Biphasic dose responses in low level light therapy

Michael R Hamblin PhD

Ying-Ying Huang. Aaron Chih-Hao Chen, James Carroll

Disclosures

Consulting fees: Lexington International, Laser Hair Therapy of North America
Sponsored research: Lexington International,
Laser Hair Therapy of North America, Palomar Medical Technologies
Scientific advisory board: Lexington International, Immunophotonics

Outline

- Introduction to mechanisms of LLLT
- Survey of biphasic dose responses in LLLT
- Mechanistic studies in mouse embryonic fibroblasts
- NF-kB activation in HEK293 cells
- HeLa cells and neurons
- Conclusions

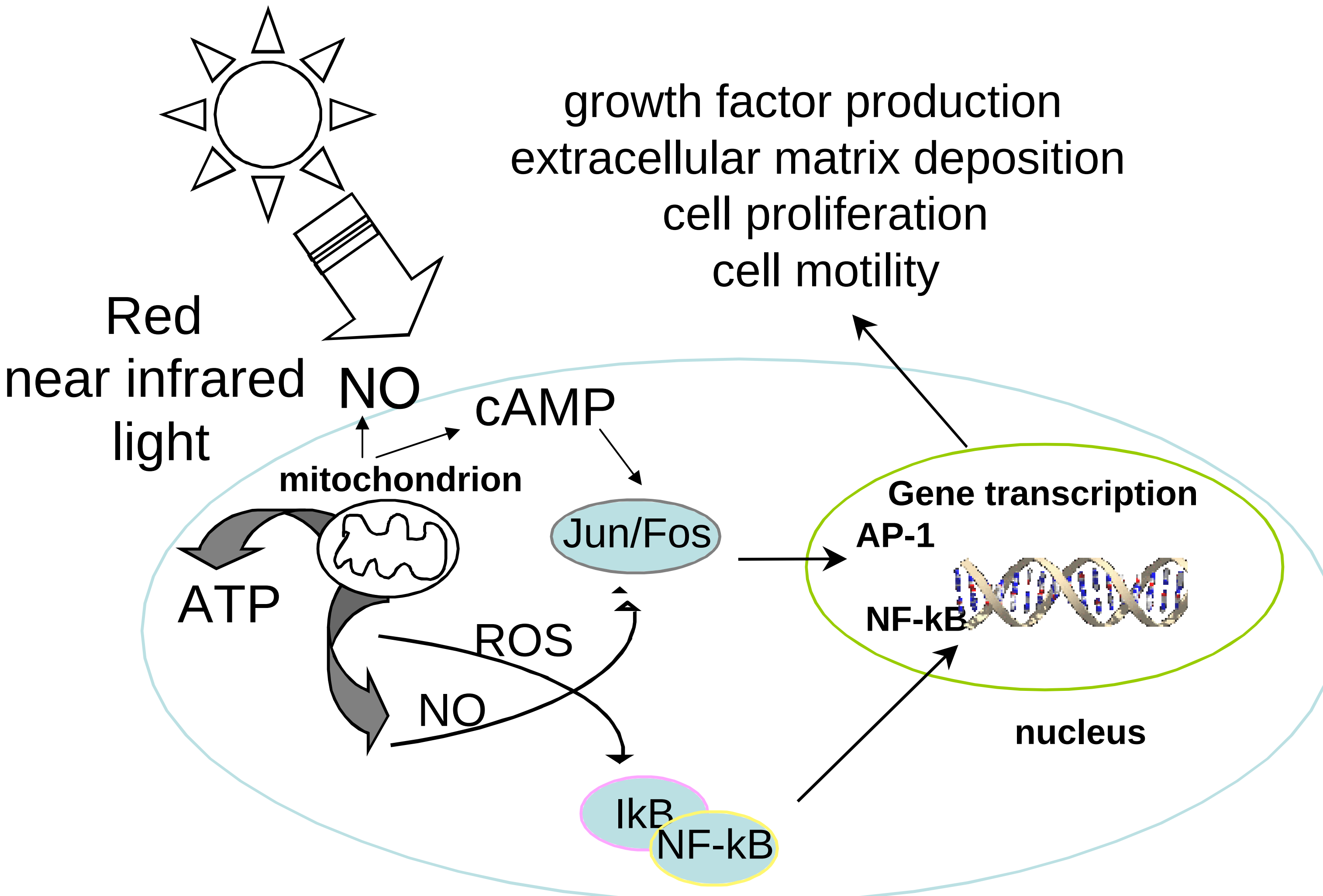
What's in a name?

Low level laser therapy
Low reactive-level laser therapy
Low intensity laser therapy
Low level light therapy
Low energy laser irradiation
Photobiomodulation
Photobiostimulation
Biomodulation
Biostimulation
Cold laser
Soft laser
Laser therapy
Phototherapy

It is called "LOW" because a little light is better than a lot of light

Biphasic dose response?

Mechanisms of LLLT



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NATURE|Vol 443|26 October 2006

POWER GAMES

There's a fight going on inside all our cells for each breath of air. **Nick Lane** sheds therapeutic light on the implications for cancer and degenerative diseases.

"The finding that the body could poison one of its own enzymes was initially shrugged off as an imperfection."

Yet over the past decade, researchers have come to appreciate that cells often use CO, and to an even greater extent NO (nitric oxide), to block respiration. Not only that, but light has striking counter-effects on cytochrome oxidase. And all these suitors to the enzyme turn out to be critical to our understanding not just

Nitric Oxide and the Control of Firefly Flashing

Barry A. Trimmer,[1]* June R. Aprille,[1] David M. Dudzinski,[2] Christopher J. Lagace,[1] Sara M. Lewis,[1] Thomas Michel,[2,3] Sanjive Qazi,[1] Ricardo M. Zayas[1]

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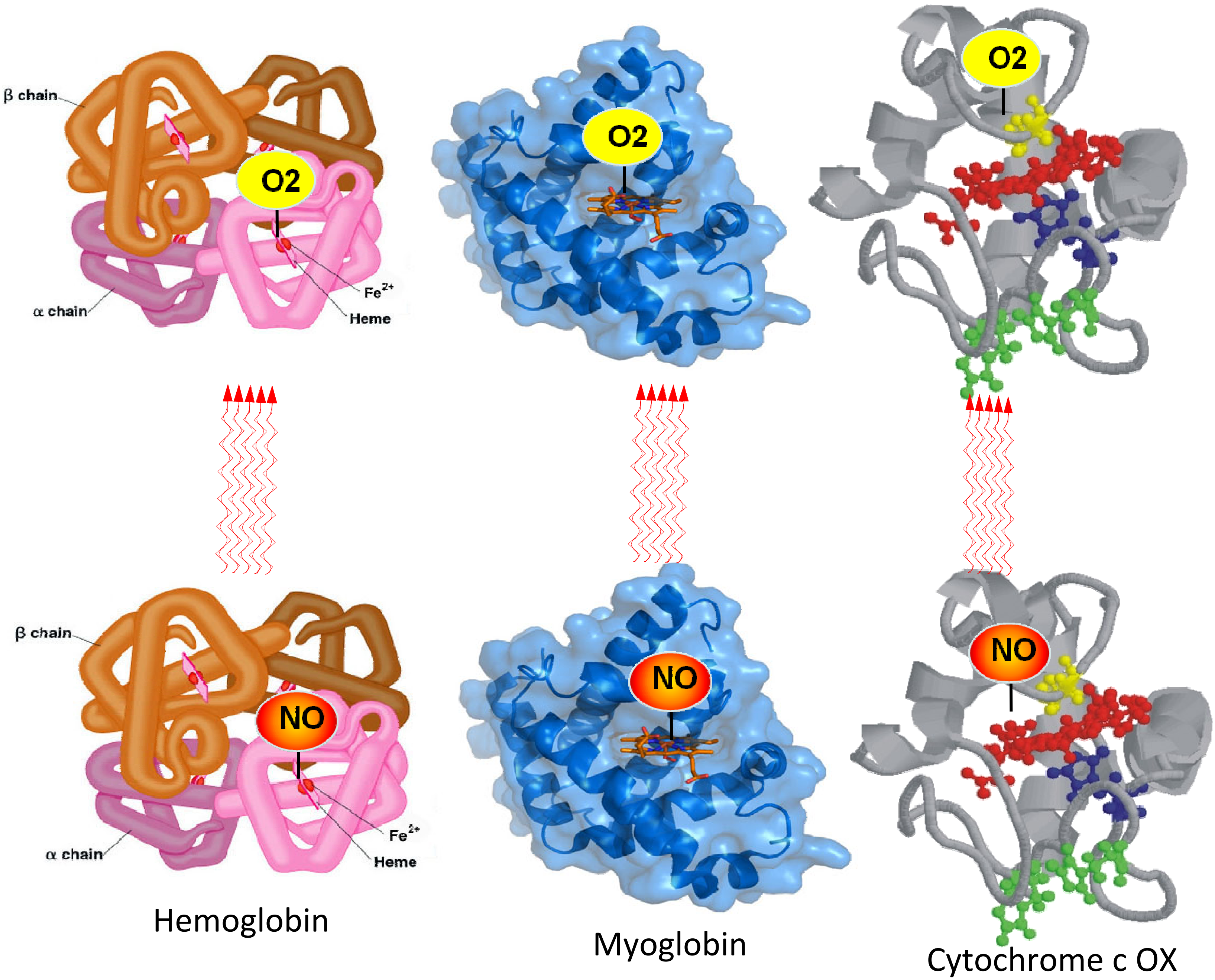
29 JUNE 2001 VOL 292 SCIENCE

The results reported here document an important role for NO in firefly flash control. It is well established that O_2 availability is the immediate biochemical trigger for light production, and we propose that the role of NO is to transiently inhibit mitochondrial respiration in photocytes and thereby increase O_2 levels in the peroxisomes. This is consistent with the distinctive spatial arrangement of NOS-containing cells, the known NO-mediated inhibition of cytochrome c oxidase (*21–23*), and the fact that firefly luminescence can be induced by cytochrome c oxidase inhibitors, such as cyanide and carbon monoxide



