investigate the ability of selected NIR micro-sensor dyes to undergo cellular internalization, demonstrate intracellular NIR fluorescent signaling, and maintain normal cellular activity. The results demonstrate that the selected NIR micro-sensor dyes undergo cellular internalization. The presence of the dyes within the cells did not affect cell viability. In addition, these dyes demonstrate changes in absorbance and fluorescence after the immune cells were challenged with a stimulant. Moreover, critical cellular functions, such as tumor necrosis factor release and superoxide production were not compromised by the internalization of the fluorescent dyes. These data suggest that selected NIR micro-sensor dyes can undergo intracellular internalization within isolated macrophages without adversely affecting various parameters of normal cellular activity.

**Key words:** Fluorescent, Dyes, Microsensor, Macrophages, Near-Infrared, Internalization

### INTRODUCTION

Environmental contamination has surged in parallel with industrial expansion. Chronic or acute exposure to these contaminants could lead to adverse tissue or intra-cellular activity. Thus, scientists have endeavored to develop innovative approaches that will indicate the occurrence of potential adverse intra-cellular activity. One such approach is the development of fluorescent microsensors that can detect intracellular changes. Fluorescence is an energy phenomenon that involves the photoemission from excited molecules as their excited electrons return to their ground states (1, 2). When light is absorbed by a photoluminescent molecule, electrons in the molecule are excited from the lowest energy

level, or ground state, to one of the excited energy levels. When the electron returns to the ground state, the absorbed energy is either emitted through a nonradiative process or a radiative one. If the process is nonradiative, the energy is either transferred to another molecule or it is released as heat. If the process is radiative, energy is released as a photon with a wavelength characteristic of the emitted energy (1, 3). Because of the nature of its photon emission, fluorescence is highly sensitive to protein structures and energy transfer to proteins. Thus the present study was designed to determine if the selected NIR dyes can undergo intracellular internalization within macrophage cultures without adversely affecting various parameters of normal cellular activity.

## MATERIALS AND METHODS

### *Isolation of Macrophages.*

Macrophages were isolated from male (retired breeder) Sprague-Dawley rats (Charles River Laboratories) using pulmonary lavage (5, 6). Briefly, the tracheas of anesthetized rats (pentobarbital) were cannulated. Bronchoalveolar lavage was performed with calcium- and magnesium-free Dulbecco's phosphate-buffered saline (DPBS) containing 1% glucose. Lungs were massaged during the recovery of lavage fluid to aid in exfoliating attached macrophages from the airway epithelium. Lavage fluid (50 ml) from both lungs was collected in polypropylene tubes (cold) and centrifuged (10 minutes, 400 x g). The cell pellets were washed twice and resuspended in DPBS. Erythrocytes contaminating the cell preparation were lysed with ammonium chloride solution. The cell suspension was then layered over a percoll gradient and centrifuged (25 minutes, 400 x g) at 25°C. Macrophages were recovered in the broad upper-band. Purity of isolated fractions was determined using peroxidase stain analysis ( $\geq 95\%$ ). In addition, viability was assessed by eosin Y exclusion analysis ( $\geq 98\%$ ). Cell yields were typically  $\geq 88 \times 10^6$  cells per animal.

### *Preparation of fluorescent dyes.*

Calcein, fluorescein, dye 780 and dye 786 are commercial dyes that were purchased from (SigmaAldrich, St. Louis, MO). Calcein and fluorescein were used as control dyes for cellular internalization, while dye 780 and 786 were the test dyes for cellular internalization. The crystalline form of the dye was dissolved in .05% DMSO and 99.95% William's Medium E. The dyes were subjected to several centrifugal elutriations in which the supernatant was retained and the pelleted debris was removed. The remaining supernatant was filtered through a Biopore filter sieve (SigmaAldrich, St. Louis, MO). The filtrate was collected, incubated at 40°C for 1 hour and then refrigerated (4°C).

### *Administration of fluorescent dyes to isolated macrophages.*

Macrophages were isolated and cultured (Williams' Medium-E containing gentamycin and supplements) in six-well plates at a density of  $1 \times 10^7$  cells/well. Either Calcein, fluorescein, dye 780 or dye 786 was added to cultures at a final concentration of 20  $\mu\text{M}/1 \times 10^7$  cells and incubated for 24 hours. After this 24 hour incubation, the culture medium was collected, sonicated and centrifuged at room temperature for 15 minutes at 120 x g to remove cellular debris, then refrigerated at -20°C. This medium was then analyzed for fluorescent dye content. Next, macrophages were removed from the wells by .02% trypsin, washed and re-suspended in PBS, then analyzed for fluorescent dye content and macrophage function.

