Biosensors for Real-Time Monitoring of Radiation-Induced Biologic Effects in Space

August 2002 Report

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I. Executive Summary

This work seeks to develop cellular biosensors based on dendritic polymers. Nanoscale polymer structures less than 20 nm in diameter will be used as the basis of the biosensors. The structures will be designed to target into specific cells of an astronaut and be able to monitor health issues such as exposure to radiation. Multiple components can be assembled on the polymers including target directors, analytical devices (such as molecular probes), and reporting agents. The reporting will be accomplished through fluorescence signal monitoring, with the use of multispectral analysis for signal interpretation. These nanosensors could facilitate the success and increase the safety of extended space flight. The design and assembly of these devices has been pioneered at the Center for Biologic Nanotechnology in the University of Michigan.

This period, synthesis of the test-bed biosensors continued. Studies were performed on the candidate fluorescent dyes to determine which might be suitable for the biosensor under development. Development continued on producing an artificial capillary bed as a tool for the use in the production of the fluorescence signal monitor. Work was also done on the *in vitro* multispectral analysis system, which uses the robotic microscope.

II. Overall Progress: Organized by technical objective and task group.

Technical Objective I: Development of dendritic polymer based biosensors capable of monitoring radiation-induced changes in lymphocyte populations.

Synthesis of Biosensor
Balazs Keszler, Ph.D., Istvan Majoros, Ph.D.

We have designed the chemical structure of a fluorescence resonance energy transfer (FRET) reagent, which will detect apoptosis via the cleavage of the peptide linker by Caspase 3.

<u>Design of a FRET</u>:

- Donor and acceptor dye molecules must be in close proximity (10-100Å).
- The absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor.
- Donor and acceptor transition dipole orientations must be approximately parallel.

The distance at which energy transfer is 50% efficient (i.e., 50% of exited donors are deactivated by FRET) is defined by Förster radius (R_o). The magnitude of R_o is dependent on the spectral properties of the donor and acceptor dyes:

$$R_o = [8.8 \times 10^{23} * \kappa^2 * n^4 * QY_D * J(\lambda)]^{1/6} \ \text{Å}$$

Where $\kappa^2 =$ dipole orientation factor (range 0 to 4; $\kappa^2 = 2/3$ for randomly oriented donors and acceptors)

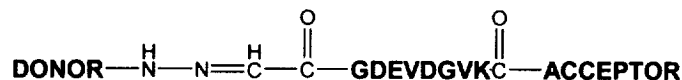$QY_D =$ fluorescence quantum yield of the donor in the absence of the acceptor

$n =$ refractive index

$J(\lambda) =$ $\int(\varepsilon_A(\lambda)*F_D(\lambda)*\lambda^4)d\lambda$,$cm^3 M^{-1}$ — spectral overlap integral of the absorption spectrum of the acceptor and fluorescence emission spectrum of the donor

where $\varepsilon_A =$ extinction coefficient of acceptor

$F_D =$ fluorescence emission intensity of donor as a fraction of the total integrated intensity

The structure of the proposed apoptosis FRET reagent is:

$$\text{DONOR}-\overset{H}{N}-N=\overset{H}{C}-\overset{\overset{\displaystyle O}{\|}}{C}-\text{GDEVDGVKC}\overset{\overset{\displaystyle O}{\|}}{}-\text{ACCEPTOR}$$

Where our donor dye is 6-carboxy-2',7'-dichlorofluorescein hydrazide (Ex: 505 nm and Em:525 nm) and our acceptor dye is 5-carboxytetramethylrhodamine (Ex: 550 nm and Em:570 nm). They have a 55 Å Förster radius.
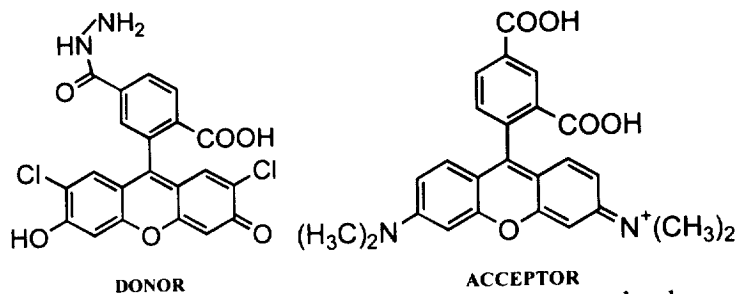
Figure 1. The structure of the donor and acceptor molecules.

The donor dye molecule will be covalently attached to the oligopeptide through the hydrazide group, while the acceptor molecule will be attached to the primary amine of lysine.
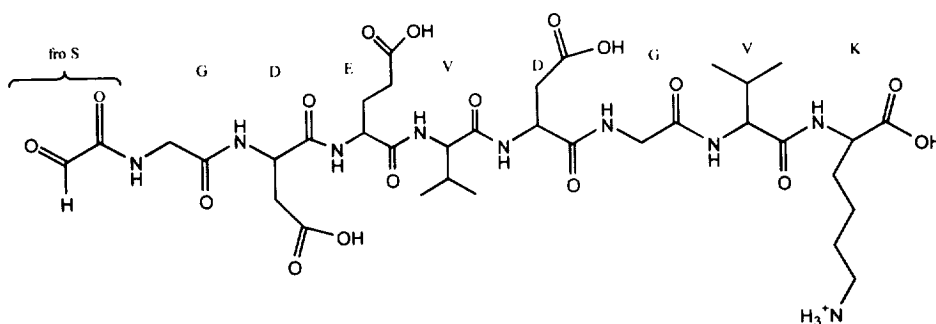

Figure 2. The structure of the peptide linker.