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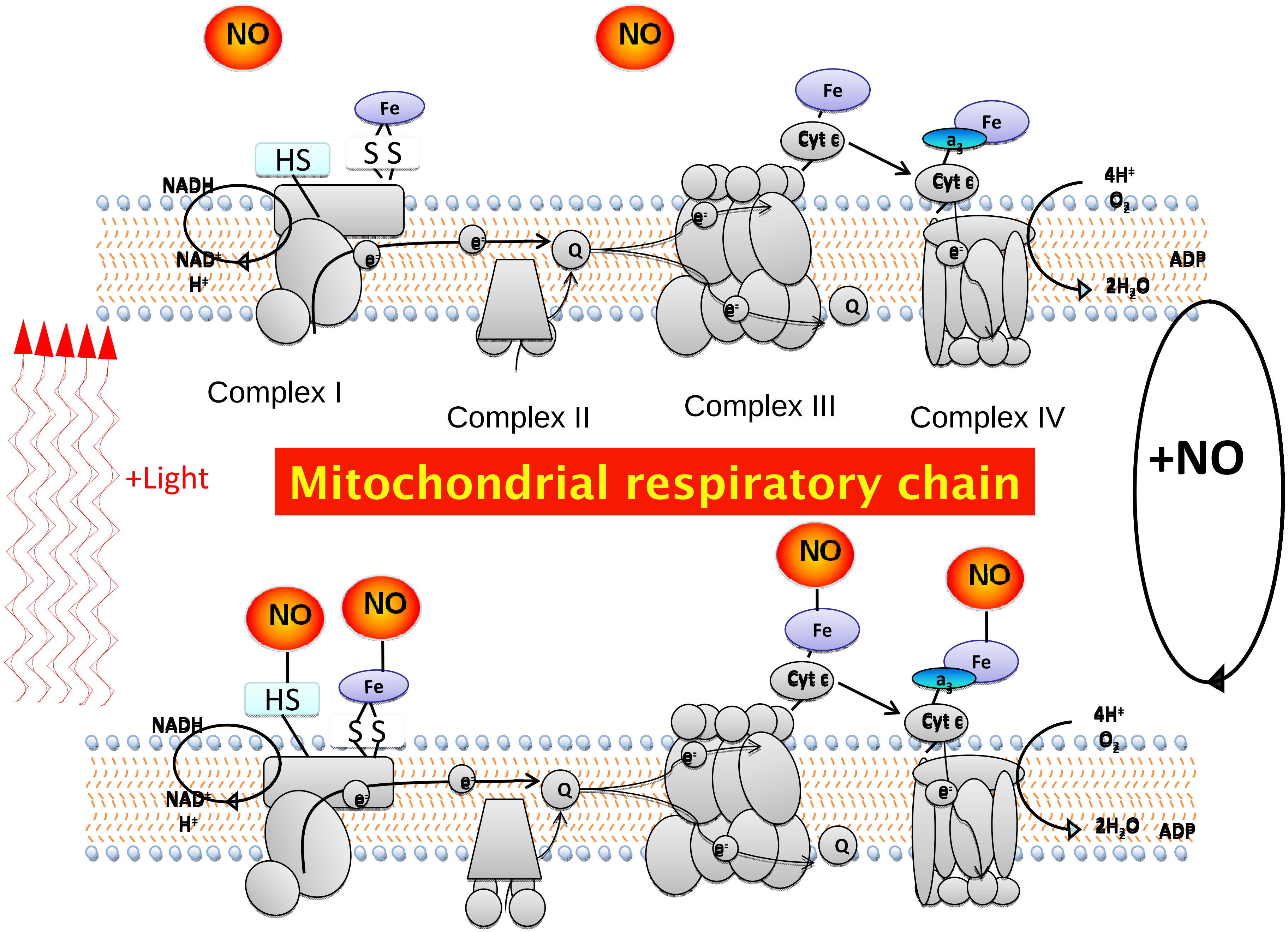

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Cytochrome c oxidase can act as a photoreceptor allowing the photolytic dissociation of any bound nitric oxide.

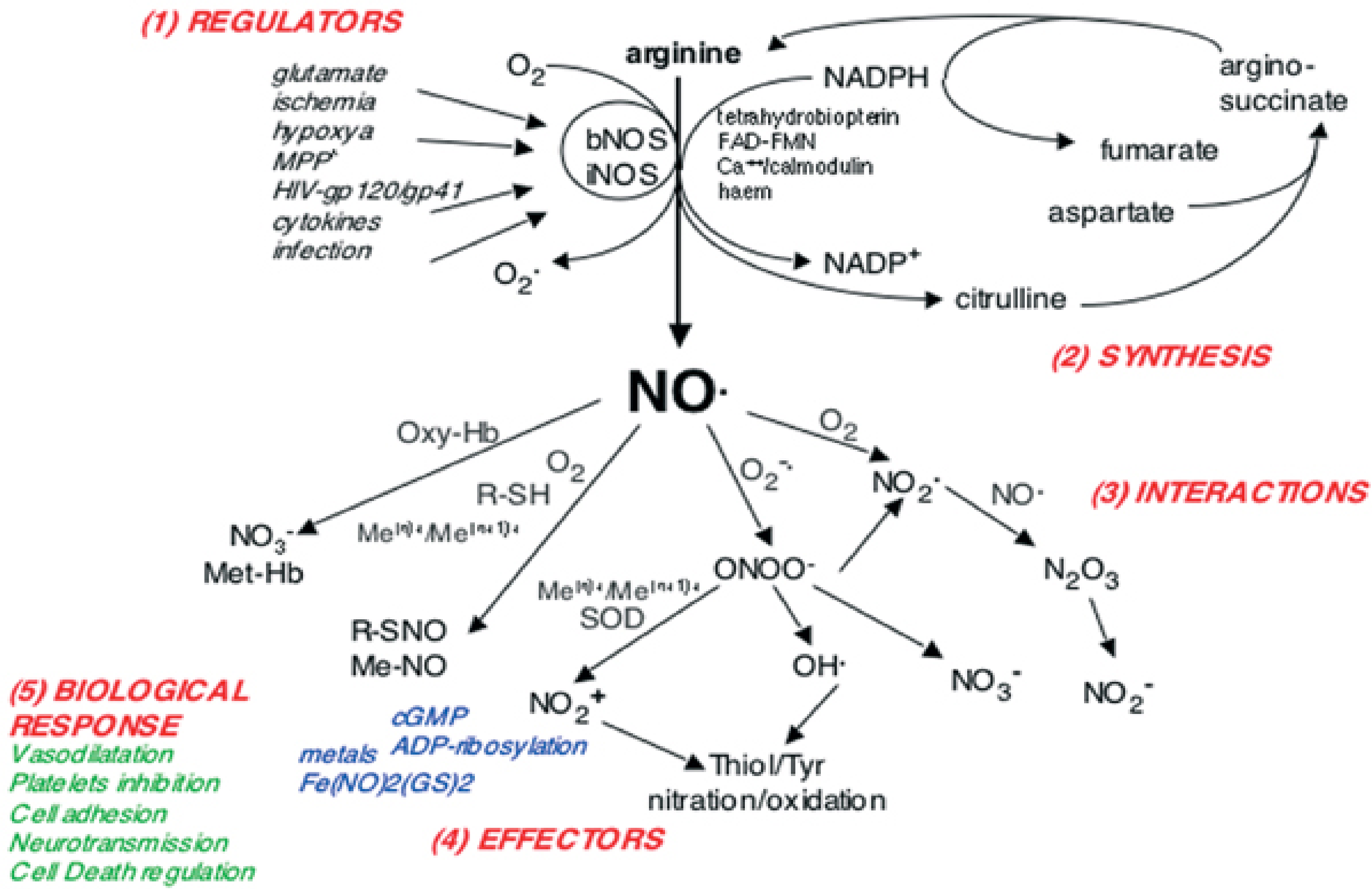
Karu, T.I., L.V. Pyatibrat, and G.S. Kalendo, Photobiological modulation of cell attachment via cytochrome c oxidase. Photochem Photobiol Sci, 2004. 3(2): p. 211-6.
Karu, T.I., et al., Absorption measurements of a cell monolayer relevant to phototherapy: reduction of cytochrome c oxidase under near IR radiation. J Photochem Photobiol B, 2005. 81(2): p. 98-106.

The nitric oxide present in the heme-CuIIa3 center of CytC ox can be photolysed by visible light.

Sarti, P., et al., Nitric oxide and cytochrome c oxidase: mechanisms of inhibition and NO degradation. Biochem Biophys Res Commun, 2000. 274(1):p. 183-7.

Near infrared light protects cardiomyocytes from hypoxia and reoxygenation injury by a nitric oxide dependent mechanism.

Zhang et al. Journal of Molecular and Cellular Cardiology 46 (2009) 4–14



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Basic mechanisms

Mitochondria are primary photoreceptors

Cytochrome c oxidase activity is increased

NO is dissociated from COX + heme proteins

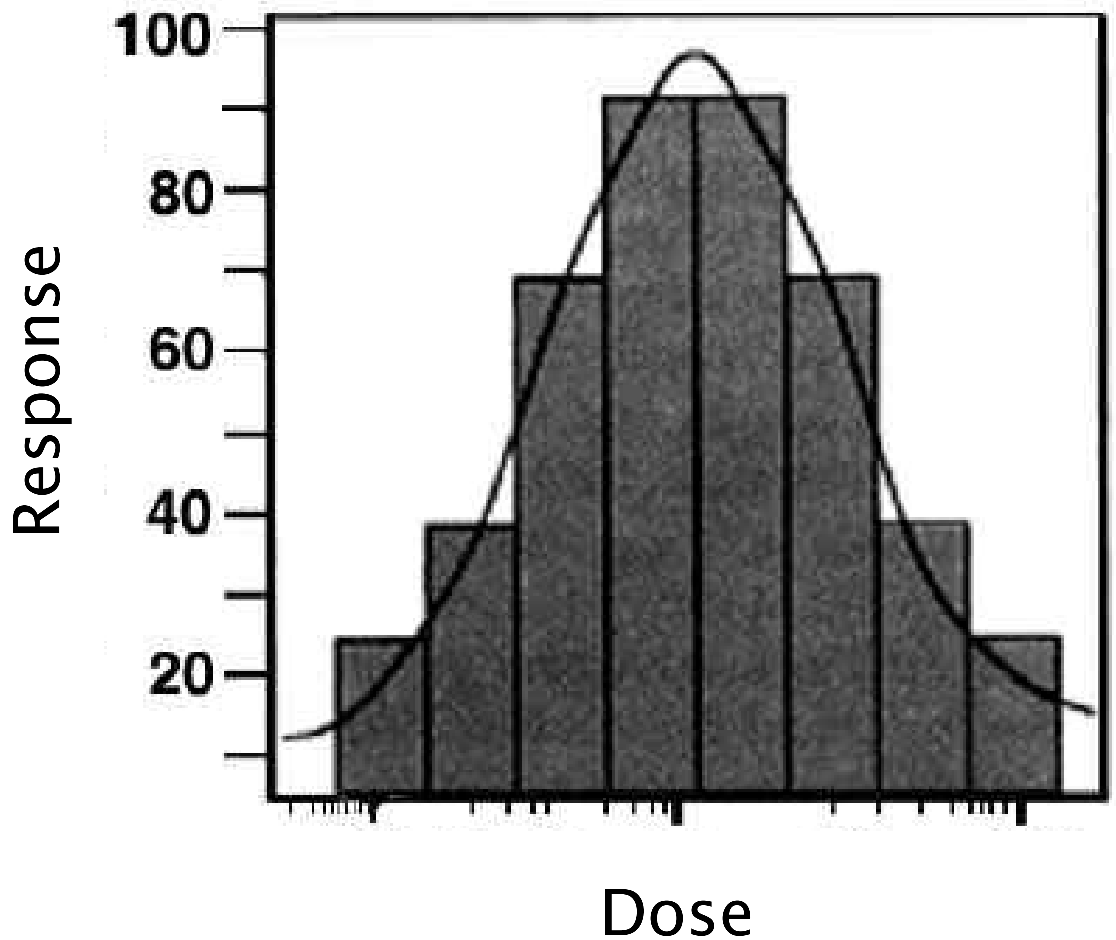
ATP and cAMP increased

Reactive oxygen species are produced

Transcription factor induction

Growth, repair, survival, less inflammation

Biphasic dose response?