### *Administration of stimulant to isolated macrophages.*

Macrophages were plated ( $1 \times 10^7$  cells/well) in Falcon 3046, six-well culture plates and allowed to adhere for 4 hours before treatment with LPS (lipopolysaccharide) at nominal concentrations of 6.25, 12.5, 25 or 50  $\mu\text{M}$  or its vehicle for 6, 12, 18, or 24 hours. After this time, the medium was collected, centrifuged at room temperature for 15 minutes at 120 x g to remove cellular debris, then refrigerated at -20°C. The conditioned medium was analyzed for LDH activity and trypan blue exclusion/eosin

Y uptake as indicators of cellular toxicity and viability, respectively.

### *Measurement of intracellular absorptivity.*

The concentration detection limits for the dyes were determined using a Cary Eclipse Fluorometer (Varian Instruments, Palo Alto, CA) equipped with a red sensitive R928S photomultiplier tube (PMT). From the detection limit concentrations, an optimal concentration range centered near  $7.5 \times 10^{-8}$  mol was adopted for this study. The macrophages were inoculated with  $10^{-7}$  molar dye solution ( $< 0.2\%$  EtOH, DMSO). In addition, fluorescence readings were performed using a Cary 100 Spectrophotometer (Varian Instruments, Palo Alto, CA). Samples were transferred by Pasteur pipette to standardized 3.4 mL disposable polystyrene fluorescence cuvetts (Fisher Scientific, Suwanee, GA).

### *Measurement of intracellular fluorescence.*

After the treatment and incubation period, separated cell solutions and medium solutions, were analyzed (triplicate) for absorbance and fluorescence. Absorbance scans (400-900 nm) were performed on both cell and medium solutions to quantitatively identify the presence of dye and possible interferents. Dyes were identified by their absorbance maximum wavelengths and their concentration in each sample was determined by their molar absorptivities (Aldrich). The absorbance maximum wavelengths were used as the excitation wavelengths in the subsequent fluorescence analysis. Maximum emission wavelengths were determined from the emission spectra obtained from scans performed on the initial sample in a triplicate set of samples. The fluorescence intensity for the remaining samples were obtained as single reads from the previously determined maximum emission wavelengths.

### *Measurement of macrophage-derived tumor necrosis factor (TNF) release.*

Macrophages ( $1.0 \times 10^7$  cells/well) were treated with vehicle, NIR or visible dyes, and incubated for 24 hours. After this incubation period the macrophage-derived conditioned medium was collected for measurement of TNF levels using ChimiKine ELISA kits (Chimicon, Temecula, CA) and a spectrophotometric plate reader (450 nm).

### *Measurement of superoxide release by macrophages.*

Macrophage-derived superoxide production after treatment with microsensors was measured by reduction of cytochrome c (100,  $\mu\text{M}$ ; horse heart, type III, SigmaAldrich, St. Louis, Mo), followed by monitoring the change spectrophotometrically (absorbance - 550 nm). The rapid reduction of cytochrome C by macrophage-derived superoxide was calculated as nmol of superoxide formed via extinction coefficient of  $21 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  but expressed as % positive control (LPS; lipopolysaccharide). The production of superoxide in response to LPS after microsensor treatment was completed after a 25 minute incubation and ended via superoxide dismutase (30 units/ml; SigmaAldrich, St. Louis, Mo) administration (10).

### *Chemotaxis of macrophages.*

A two chambered chemotaxis system was used to determine if internalization of microsensor dyes would alter macrophage chemotaxis. The upper chamber, a Falcon 3181 Cell Culture Insert (Becton Dickinson, Franklin Lakes, NJ), was separated from the lower chamber, a Falcon 3043 Multiwell Culture Plate (Becton Dickinson, Franklin Lakes,

NJ), by a cyclopore polyethylene terephthalate membrane located at the base of the insert. Macrophages ( $1.5 \times 10^6$  cells) were placed in the upper chamber, and medium (1.0 mL) containing a chemotactic agent (concanavalin A/stimulus) was placed in the lower chamber. In response to the chemotactic agent, the macrophages migrate through the membrane to the lower chamber. One hour after both chambers were loaded, the degree of chemotaxis was determined by counting the macrophages present in the lower chamber. Concanavalin A (Con A [50 nM]) was used as the positive control stimulus for chemotaxis of macrophages. Chemotaxis was expressed as % of this positive control.