Conjugation of MitoTracker® dyes to G5 dendrimer:

In this period we have conjugated MitoTracker® Red CMXRos (M-7512) and MitoTracker® Deep Red (M-22426) mitochondrion-selective dyes to the dendrimer. We have followed the procedure we developed for the model reaction described in our previous report. We have prepared these biosensors with folic acid assuring specific targeting. We also prepared G5-dye conjugates without specific targeting moiety. These materials serve as control material.

Conjugation of MitoTracker® Red CMXRos (M-7512) to G5 dendrimer: We used partially acylated G5 dendrimer for the conjugation, with 86 out of 110 primary amine groups acylated. 0.00182 g (6.14×10^{-8} mol) of G5-$(NH_2)_{24}$-$(OCH_3)_{86}$ was dissolved in 1 mL of PBS buffer (pH=8). First 0.00026 mL (1.84×10^{-6} mol, 10x molar excess of the dye) of Et$_3$N, then 100 μg (1.84×10^{-7} mol) of dye dissolved in 0.5 mL of DMSO was added to the dendrimer solution. The mixture was stirred for 48 hours, dialyzed in water-methanol (2:1), then in DI water. After dialysis, TLC showed no traces of free dye. The dye-to-dendrimer ratio was determined by UV spectroscopy to be 1:1.

Conjugation of MitoTracker® Deep Red (M-22426) to G5 dendrimer: We used partially acylated G5 dendrimer for the conjugation, with 86 out of 110 primary amine groups acylated. 0.00186 g (6.27×10^{-8} mol) of G5-$(NH_2)_{24}$-$(OCH_3)_{86}$ was dissolved in 1 mL of PBS buffer (pH=8). First 0.00026 mL (1.84×10^{-6} mol, 10x molar excess of the dye) of Et$_3$N, then

0.0001 g (1.84×10^{-7} mol) of dye dissolved in 0.5 mL of DMSO was added to the dendrimer solution. The mixture was stirred for 48 hours, dialyzed in water-methanol (2:1), then in DI water. After dialysis, TLC showed no traces of free dye. The dye-to-dendrimer ratio was determined by UV spectroscopy to be 1:1.

Conjugation of MitoTracker® Red CMXRos (M-7512) to G5-$(NH_2)_{21}(OCH_3)_{86}$-FA$_3$ dendrimer: The G5 dendrimer had 3 folic acid units for specific cell targeting. 0.00192 g (6.21×10^{-8} mol) of G5-$(NH_2)_{21}$-$(OCH_3)_{86}$-FA$_3$ was dissolved in 1 mL of PBS buffer (pH=8). First 0.00026 mL (1.84×10^{-6} mol, 10x molar excess of the dye) of Et$_3$N, then 0.0001 g (1.84×10^{-7} mol) of dye dissolved in 0.5 mL of DMSO was added to the dendrimer solution. The mixture was stirred for 48 hours, dialyzed in water-methanol (2:1), then in DI water. After dialysis, TLC showed no traces of free dye. The dye-to-dendrimer ratio was determined by UV spectroscopy to be 1:1.

Conjugation of MitoTracker® Deep Red (M-22426) to G5-$(NH_2)_2$-$(OCH_3)_{86}$-FA$_3$ dendrimer: The partially acylated G5 dendrimer had 3 folic acid units as specific targeting moiety. 0.00195 g (6.22×10^{-8} mol) of G5-$(NH_2)_{21}$-$(OCH_3)_{86}$-FA$_3$ was dissolved in 1 mL of PBS buffer (pH=8). First 0.00026 mL (1.84×10^{-6} mol, 10x molar excess of the dye) of Et$_3$N, then 0.0001 g (1.84×10^{-7} mol) of dye dissolved in 0.5 mL of DMSO was added to the dendrimer solution. The mixture was stirred for 48 hours, dialyzed in water-methanol (2:1), then in DI water. After dialysis, TLC showed no traces of free dye. The dye-to-dendrimer ratio was determined by UV spectroscopy to be 1:1.

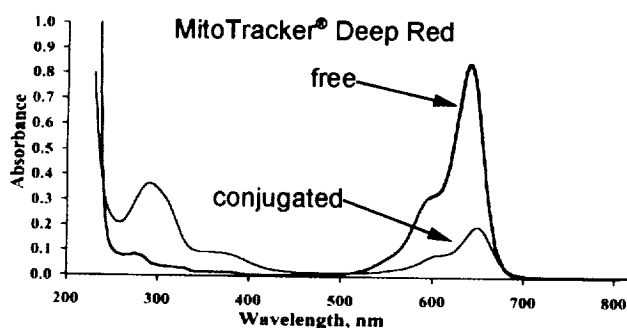Upon conjugation to dendrimer, the absorption maximum of the dyes (A_{max}) shifted to a longer wavelength.