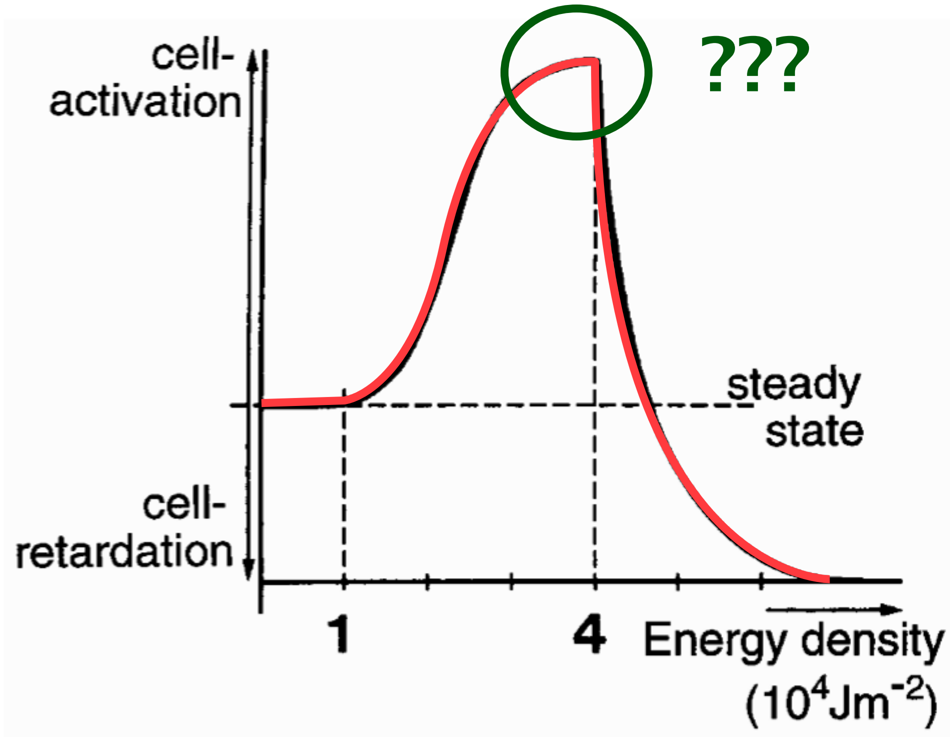
How is dose measured?

Power (W) x Time (sec) = Energy (Joules)

$$\frac{\text{Power mW}}{\text{Beam Area cm2}} = \text{(irradiance) x time = fluence (J/cm2)}$$

Arguments have been made for total energy, fluence, irradiance and illumination time to be most important parameters in measuring dose

• Dose response curve



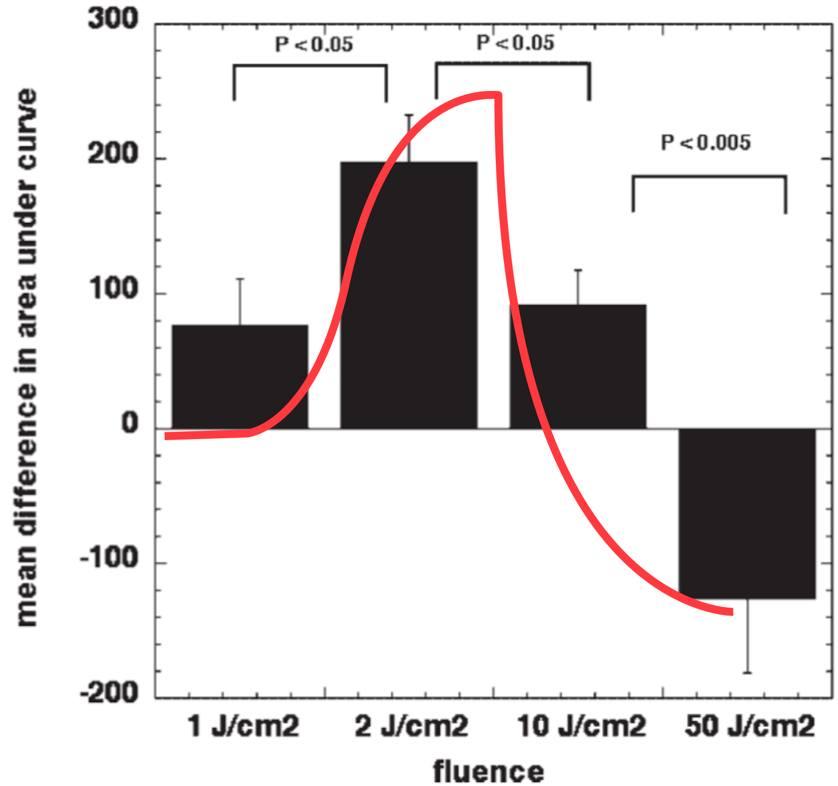
increase energy

Arndt-Schulz curve

Sommer AP, Pinheiro AL, Mester AR, Franke RP, Whelan HT. (2001) Biostimulatory windows in low-intensity laser activation: lasers, scanners, and NASA's light-emitting diode array system. J Clin Laser Med Surg. Feb;19(1):29-33

Stimulation/Inhibition of Wound Healing in Mice

635-nm Laser for wound healing in mice



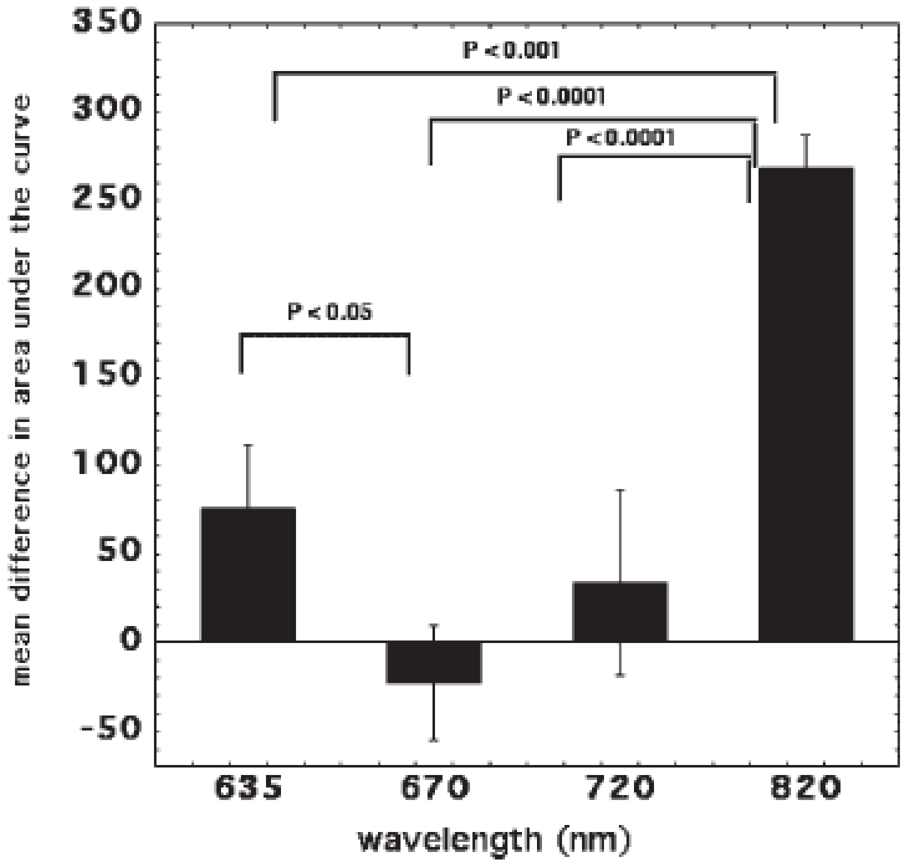
Lasers in Surgery and Medicine 39:706–715 (2007)

Low-Level Light Stimulates Excisional Wound Healing in Mice

Tatiana N. Demidova-Rice, BS,[1,2] Elena V. Salomatina, BS,[1] Anna N. Yaroslavsky, PhD,[1,3] Ira M. Herman, PhD,[2*] and Michael R. Hamblin, PhD[1,3,4]**

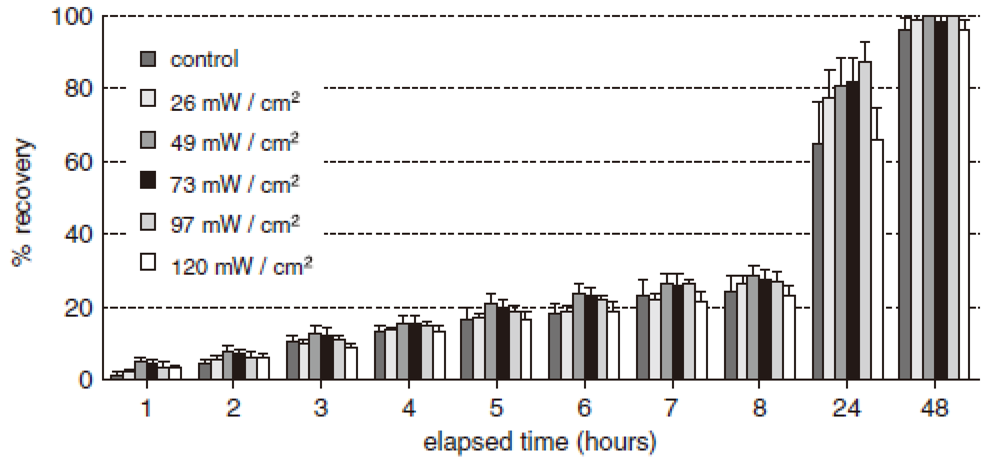
[1]Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, Massachusetts 02114
[2]Graduate Program in Cell Molecular and Developmental Biology, Sackler School of Graduate Biomedical Sciences, Tufts University School of Medicine, Boston, Massachusetts 02111
[3]Department of Dermatology, Harvard Medical School, Boston, Massachusetts 02115
[4]Harvard-MIT Division of Health Sciences and Technology, Cambridge, Massachusetts 02139

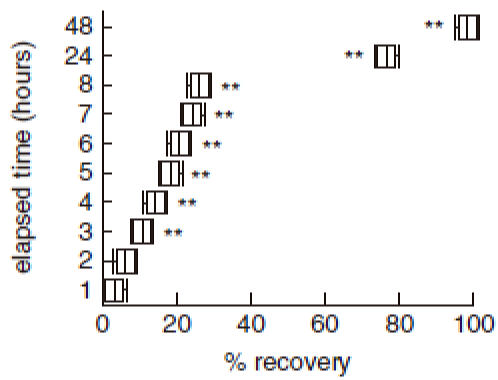
Wavelength response (action spectrum?)

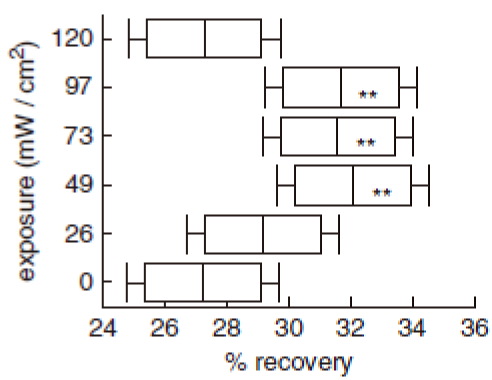


Biphasic dose response may be different at each wavelength

Wound healing in vitro 980-nm laser - scratch in fibroblast monolayer



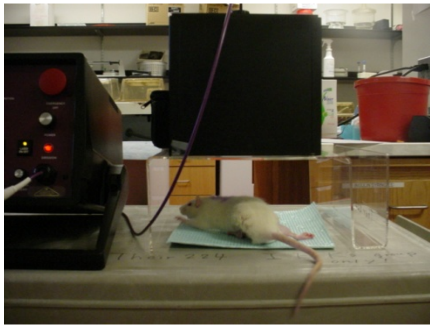


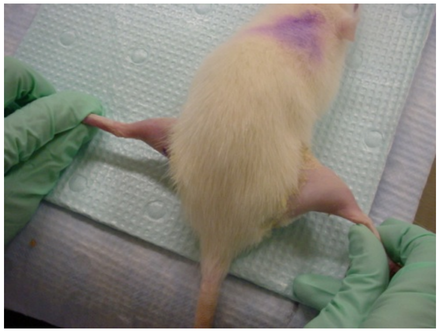


Constant time - irradiance and fluence vary

Mark D. Skopin & Scott C. Molitor
Effects of near-infrared laser exposure in a cellular model of wound healing
Photodermatol Photoimmunol Photomed, ***25, 75-80.(2009).***