*Statistical analysis.*

Results are expressed as mean  $\pm$  SEM. Data were analyzed by analysis of variance (ANOVA), and individual means were compared using Dunnett's test. Appropriate transformations were performed on data for which variances were not homogeneous. For each treatment group, cell populations were prepared from different animals and different cell isolations, respectively;  $N = 8$  for all studies unless otherwise indicated. The criterion for significance was  $p \leq 0.05$  (5).

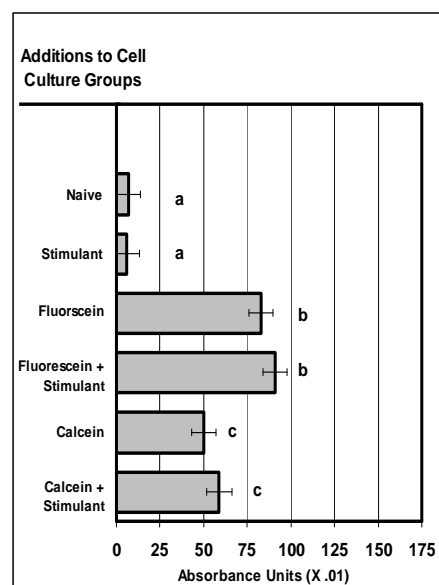
**RESULTS**

*Internalization of VIS micro-sensor dyes by macrophages*

Applying the Beers-Lambert law, the molar absorptivity of the VIS micro-sensor dyes can be used to determine the concentration of dyes associated with macrophage internalization. The relationship between the absorbance and concentration is given by (1), where  $a$  is the path length in centimeters,  $c$  is the concentration expressed as mols/mL,  $\epsilon$  is the molar absorptivity, and  $abs$  is the absorbance.

$$ac\epsilon = abs(1)$$

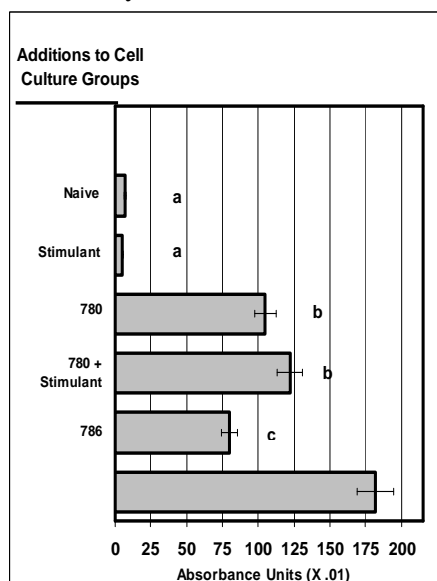
Equation 1 can be rearranged and the concentration of dyes can be calculated using the measured absorbance values associated with dye internalization by isolated macrophages. The determined concentration of the dyes indicated no significant difference in the internalization of calcein and fluorescein in non-stimulated cells. For the stimulated cells, a moderate increase was observed for fluorescein internalization when compared to calcein internalization.



**Figure 1.** VIS micro-sensor dyes are internalized isolated macrophages. Macrophage cultures were treated with either vehicle or visible-spectra micro-sensor dyes (fluorescein or calcein; 20 ug/10<sup>6</sup> cells) and incubated for 24 hours. 12 hours after treatment with the sensor dyes, the cultures were stimulated with LPS. After the complete 24-hour incubation period, macrophage cultures were analyzed for micro-sensor dye uptake. Spectral absorptivity was used as the endpoint for indicating cellular uptake. Percent values are expressed as the mean  $\pm$  SEM,  $N = 8$ . Statistical significance (a), (b) and (c) was expressed as  $P \leq 0.05$  in comparison to each other.

*Internalization of NIR micro-sensor dyes by macrophages*

When comparing the macrophage internalization of near-infrared dyes to that of the visible dyes, the internalization of near-infrared dyes was comparable or greater to that of the visible control dyes, fluorescein and calcein.