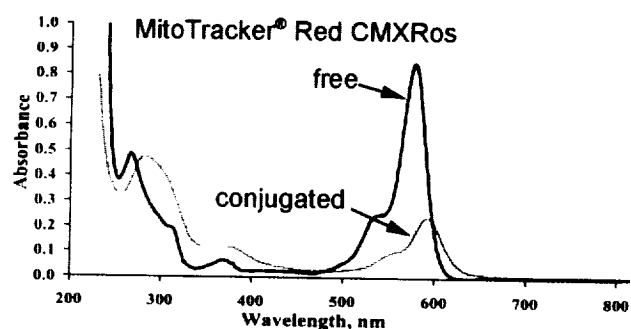Figure 3. The A_{max} of MitoTracker® Deep Red is shifted from 640 nm to 649 nm when it is conjugated to dendrimer.

Figure 4. The A_{max} of MitoTracker® Red CMXRos is shifted from 578 nm to 592 nm when it is conjugated to dendrimer.

The reaction conditions used in the model reaction must be modified to obtain higher dye/dendrimer molar ratios.

Activities planned for the next reporting period
In the next period we will modify the donor molecule (to form a hydrazide reactive group), purchase oligopeptide, and modify for coupling of the donor molecule.

In the next period well increase the mitochondrion-selective dye to dendrimer molar ratio. We will also conjugate the apoptosis indicator (Rhodamine 110, bis-(L-aspartic acid) amide) dye to the dendrimer.

Technical Objective II: The analysis and verification of the radiation sensing capability in lymphocytes in vitro.
The main goal of this technical objective is to characterize and measure the efficacy of dendrimer-based biosensors as signal carriers. This includes a systematic analysis of single or multiple conjugates with intracellular spectral probes.

Testing of the caspase-3 substrate, rhodamine 110, bis-(L-aspartic acid amide), trifluoroacetic acid salt (R110), *in vitro* as a monitor of apoptosis *in vivo*
Andrzej Myc, Ph.D., Jolanta Kukowska-Latallo, Ph.D., Alina Kotlyar, M.S., Katarzyna Janczak, M.S., Jeffrey Landers, B.S.

Caspase-3 is one of the cysteine proteases most frequently activated during the process of apoptosis or Programmed Cell Death (PCD). In response to pro-apoptotic stimuli, the 32 kDa pro-Caspase-3 is processed to an active enzyme consisting of two subunits of 17 and 12 kDa. Activated caspase-3 is essential for the progression of apoptosis, resulting in the degradation of cellular proteins, apoptotic chromatin condensation, and DNA fragmentation.

The bis-L-aspartic acid amide of R110 contains the rhodamine 110 fluorophore flanked by aspartic acid residues and in this form does not fluoresce (Figure 5). Activated caspase-3
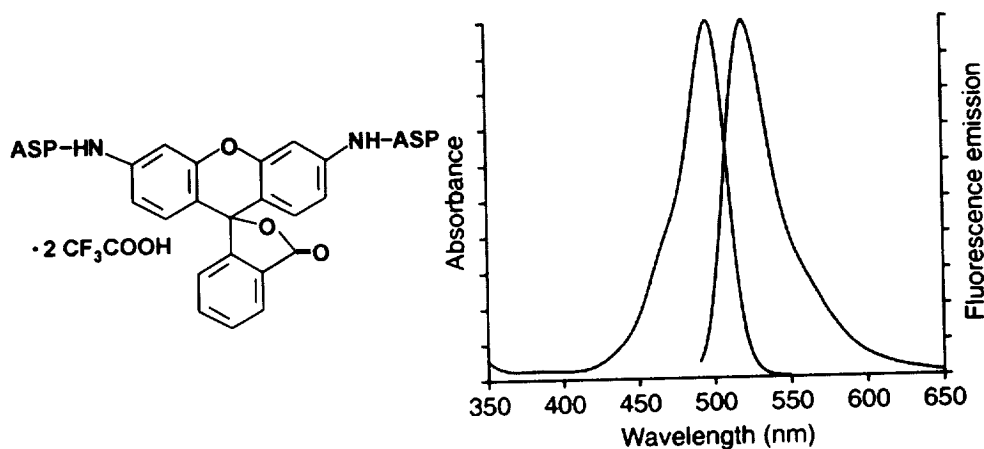
Figure 5. Molecular structure and absorption/emission spectra of rhodamine 110, bis-(L-aspartic acid amide), trifluoroacetic acid salt. $C_{22}H_{26}F_6N_4O_{13}$ Mol. Wt. 788.57

specifically cleaves the aspartic acid moieties from the compound and releases the highly fluorogenic rhodamine 110 fluorophore. The fluorescence of rhodamine 110 can be detected and quantified in apoptotic cells by flow cytometry. Since the R110 does not require any

invasive techniques, such as osmotic shock, to gain entrance into the cytoplasm, it may be used to detect apoptosis in living cells.