810-nm laser for arthritis in rats





	3 J/cm2	30 J/cm2
5 mW/cm2	10 minutes	100 minutes
50 mW/cm2	1 minute	10 minutes

Hypothesis: length of illumination is more important than total fluence or irradiance in LLLT effect

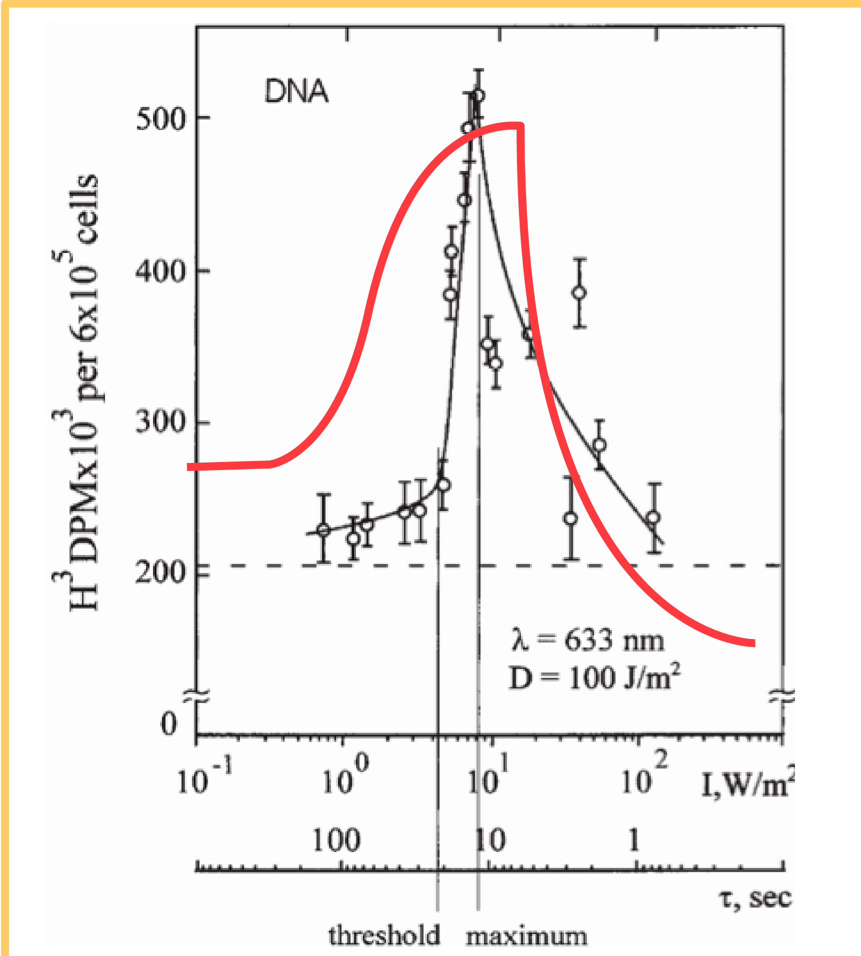
Lasers in Surgery and Medicine 39:543–550 (2007)

Low-Level Laser Therapy for Zymosan-Induced Arthritis in Rats: Importance of Illumination Time

Ana P. Castano, MD,[1,2] Tianhong Dai, PhD,[1,2] Ilya Yaroslavsky, PhD,[3] Richard Cohen, MD,[3] William A. Apruzzese, PhD,[3] Michael H. Smotrich, PhD,[3] and Michael R. Hamblin, PhD[1,2,4]*
[1]*Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, Massachusetts 02114*
[2]*Department of Dermatology, Harvard Medical School, Boston, Massachusetts 02115*
[3]*Palomar Medical Technologies Inc., Burlington, Massachusetts 01803*
[4]*Harvard-MIT Division of Health Sciences and Technology, Cambridge, Massachusetts 02139*

• Dose response curve

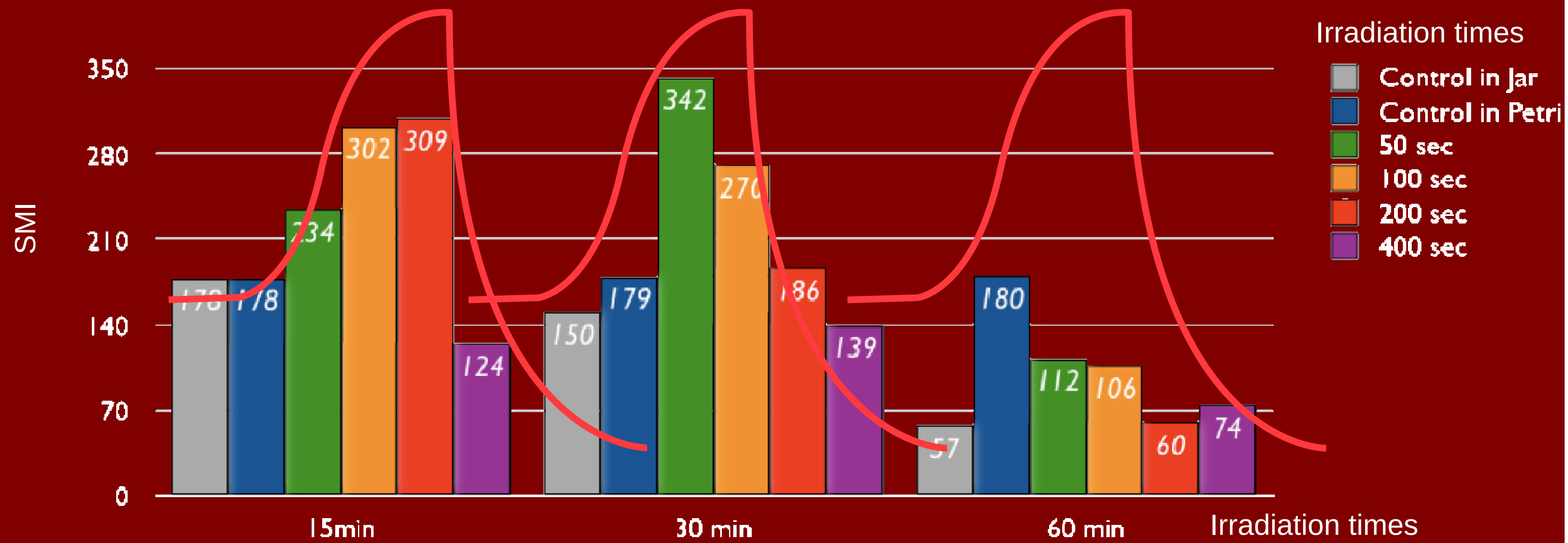
HeLa DNA synthesis
633nm
0.1J/cm2
10 - 1000mW/cm2



increase power
reduce time
(same energy)

Karu TI, Kolyakov SF. (2005) Exact action spectra for cellular responses relevant to phototherapy. Photomed Laser Surg. Aug;23(4):355-61.

Motility changes over time
Fresh Semen
LED 45mW/cm2



Motility changes over time
with different irradiation times using
104 x LED Cluster mixed 660nm & 850nm
45mW/cm2

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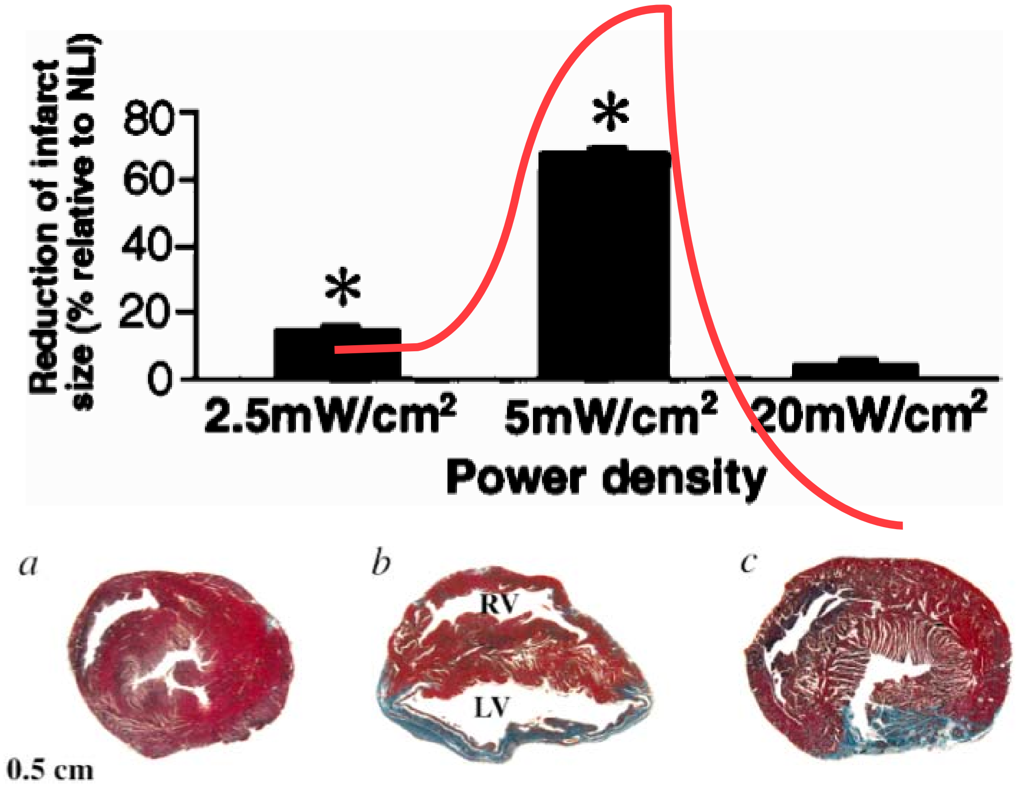
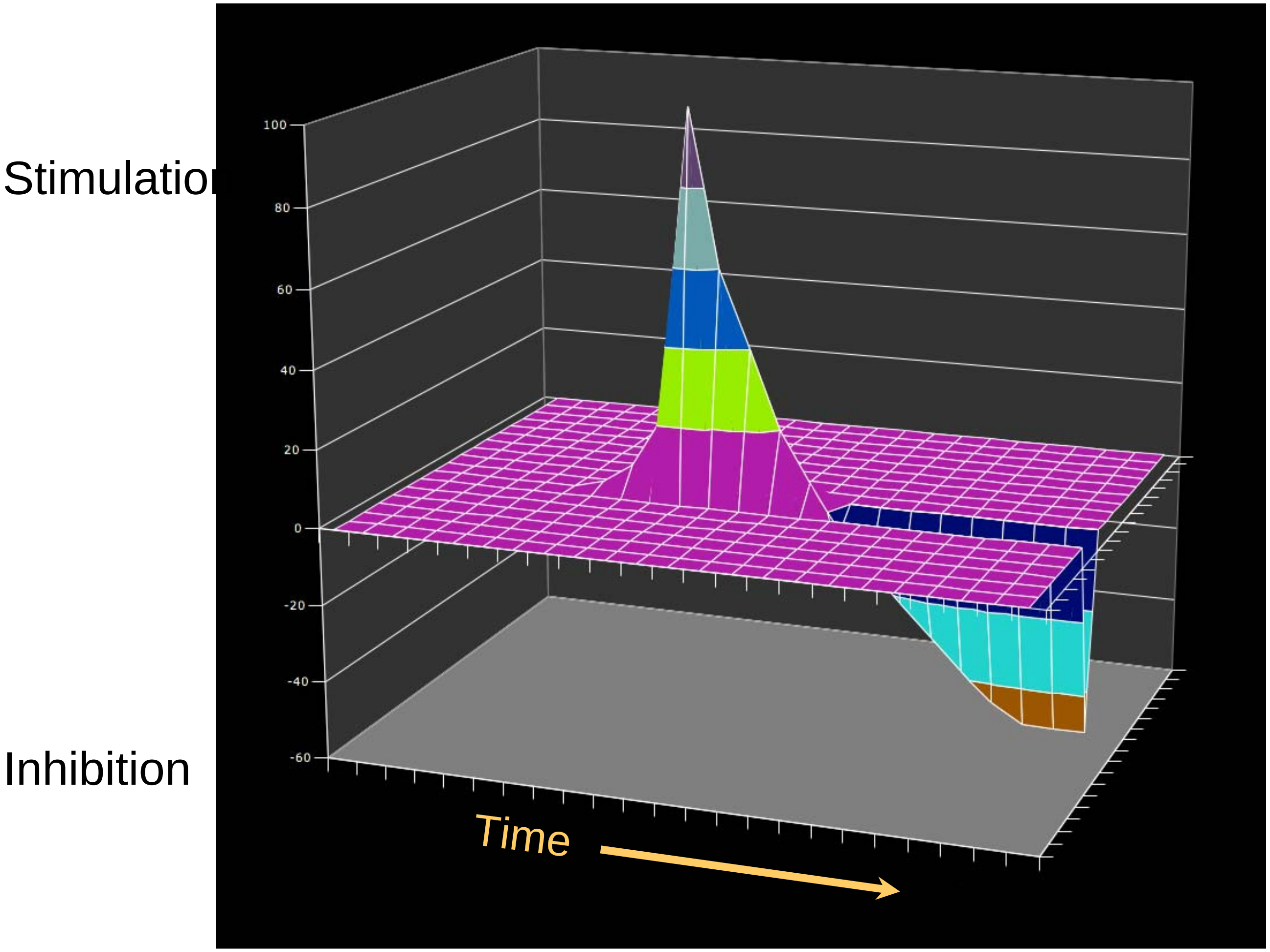
Reduction of infarct size (heart attack) by low-energy laser