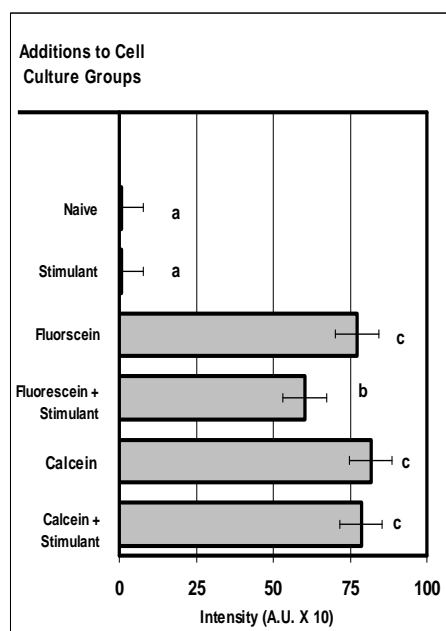


**Figure 2.** NIR micro-sensor dyes are internalized isolated macrophages. Macrophages cultures were treated with either vehicle or NIR-spectra micro-sensor dyes (NIR-786 or NIR-780; 20  $\mu\text{g}/10^6$  cells, respectively) and incubated for 24 hours. 12 hours after treatment with the sensor dyes, the cultures were stimulated with LPS. After the complete 24-hour incubation period, macrophage cultures were analyzed for micro-sensor dye uptake. Spectral absorptivity was used as the endpoint for indicating cellular uptake. Percent values are expressed as the mean  $\pm$  SEM, N = 8. Statistical significance (a), (b) (c) and (d) was expressed as  $P \leq 0.05$  in comparison to each other.

The internalization of NIR-spectra micro-sensor dyes was as much as 3-fold greater than that of visible-spectra micro-sensor dyes. While the internalization of dye 780 and dye 786 were of similar magnitude for non-stimulated macrophages, dye 786 demonstrated significantly more internalization than dye 780. for the stimulated group of macrophages.

#### *Intracellular fluorescence of VIS micro-sensor dyes after internalization by macrophages.*

The fluorescence exhibited by calcein after its internalization by macrophages, correlated well with the concentrations of calcein dye associated with dye internalization. There was no significant difference between the fluorescence associated with calcein for non-stimulated and stimulated macrophages.



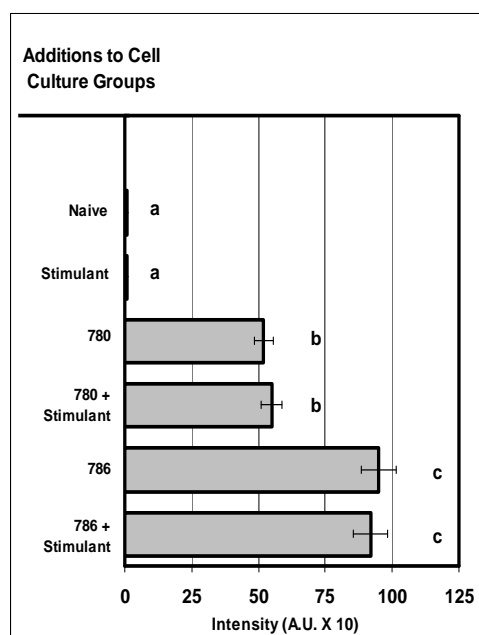
**Figure 3.** VIS micro-sensor dyes demonstrated intracellular fluorescence after internalization by macrophages. Macrophage cultures were treated with either vehicle or VIS micro-sensor dyes (fluorescein or calcein; 20  $\mu\text{g}/10^6$  cells), and incubated for 24 hours. 12 hours after treatment with the sensor dyes, the cultures were stimulated with LPS. After the complete 24-hour incubation period, intracellular

fluorescence of the VIS micro-sensor dyes was analyzed. Percent values are expressed as the mean  $\pm$  SEM, N = 8. Statistical significance (a), (b) and (c) was expressed as  $P \leq 0.05$  in comparison to each other.

Fluorescein demonstrated fluorescence intensities that did not correlate with the concentration of fluorescein associated with dye internalization. The fluorescence intensity of fluorescein demonstrated significant difference between non-stimulated and stimulated macrophages. The fluorescence associated with fluorescein in non-stimulated cells was significantly higher than the fluorescence of fluorescein in stimulated macrophages.

#### *Intracellular fluorescence of NIR micro-sensor dyes after internalization by macrophages*

In comparison to control groups, a significant degree of fluorescence was generated by visible and NIR micro-sensor dyes. The fluorescence intensity displayed by the two micro-sensor dye groups was comparable. The fluorescence intensity demonstrated by the near-infrared correlated very well with their concentration levels that were associated with macrophage internalization.