The R110 was evaluated *in vitro* to assess its potential to detect and quantitate apoptosis in Jurkat cells. Jurkat cells were incubated with 1 μM staurosporine for 6 h and stained afterwards with R110 for 20 min. The fluorescence was quantified by flow cytometry using the 488 nm excitation laser (Figure 6). Emission spectra were detected using four different photo multipliers (FL1- 525 nm, FL2- 575 nm, FL3- 620 nm, and FL4 - 675 nm). Although R110 has a very broad emission spectrum (Figure 5), the highest fluorescence was recorded at wavelength of 525 nm (Figure 7). At this wavelength, we also observed the greatest resolution between non-apoptotic (control cells) and apoptotic cells. In our next experiment,
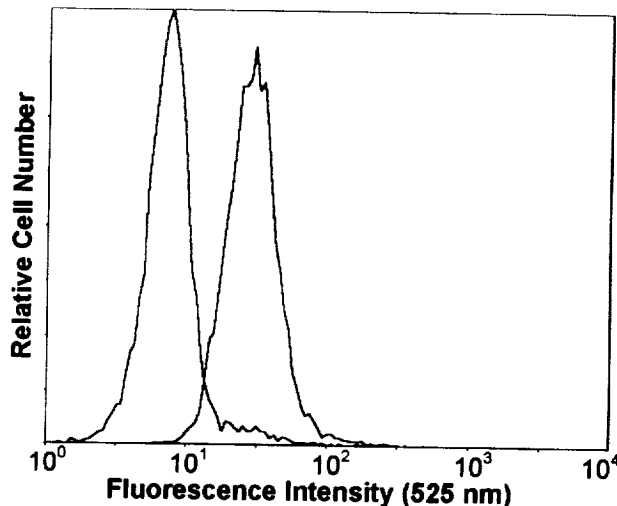
Figure 6. Histograms of control (red) and apoptotic (blue) Jurkat cells stained with Rh110 (10 μM). Cells were treated with either staurosporine (1 μM) or DMSO (control) for 6 h, stained with Rh110 for 20 min and washed. Fluorescence of 10,000 cells was measured using flow cytometer at the wavelength of 525 nm.
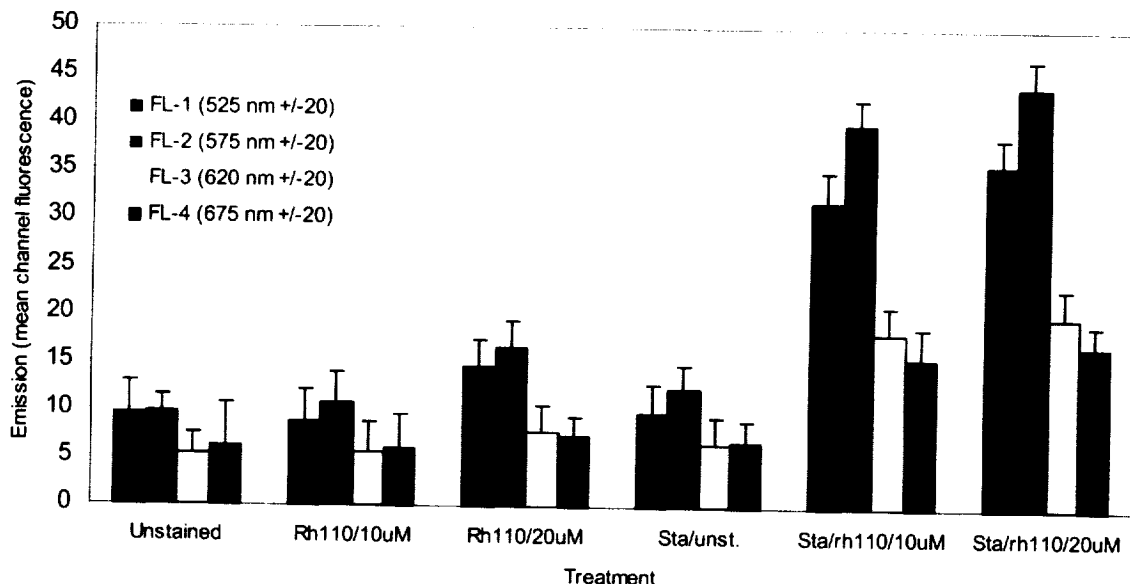
Figure 7. Detection of apoptosis in Jurkat cells using Rh110. Jurkat cells were treated for 6 h with 1μM of Staurosporine to induce apoptosis and subsequently stained with Rh110 for 20 min. Cell fluorescence was measured with a flow cytometer using four different photo multipliers (FL1- 525 nm, FL2- 575 nm, FL3- 620 nm, and FL4 - 675 nm). The controls included cells unstained with Rh110 (Unstained), stained with 10 μM Rh110 (Rh110/10μM), cells stained with 20 μM Rh110 (Rh110/20μM) and Staurosporine treated unstained cells (Sta/unst.) The Staurosporine treated apoptotic cells were stained with 10 μM Rh110 (Sta/rh110/10μM) and with 20 μM Rh110 (Sta/rh110/20μM).

we tested if R110 could be used to measure the kinetics of apoptosis. There is a linear correlation between the progression of apoptosis and the fluorescence intensity of rhodamine 110 (Figure 8).
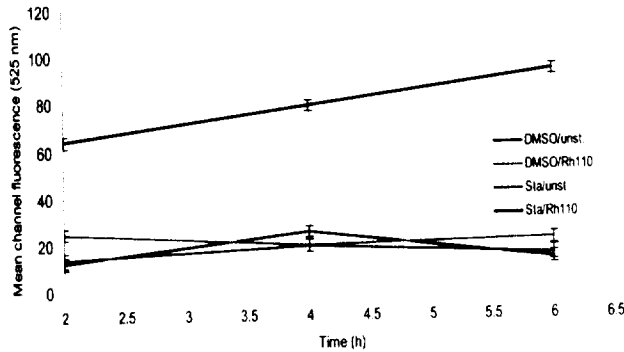
Figure 8. Kinetics of apoptosis. Jurkat cells were treated with Staurosporine for 2, 4, and 6 hours, then stained with Rh110 (Sta/Rh110). The cell fluorescence was measured using a flow cytometer. Controls included Staurosporine treated unstained cells (Sta/unst.), DMSO treated Rh110 stained (DMSO/Rh110) and DMSO treated unstained Jurkat cells (DMSO/unst.).