Oron U, et al
Attenuation of infarct size in rats and dogs after myocardial infarction by low-energy laser irradiation. Lasers Surg Med.;28(3):204-11.

- Dose response curve



Time
(same Power)

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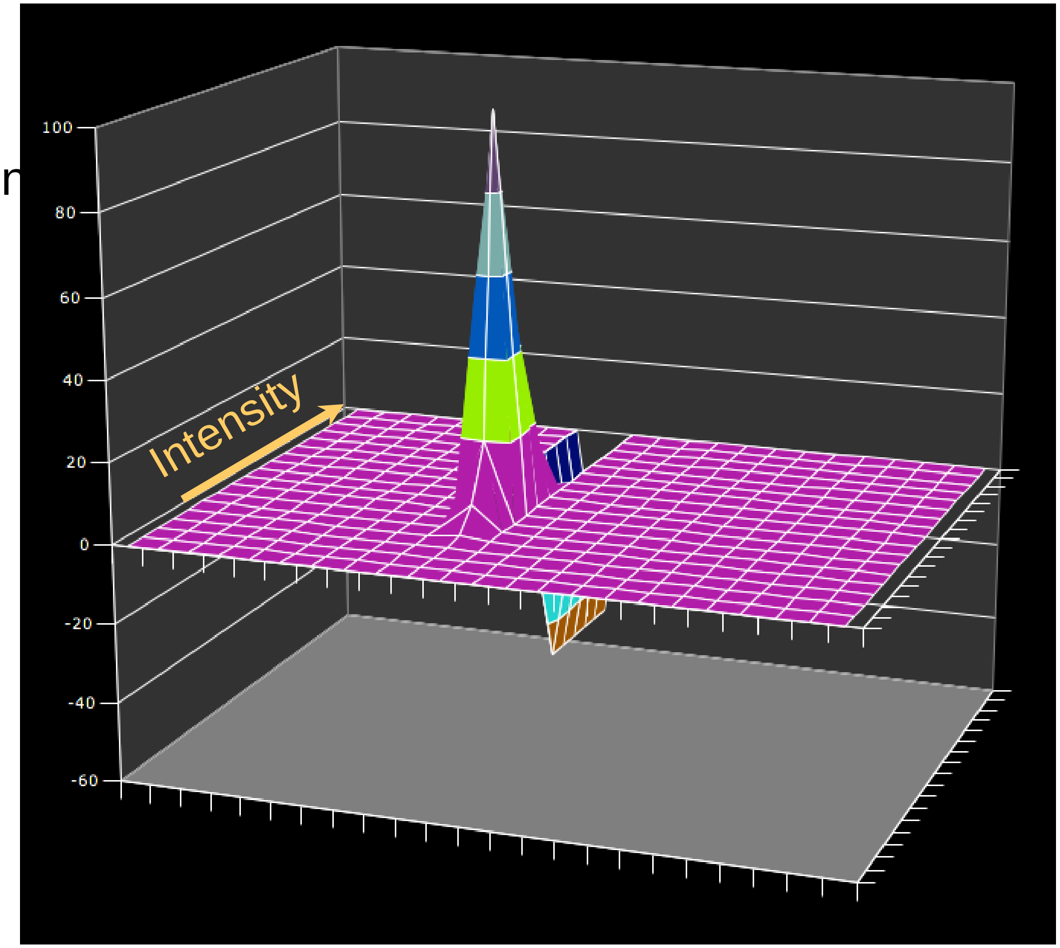
• Dose response curve

Stimulation

Inhibition



Intensity
(same time)

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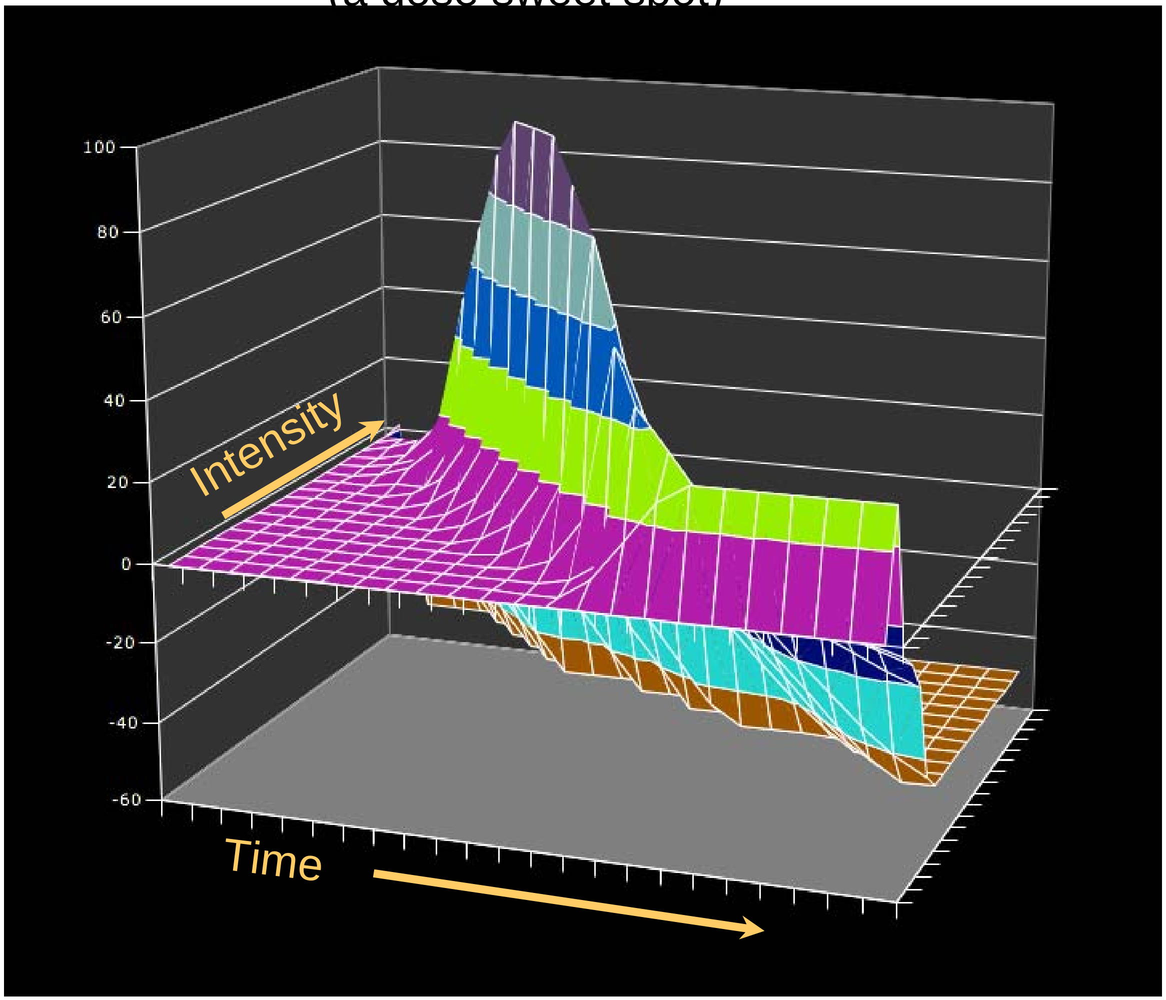
A 3D model for Low Level Laser / LED therapy
Biostimulation and Bioinhibition
(a dose sweet spot)

Stimulation



Inhibition

Anders Island

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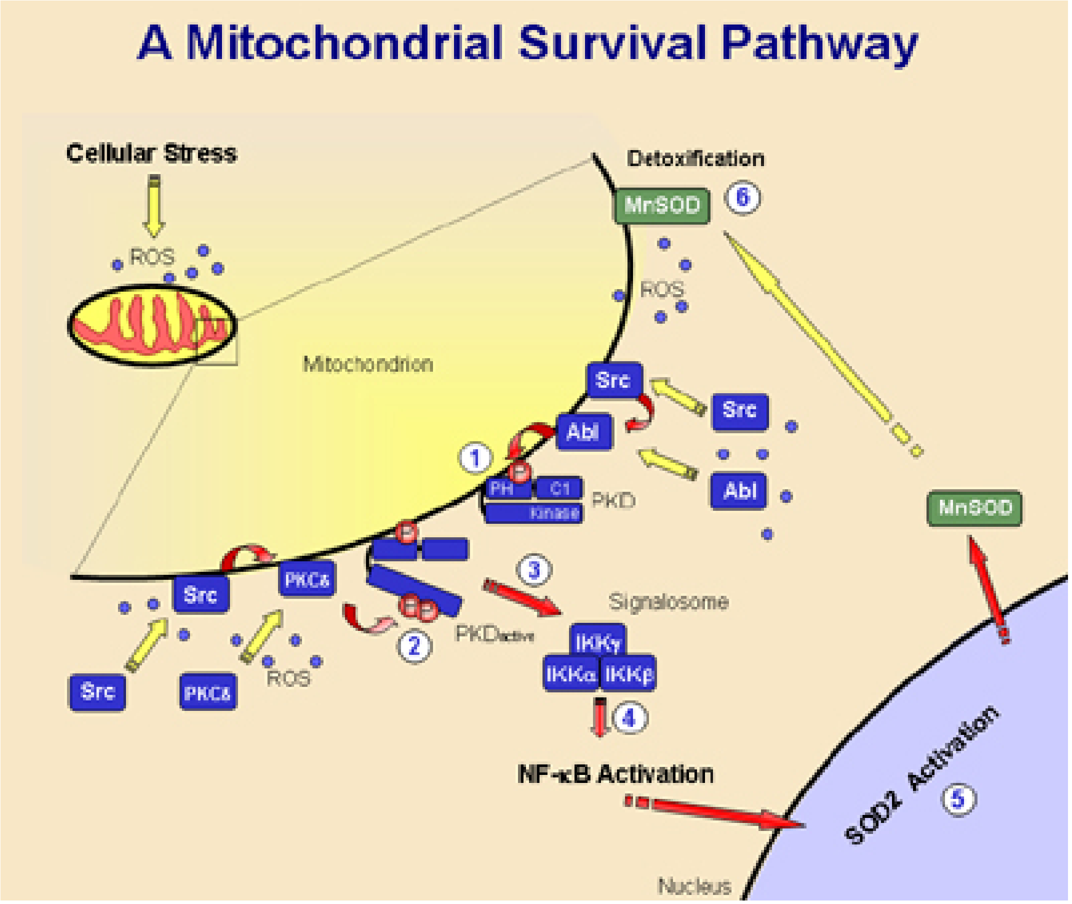
Mitochondrial ROS induces transcription factor NF-kB