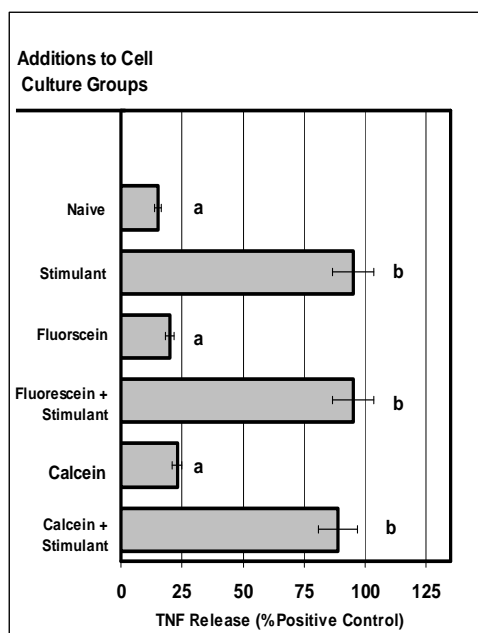


**Figure 4.** NIR micro-sensor dyes demonstrated intracellular fluorescence after internalization by macrophages. Macrophage cultures were treated with either vehicle or NIR micro-sensor dyes (NIR-786 or NIR-780; 20  $\mu\text{g}/10^6$  cells) and incubated for 24 hours. 12 hours after treatment with the sensor dyes, the cultures were stimulated with LPS. After the complete 24-hour incubation period, intracellular fluorescence of the micro-sensor dyes was analyzed. Percent values are expressed as the mean  $\pm$  SEM, N = 8. Statistical significance (a), (b) and (c) was expressed as  $P \leq 0.05$  in comparison to each other.

No significant difference between stimulated and non-stimulated cells was observed for the fluorescence intensity of either IR780 or IR786. The fluorescence intensity of IR786 was greater than the fluorescence intensity determined for IR780 in both cell populations.

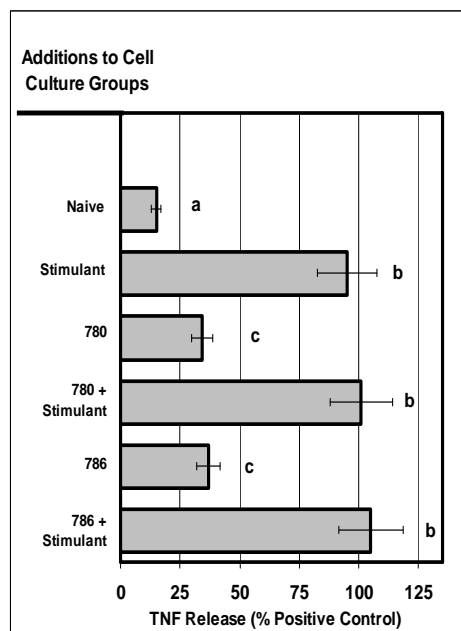
*TNF release by macrophages after internalization NIR micro-sensor dyes*

Internalization of NIR micro-sensor dyes did not alter macrophage TNF release in response to stimuli.



**Figure 5.** Internalization of VIS micro-sensor dyes did not alter TNF release in response to stimuli. Macrophage cultures were treated with either vehicle or visible micro-sensor dyes (fluorescein or calcein; 20 ug/10<sup>6</sup> cells) and incubated for 24 hours. 12 hours after treatment with the sensor dyes, the cultures were stimulated with LPS. After the complete 24-hour incubation period, the ability of macrophages to release TNF was analyzed. TNF release was used as a marker of normal macrophage function. Percent values are expressed as the mean ± SEM, N = 8. Statistical significance (a) was expressed as P ≤ 0.05.

The macrophages containing the micro-sensor dyes demonstrated a very moderate degree of TNF release similar to that of macrophages void of the microsensor-dyes.



**Figure 6.** Internalization of NIR micro-sensor dyes did not alter TNF release in response to stimuli. Macrophage cultures were treated with either vehicle or NIR-spectra micro-sensor dyes (NIR-786 or NIR-780; 20 ug/10<sup>6</sup> cells) and incubated for 24 hours. 12 hours after treatment with the sensor dyes, the cultures were stimulated with LPS. After the complete 24-hour incubation period, the ability of macrophages to release TNF was analyzed. TNF release was used as a marker of normal macrophage function. Percent values are expressed as the mean ± SEM, N = 8. Statistical significance (a) was expressed as P ≤ 0.05.