In conclusion, the R110 substrate gains entrance into the cytoplasm of living cells and therefore can detect the apoptosis *in situ*. The R110 will be used to stain primary mouse splenocytes *ex vivo* and to examine the detection of apoptosis *in vivo* using two-photon fluorescence measurement through optical fibers.

Technical Objective III: The development of a noninvasive laser analysis system to monitor biosensor signals from lymphocytes with radiation-induced damage.
The primary goal of this component of the research program is to develop ultrasensitive *in vivo* fluorescence detection technologies and methodologies for real-time monitoring of radiation-induced biologic effects in space.

Testing Using Microscopic Multispectral Analysis
Felix de la Iglesia, MD, Timothy Sassanella, M.S.

The development of the image quantitation procedure has progressed significantly, and should be completed during the next month. Several useful macros have been obtained for the Image Pro software, and progress has been made in the areas of background elimination, average pixel intensity determination, and the transfer of data to appropriate analytical software for manipulation and graphing.
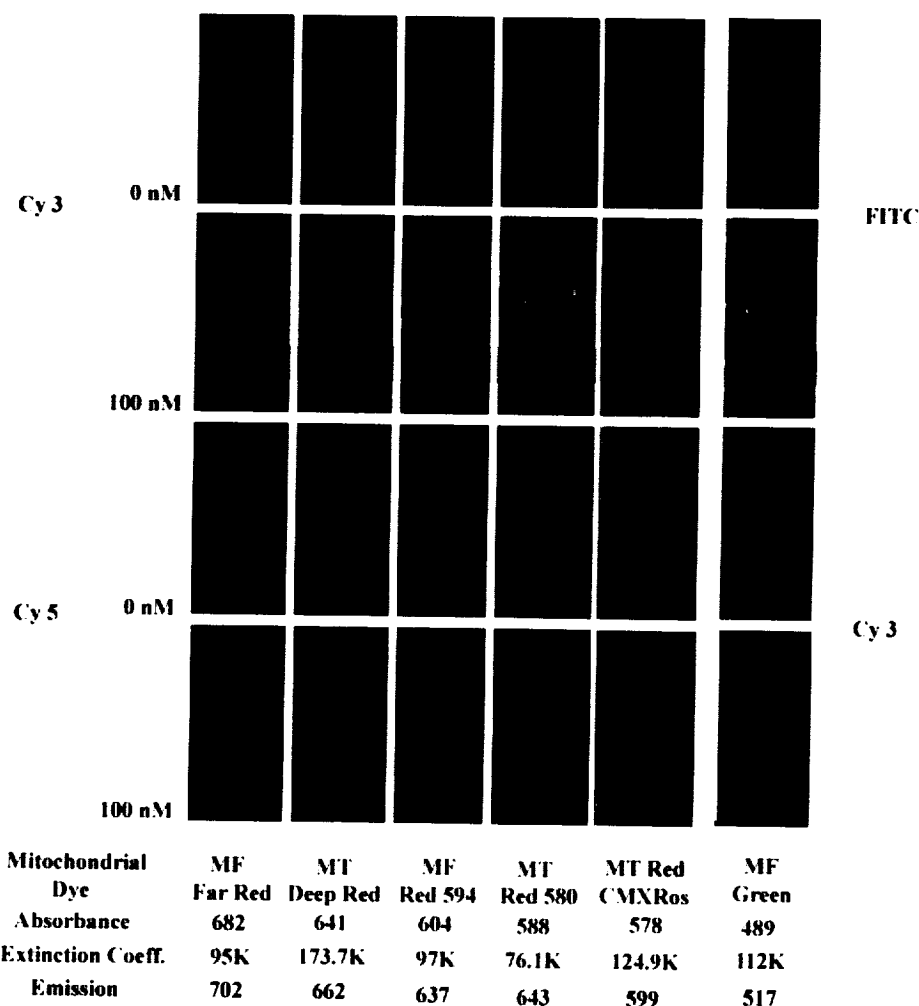
The IRB protocol for blood use for the project has been submitted.

KB cells were tested with six mitochondrial monitoring dyes (including MitoTracker® Red CMXRos and MitoTracker® Deep Red) to verify cellular uptake and localization of the free dye in culture. The six dyes employed were those previously selected as potential candidates for conjugation. All dyes fluoresced in live cells under the conditions tested. The optimal concentration range was determined. Qualitative analysis indicates mitochondrial localization, but the intensity of fluorescence varied significantly, though the dyes were tested

at the same concentrations and with the same methods. MitoTracker® CMXRos appeared to be the most intensely fluorescent (Figure 9). The absorbance spectra in water for the six dyes was also determined (Figure 10, next page).

| Mitochondrial Dye	MF Far Red	MT Deep Red	MF Red 594	MT Red 580	MT Red CMXRos	MF Green
Absorbance	682	641	604	588	578	489
Extinction Coeff.	95K	173.7K	97K	76.1K	124.9K	112K
Emission	702	662	637	643	599	517

Figure 9. Summary of mitochondria-specific dye testing in KB cells. Six dyes were tested: five were in the red range, and the Cy3 and Cy5 filter sets were used; the sixth dye was in the green range, and the FITC and Cy3 filer sets were used. The concentration of dye was 100 nM.