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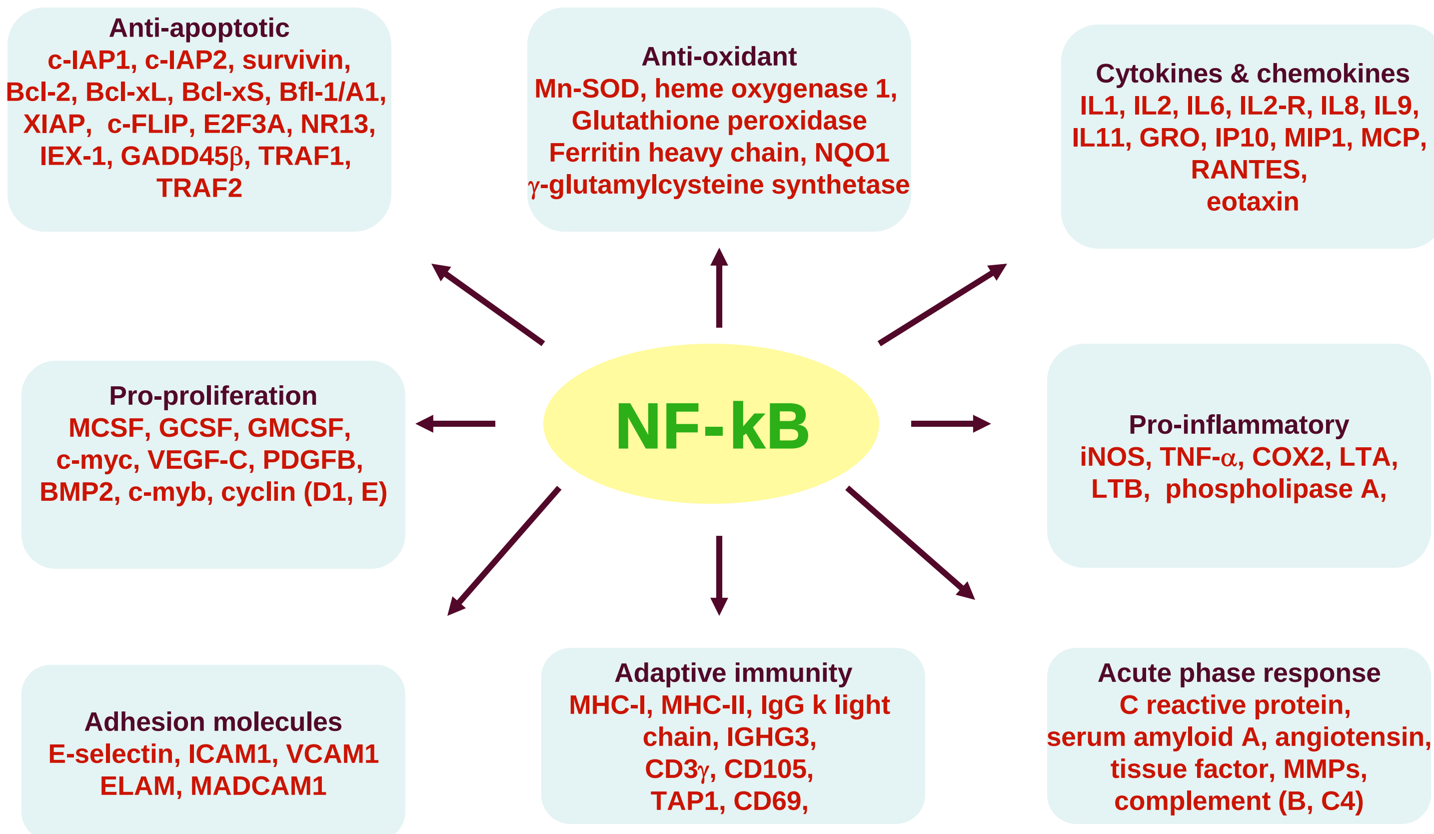
NF-kB target genes



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Use of luciferase and bioluminescence

Luciferase is a generic name for enzymes commonly used in nature for bioluminescence.

Firefly luciferase (EC 1.13.12.7) from the firefly Photinus pyralis.

Light is produced by the oxidation of luciferin substrate consuming adenosine triphosphate (ATP) and catalyzed by luciferase

$$\text{luciferin} + \text{ATP} \xrightarrow{\text{luciferase}} \text{luciferyl adenylate} + \text{Ppi}$$

$$\text{luciferyl adenylate} + O_2 \rightarrow \text{oxyluciferin} + \text{AMP} + \text{light}$$

ELSEVIER

Mutation Research xxx (2004) xxx–xxx

www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Review

Molecular imaging of the transcription factor NF-κB, a primary regulator of stress response

Harald Carlsen, George Alexander, Liv M.I. Austenaa, Kanae Ebihara, Rune Blomhoff*

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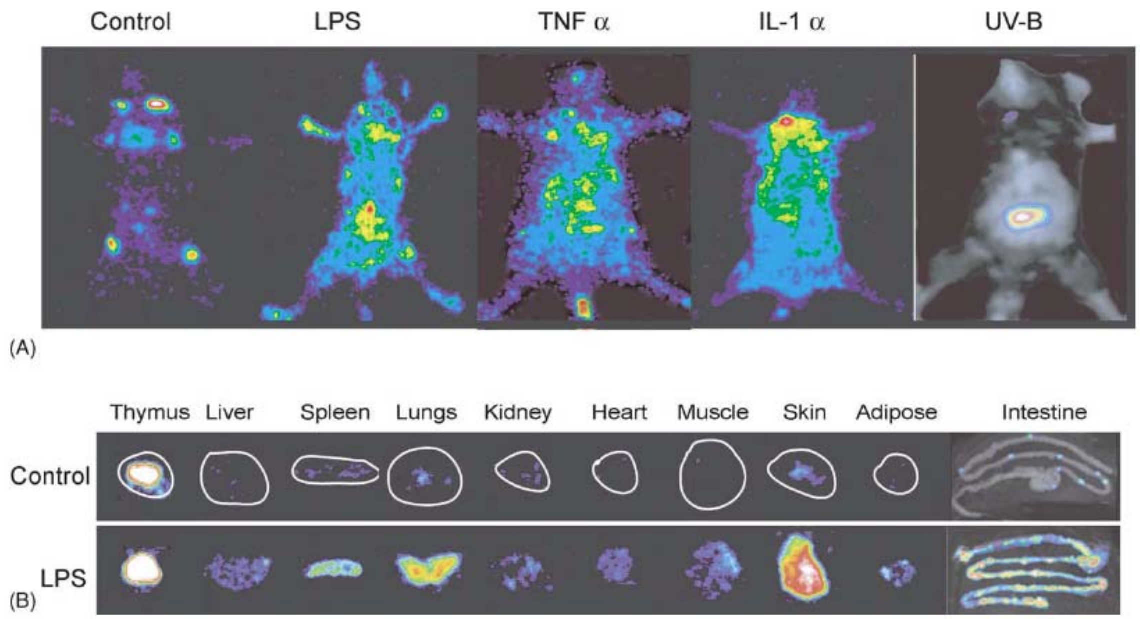
Received 3 October 2003; received in revised form 23 February 2004; accepted 23 February 2004

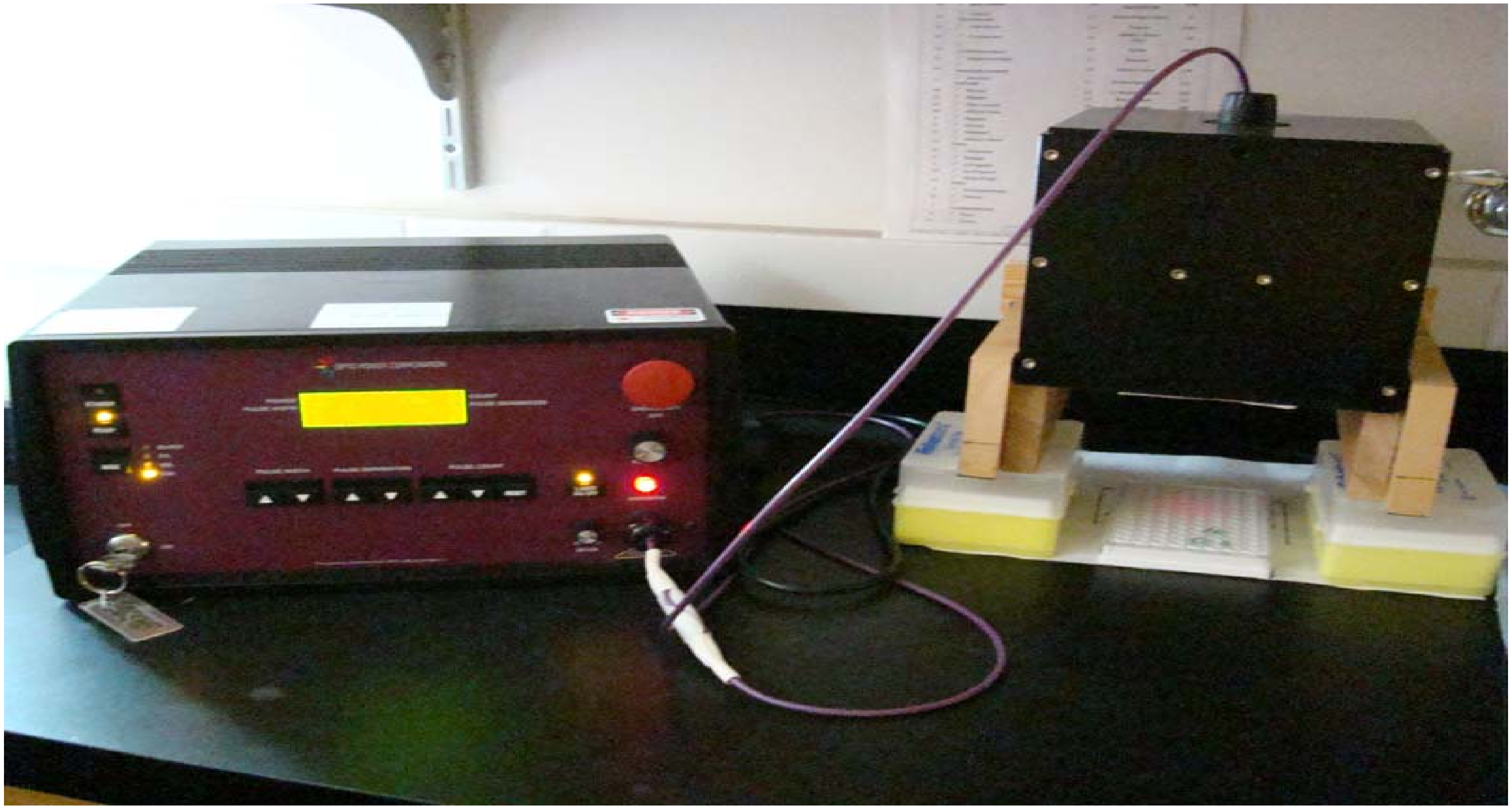


NF-kB response element on Ig-k light chain promoter drives luciferase

Does LLLT activate NF-kB?