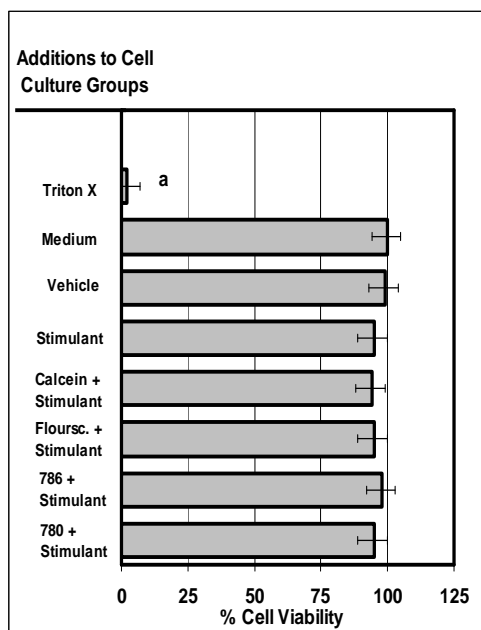
*The effect of VIS and NIR dyes on macrophage viability*

The internalization of VIS or NIR micro-sensor dyes did not alter normal cell viability (Figure 7). The macrophage populations containing the micro-sensor dyes did not exhibit any cytotoxicity as measured through the release of LDH (marker of cytotoxicity). The presence of intracellular micro-sensor dyes did not cause cellular lethality as indicated by trypan blue exclusion and eosin y uptake (markers of cell death).

*The effect of internalized dyes on macrophage super oxide production*

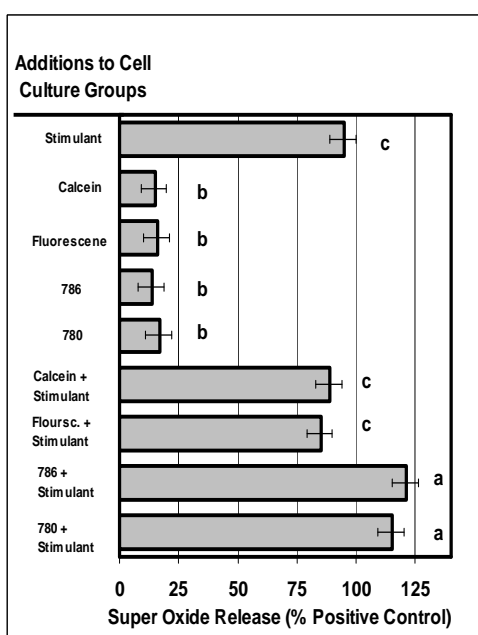
The internalization of VIS or NIR micro-sensor dyes did not alter super oxide production by macrophages in response to stimuli (Figure 8). Macrophages that had internalized the micro-sensor dyes were able to super oxide anion at levels similar to those of control macrophages.





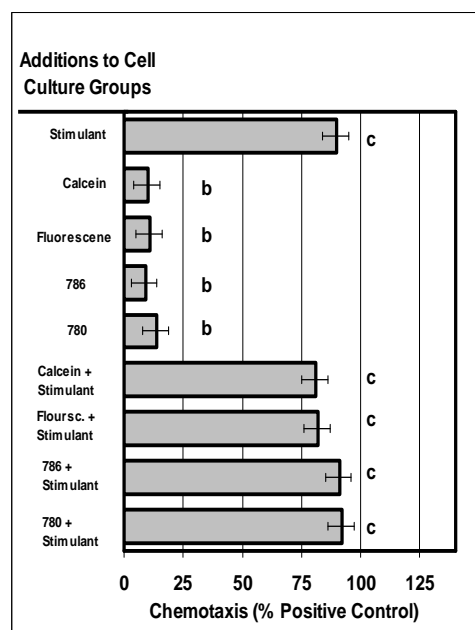
**Figure 7.** The internalization of VIS or NIR micro-sensor dyes did not alter normal cell viability. Macrophage cultures were treated with either vehicle, visible micro-sensor dyes (fluorescein or calcein; 20 ug/10<sup>6</sup> cells), or NIR micro-sensor dyes (NIR-786 or NIR-780; 20 ug/10<sup>6</sup> cells) and incubated for 24 hours. 12 hours after treatment with the sensor dyes, the cultures were stimulated with LPS. After the complete 24-hour incubation period, macrophages were analyzed for viability. Trypan blue exclusion and eosin Y uptake were used as endpoints for the determination of lethality. Percent values are expressed as the mean ± SEM, N = 8. Statistical significance (a) was expressed as P ≤ 0.05 in comparison to other groups.