Two dyes, MitoTracker® Red CMXRos and MitoTracker® Deep Red, have been conjugated to dendrimers, both with and without a targeting agent, and have been received recently from the chemistry group. Experimental preparations are underway to test these conjugates.

Preparation, function testing, and calibration testing of the robotic microscope and individual components continued in August.

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Mitochondrial Dye Absorbance
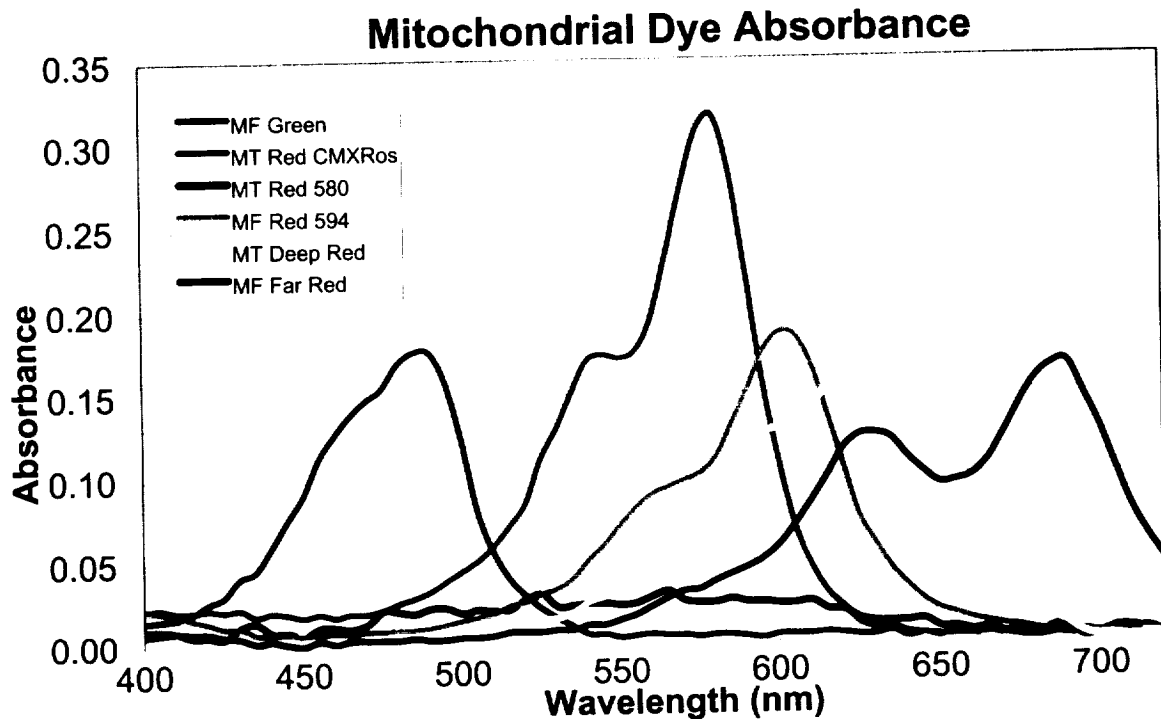
Figure 10. Absorbance spectra of the six selected mitochondria-specific dyes. The spectra of free and dendrimer conjugated dyes will be used to select or acquire appropriate excitation, dichroic, and emission filters for photomicrography.

Activities planned for the next reporting period:

1) The quantitative protocol for micrographs will continue to be developed. When completed, microscope calibrations will be conducted.
2) Quantitative analysis on these micrographs will be done in the coming month as the technique is developed.
3) A photobleaching study using MitoTracker® Red CMXRos and MitoTracker® Deep Red will be completed.
4) Cell toxicity under single and dual dye conditions will be tested.
5) Dendrimer conjugates with MitoTracker® Red CMXRos and MitoTracker® Deep Red will be tested as soon as the appropriate controls and parameters are determined.

Flow Cytometer-Laser System Development
Theodore Norris, Ph.D., Jingyong Ye, Ph.D., Cheng Frank Zhong, B.S.

Integrated Microfluidics System

We are designing a specific microfluidics chip to simulate blood flow through a capillary bed. We will use small samples of human blood. The dimension of the channels on the chip should be over 20 microns. We have contact with the MEMS-Exchange, an on-campus microfrabication facility. They suggest that we use a chip with a single-level depth. Making separated wafers and each having channels of a uniform depth, is a straightforward task. The process sequence is shown below:

1. Contact photolithography
2. Deep RIE
3. $Si O_2$ CVD or thermally grow a thin layer of $Si O_2$

Both methods of producing a SiO_2 layer will cause the mouth of the trench to pinch closed slightly. The CVD method will also cause the layer to thin towards the bottom of the trench. But since the thickness is not so important for us, this should not present a problem.