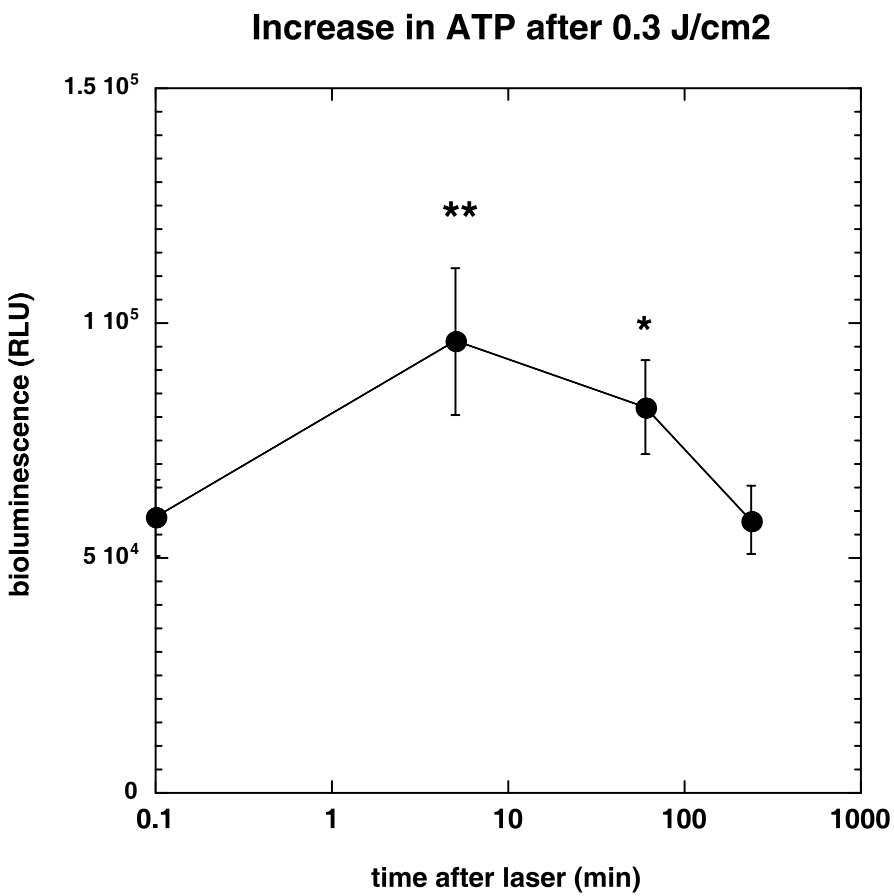
1. Establish a fibroblast cell line (3T3 protocol) from NF-kB luciferase reporter mice (HLL)
2. Deliver different fluences of 810-nm light from a laser (or other light source)
3. Keep illumination time constant at 5 min (vary irradiance)
4. After various times assay for luciferase expression (NF-kB activation) and cellular ATP

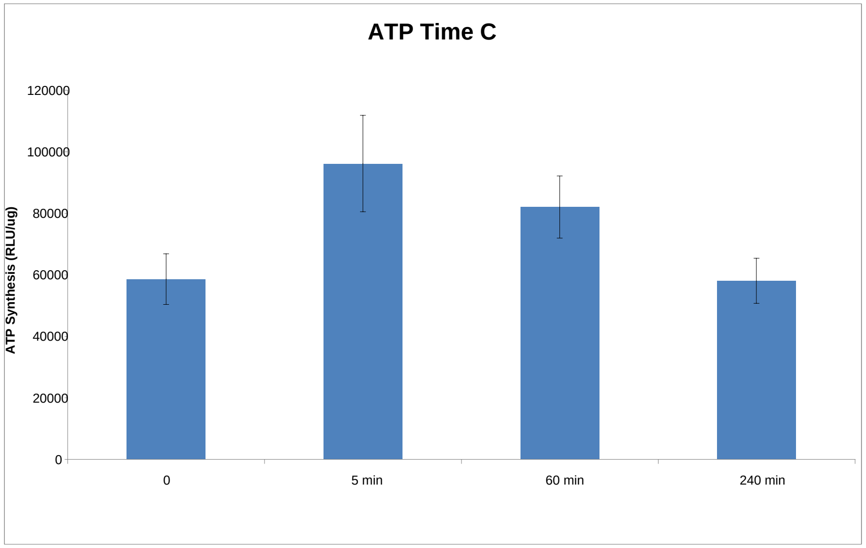
810-nm laser



1. Effects on ATP production

0.3 J/cm^2 810-nm delivered over 5 min





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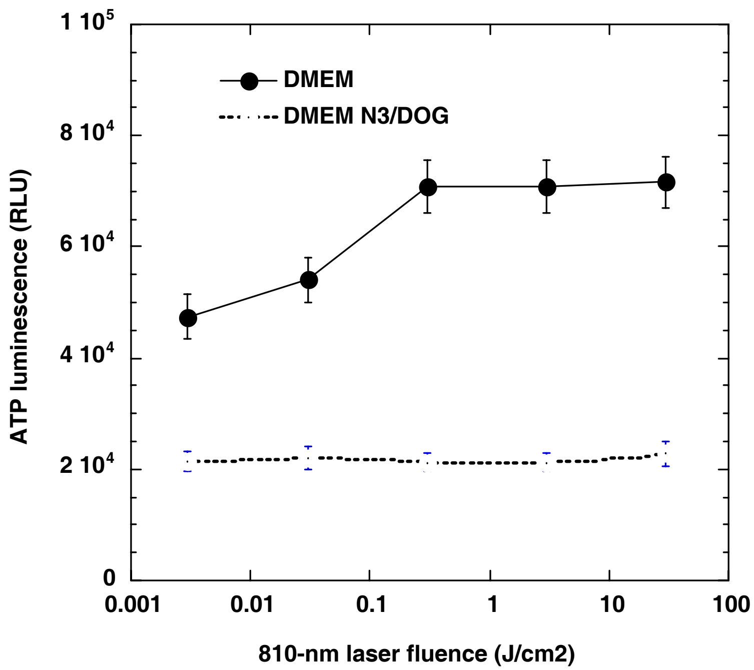
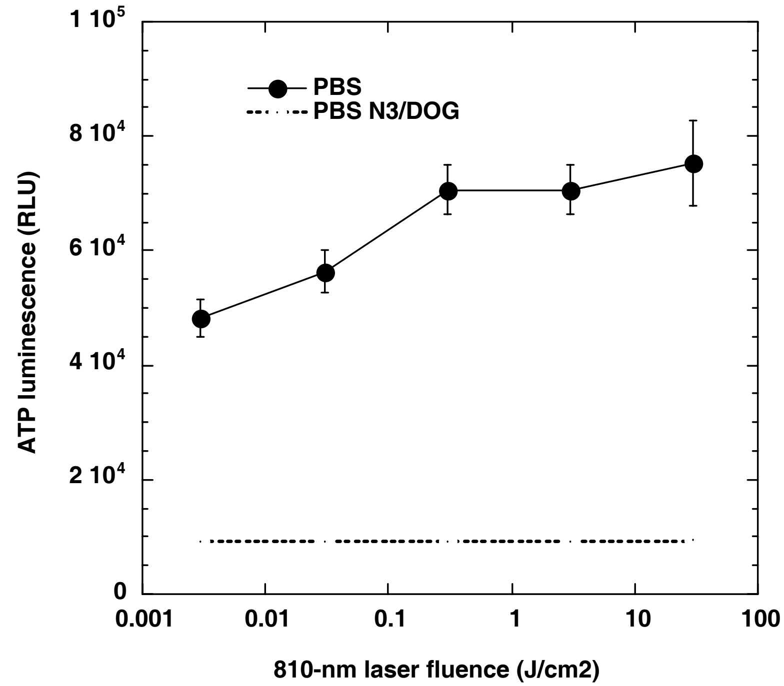
Fluence effect (dose-response) of ATP increase

(measured at 5 min post laser)

Azide/deoxyglucose mimics hypoxia

0.003, 0.03, 0.3, 3 and 30 J/cm^2





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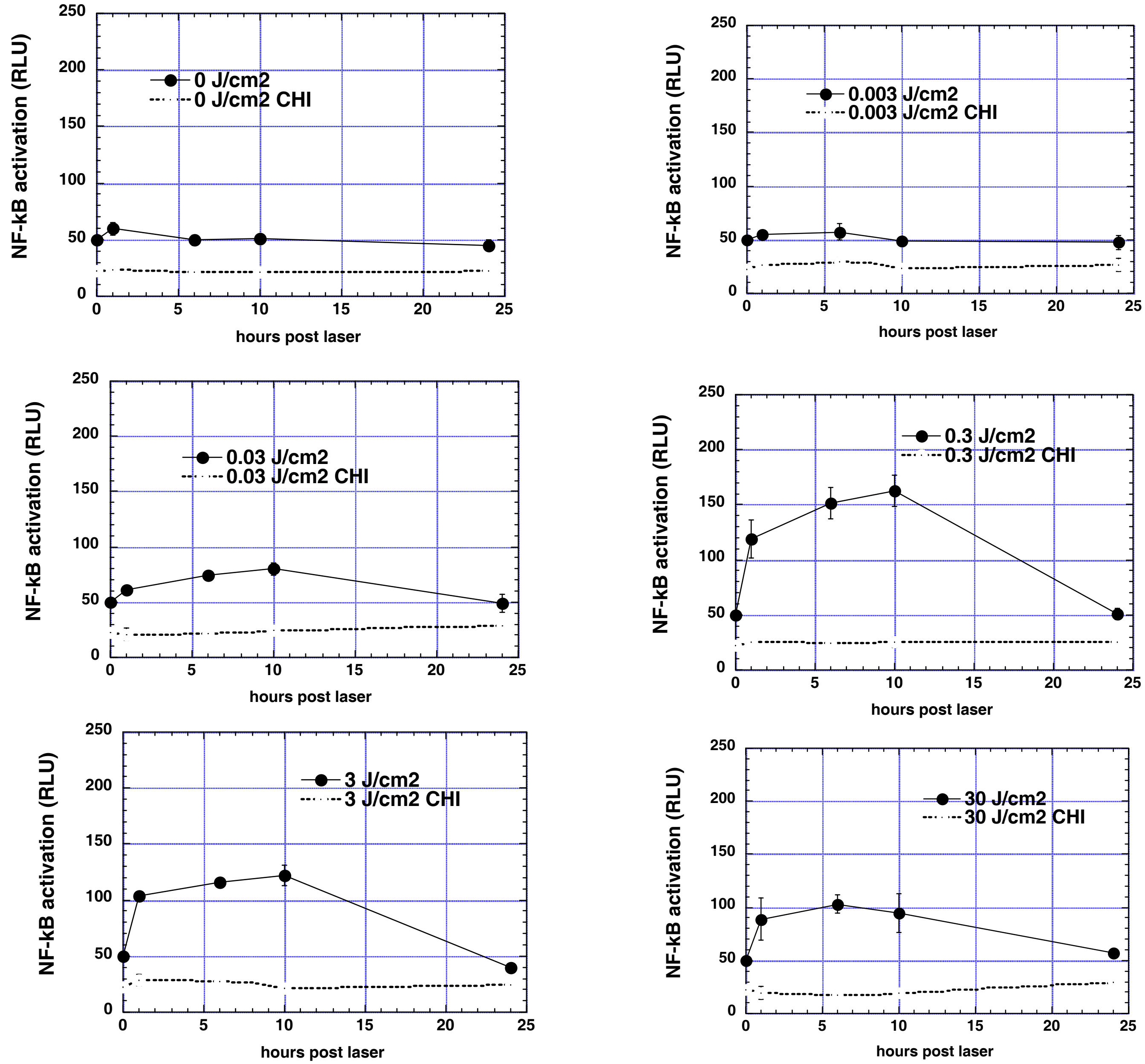
2. Time course of NF-kB activation