*The effect of internalized dyes on macrophage chemotaxis*



**Figure 8.** The internalization of VIS or NIR micro-sensor dyes did not alter super oxide production by macrophages in response to stimuli. Macrophage cultures were treated with either vehicle, VIS micro-sensor dyes (fluorescein or calcein; 20 ug/10<sup>6</sup> cells), or NIR micro-sensor dyes (NIR-786 or NIR-780; 20 ug/10<sup>6</sup> cells) and incubated for 24 hours. 12 hours after treatment with the sensor dyes, the cultures were stimulated with LPS. After the complete 24-hour incubation period, the ability of macrophages to produce super oxide was analyzed. Super oxide production was also used as a marker of normal macrophage function. Percent values are expressed as the mean ± SEM, N = 8. Statistical significance (a), (b) and (c) was expressed as P < 0.05 in comparison to each other.

The internalization of VIS or NIR micro-sensor dyes did not alter the chemotaxis of macrophages in response to stimuli (Figure 9). Macrophages that had internalized the micro-sensor dyes were able to demonstrate a normal degree of chemotaxis in comparison to control macrophages.



**Figure 9.** The internalization of VIS or NIR micro-sensor dyes did not alter chemotaxis of macrophages in response to stimuli. Macrophage cultures were treated with either vehicle, VIS micro-sensor dyes (fluorescein or calcein; 20 ug/10<sup>6</sup> cells), or NIR micro-sensor dyes (NIR-786 or NIR-780; 20 ug/10<sup>6</sup> cells) and incubated for 12 hours. After this incubation, the macrophage chemotaxis was analyzed using a two-chambered chemotaxis system. Chemotaxis was determined when macrophages migrated from the upper chamber to the lower chamber. Chemotaxis was used as a marker of normal macrophage function. Percent values are expressed as the mean ± SEM, N = 8. Statistical significance (a), (b) and (c) was expressed as P < 0.05 in comparison to each other.

**DISCUSSION**

The current study has demonstrated that the internalization of NIR micro-sensor dyes (NIR micro-sensor dyes 780 and 786) by isolated

macrophages did not result in a cellular lethality. In addition, this internalization did not alter the normal responses of the macrophages to stimuli. Moreover, once intracellular distribution of the microsensor dyes has occurred, the spectral property of fluorescence was easily measured. These observations have demonstrated that the NIR micro-sensor dyes are not harmful once internalization within macrophage populations has occurred. Moreover, it is conceivable that the aforementioned NIR micro-sensor dyes could be modified to detect or monitor changes/alterations of intracellular activity without adversely effecting cellular homeostasis. The use of fluorescence and fluorescent constructs as indicators of intra- and extra-cellular activity is a widely used application (7, 8). The parameter of concern regarding these applications is the ability of the constructs to distribute into the intracellular environment without compromising normal cellular homeostasis. Studies conducted by Fehr (2002) have demonstrated the use of fluorescent nanosensors to monitor transport of maltose inside yeast cell populations. Another study conducted by Sato (2002) used fluorescent sensors constructs (phocuses) to indicate the degree of phosphorylation within living cell populations. These studies have provided very intriguing insights into the development and use of fluorescent sensors to monitor intracellular events with minimal cytotoxicity. One concern, regarding the construction and application of many fluorescent nanosensors is the difficulty in constructing sensors that demonstrate fluorescence or increased fluorescent intensity for specific intracellular events only, and are not committed to a constant/continuous fluorescent state. In an attempt to address this concern, a study conducted by Heo (2003) was able to construct a fluorescent dye (BCECF-AM) that exhibits extra-cellular non-fluorescence, until translocation across the cellular membrane. Once this intracellular translocation occurs, the BCECF-AM is converted into a fluorescent form (BCECF) for the analysis of intracellular activity. These developments demonstrate promise for the continued development and application of novel biosensors to detect intracellular changes after exposures to toxic agents.

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