Multi-dye detection

We changed the optical fiber based detection system to a free-space detection system. Using this system, we did a set of experiments to compare the fluorescence signal of five free dyes, each as a solution in DSMO, and at the same concentration of 30 µM. The dyes tested were: Mito-Tracker® Red CMXRos, MitoTracker® Red 580, MitoTracker® Deep Red 633, 6-TAMRA, and FITC (Figure 11). Results show that MitoTracker® Deep Red 633 gives the strongest two-photon fluorescence excitation signal. We repeated this dye comparison experiment using a 76-MHz fs pulse laser with a pulse duration of 10 fs and wavelength of 790 nm. The result is almost the same, with MitoTracker® Deep Red 633 giving the strongest two-photon fluorescence excitation signal.

We did another set of measurements on solutions of MitoTracker® Deep Red 633 at different concentrations ranging from
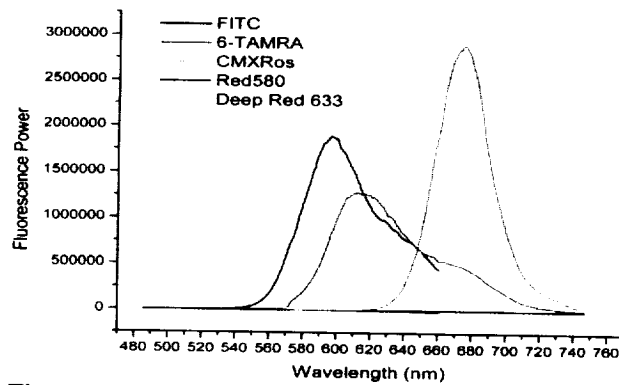
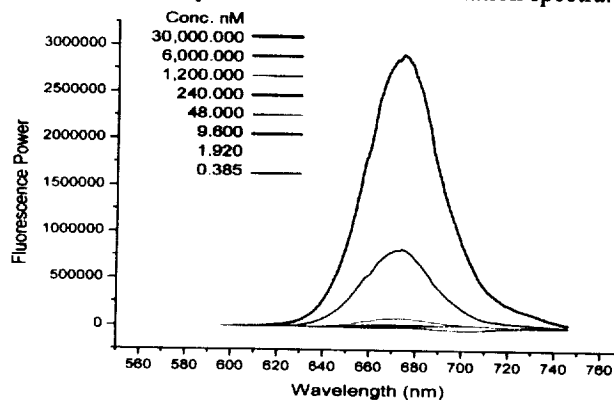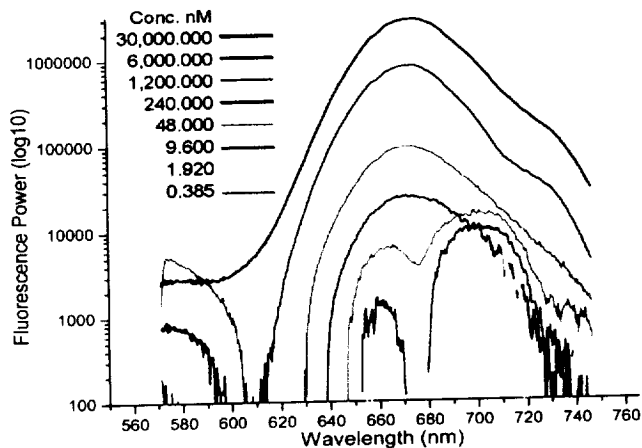**Figure 11.** Two-photon fluorescence excitation spectra.

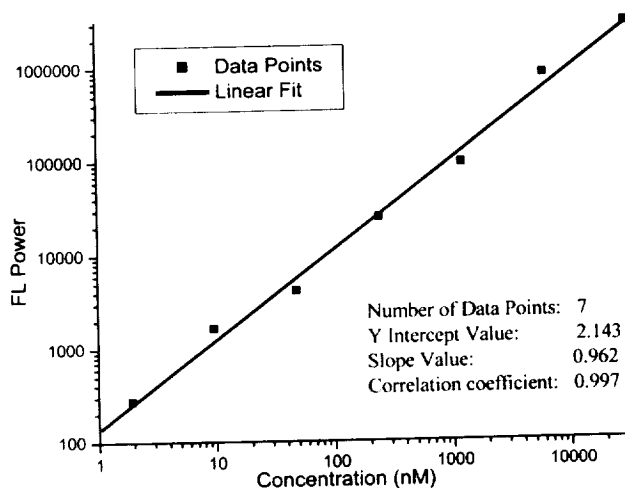**Figure 12.** Two-photon fluorescence excitation spectrum of MitoTracker® deep red 633.

Figure 13. Logarithmic plots of two-photon fluorescence excitation spectrum of MitoTracker® Deep Red 633.

Figure 14. Logarithmic plot of the dependence of relative two-photon induced fluorescence on dye concentration for solutions of Mito Tracker Deep Red 633 in DSMO.

Figure 15. Background spectrum of DMSO.

30 µM to 0.385 nM, with each consecutive sample being 80% less concentrated than the previous one (Figure 12, previous page). This gives us a detailed view of the signal level of this dye in solution at lower concentrations (Figure 13). The two-photon fluorescence excitation signals are peaked around 670 nm. Choosing the maximum fluorescence signal of each spectrum (except for 0.385 nM because we did not see any signal), we drew a linear fit of relative two-photon induced fluorescence vs dye concentration for the solutions of MitoTracker® Deep Red 633 in DSMO (Figure 14). The correlation coefficient of 0.997 is very close to 1, and the Y-axis intercept value of 2.143 is small compared with the signal level. This result shows that the two-photon fluorescence excitation signal is proportional to the dye concentration within the limits of experimental error.
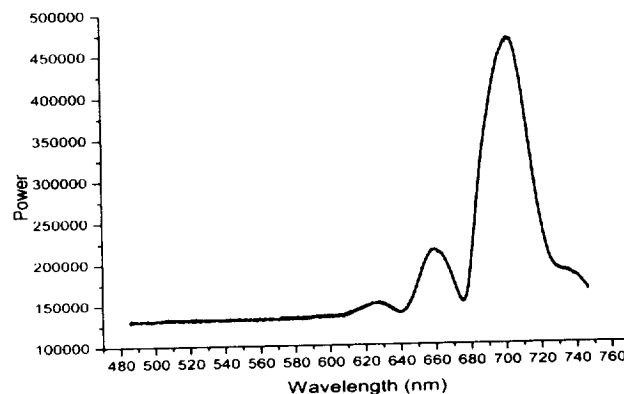
The shape of the two-photon fluorescence excitation spectrum at lower concentrations (less than 48 nM, Figure 13) is not as expected. From the linearity of the logrithmic plot of fluorescense vs dye concentration (Figure 14), we expected the lower concentrations to give us curves similar to the curves for higher concentrations. However, they have a peak larger than expected around 700 nm. This is due to the high background signal of DMSO, relative to the low dye signal. DMSO has a peak around 700 nm (Figure 15).

III. Problems encountered and their resolution.

The existing desktop computer that is used to process images generated by the robotic microscope takes one day to a day and a half to process one batch of images. Being able to process such images in hours rather than a few days will have a significant effect on productivity and efficiency. It will also enhance our capability to select and screen different dyes and conjugated materials for acceptability on a quantitative basis, as well as to investigate expeditiously the intracellular localization and effects of the new experimental materials.

We submitted a Contrating Officer's Authorization, on July 22, 2002, to request rebudgeting of $10,000 for the purchase of a high-end graphics workstation. The selected workstation would decrease deconvolution time by at least 50%, significantly enhancing our ability to digitally analyze complex, multispectral images with the multimode fluorescent microscope.

VI. Copies of manuscripts (published or unpublished) derived from the research and copies of all abstracts, manuscripts, preprints and publications.
None.

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REPORT DOCUMENTATION PAGE		Form Approved OMB NO. 0704-0188

Public Reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comment regarding this burden estimates or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188,) Washington, DC 20503.

1. AGENCY USE ONLY (Leave Blank)	REPORT DATE August 2002	3. REPORT TYPE AND DATES COVERED Monthly: 07/26/02-08/25/02
4. TITLE AND SUBTITLE Biosensors for Real-time Monitoring of Radiation Induced Biologic Effects in Space		5. FUNDING NUMBERS C-NAS2-02069
6. AUTHOR(S) James R. Baker, Jr., MD, Lajos Balogh, Ph.D., Istvan Majoros, Ph.D., Balazs Keszler, Ph.D., Andrzej Myc, Ph.D., Jolanta Kukowska-Latallo, Ph.D., Theodore Norris, Ph.D., Felix de la Iglesia, MD Compiler/editor: Nicholas W. Beeson, Ph.D.		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Center for Biologic Nanotechnology University of Michigan 9220 MSRB III, 1150 W. Medical Center Drive Ann Arbor, MI 48109-0648		8. PERFORMING ORGANIZATION REPORT NUMBER NAS2-02069-4
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) NASA-Ames Research Center Paul Fung, Ph.D. MS 19-20 Moffett Field, CA 94035-1000		10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES None		
12 a. DISTRIBUTION / AVAILABILITY STATEMENT See Handbook NHB 2200.2		12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

This work seeks to develop cellular biosensors based on dendritic polymers. Nanoscale polymer structures less than 20 nm in diameter will be used as the basis of the biosensors. The structures will be designed to target into specific cells of an astronaut and be able to monitor health issues such as exposure to radiation. Multiple components can be assembled on the polymers including target directors, analytical devices (such as molecular probes), and reporting agents. The reporting will be accomplished through fluorescence signal monitoring, with the use of multispectral analysis for signal interpretation. These nanosensors could facilitate the success and increase the safety of extended space flight. The design and assembly of these devices has been pioneered at the Center for Biologic Nanotechnology in the University of Michigan.

14. SUBJECT TERMS Nanotechnology/ Optics Polymer-based platforms Biosensors (implantable)			15. NUMBER OF PAGES 12
			16. PRICE CODE
17. SECURITY CLASSIFICATION OR REPORT UNCLASSIFIED	18. SECURITY CLASSIFICATION ON THIS PAGE UNCLASSIFIED	19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED	20. LIMITATION OF ABSTRACT UL

NSN 7540-01-280-5500
Standard Form 298 (Rev.2-89)
Prescribed by ANSI Std. 239-18
298-102