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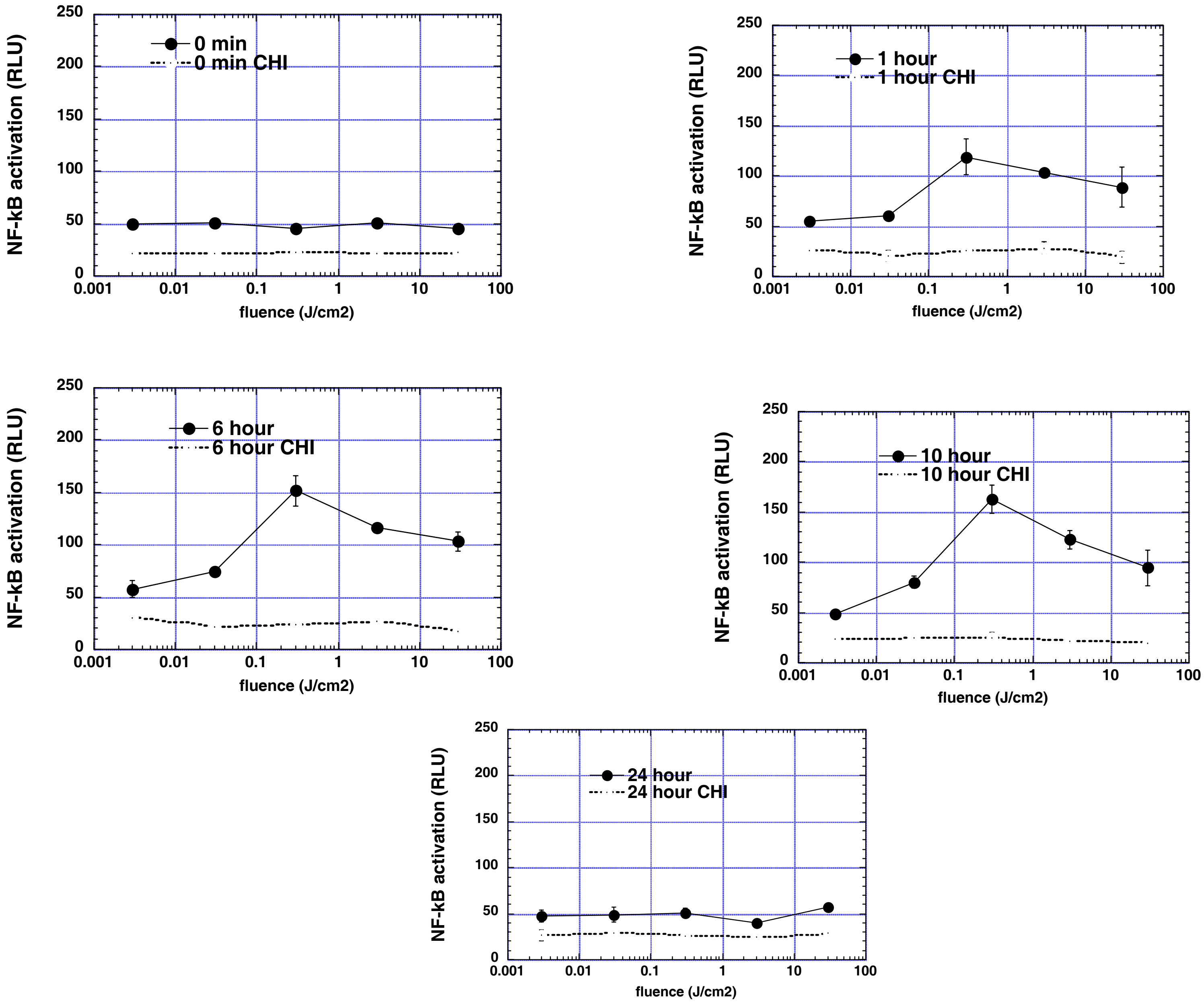
Fluence response of NF-kB activation



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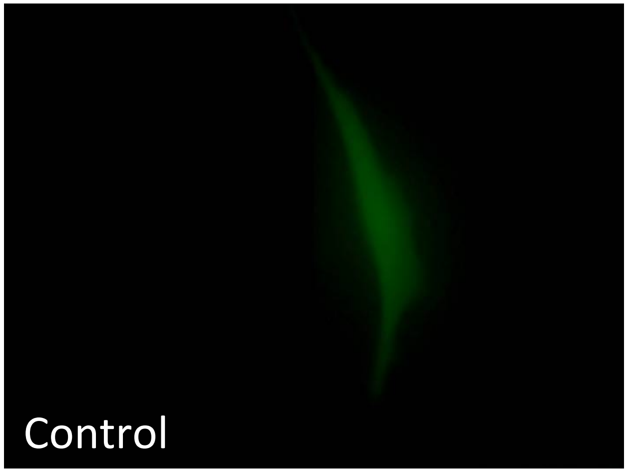
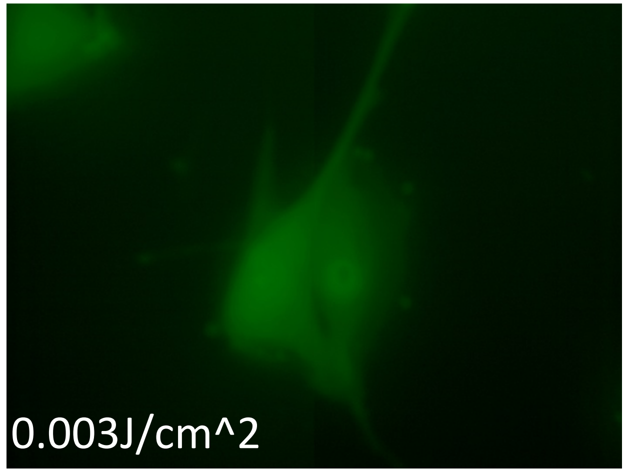
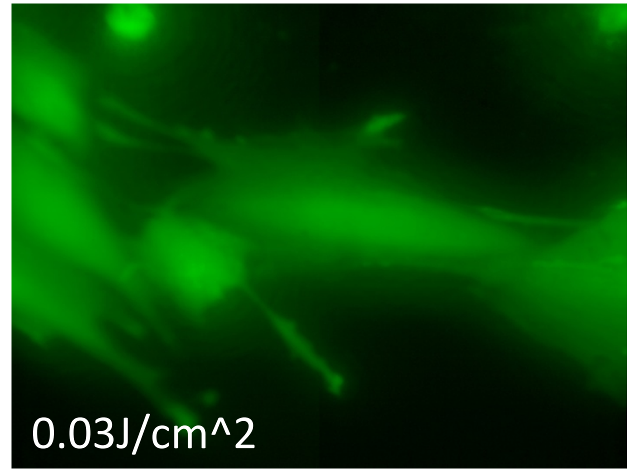
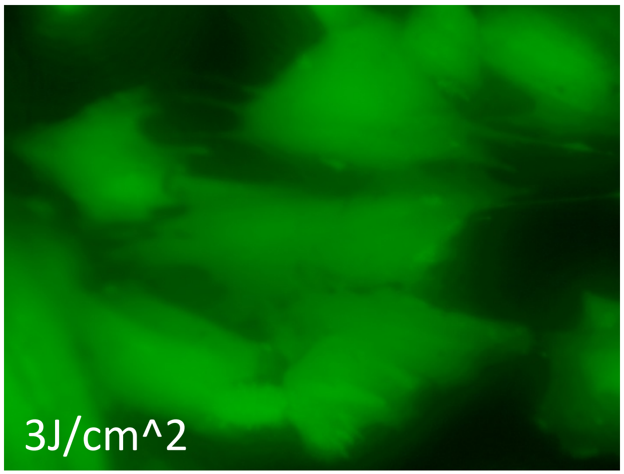
What is mechanism of NF-kB activation?

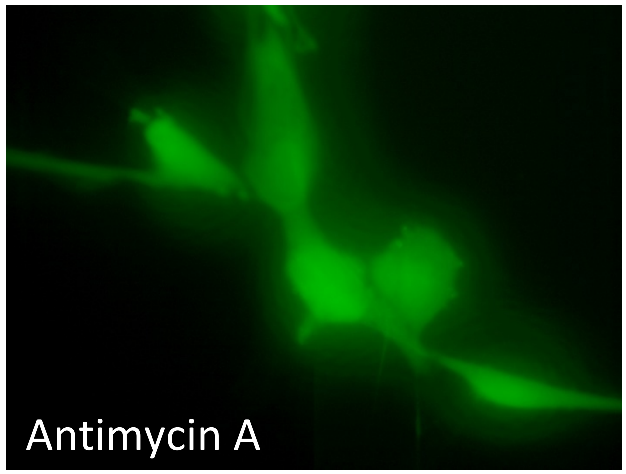
Hypothesis is reactive oxygen species

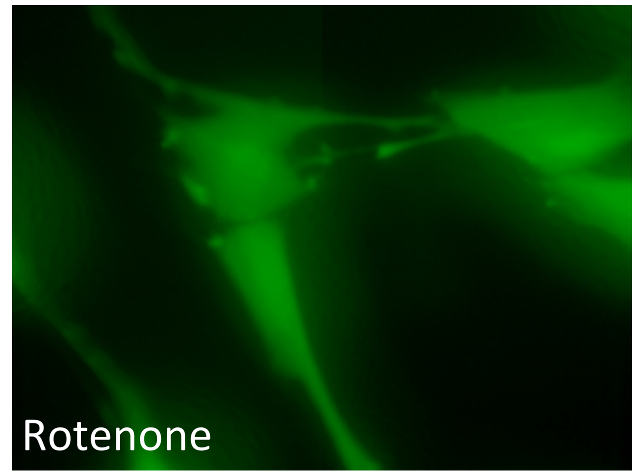


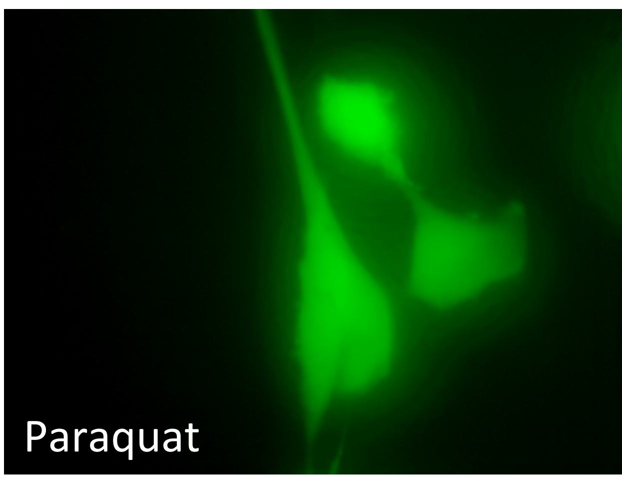






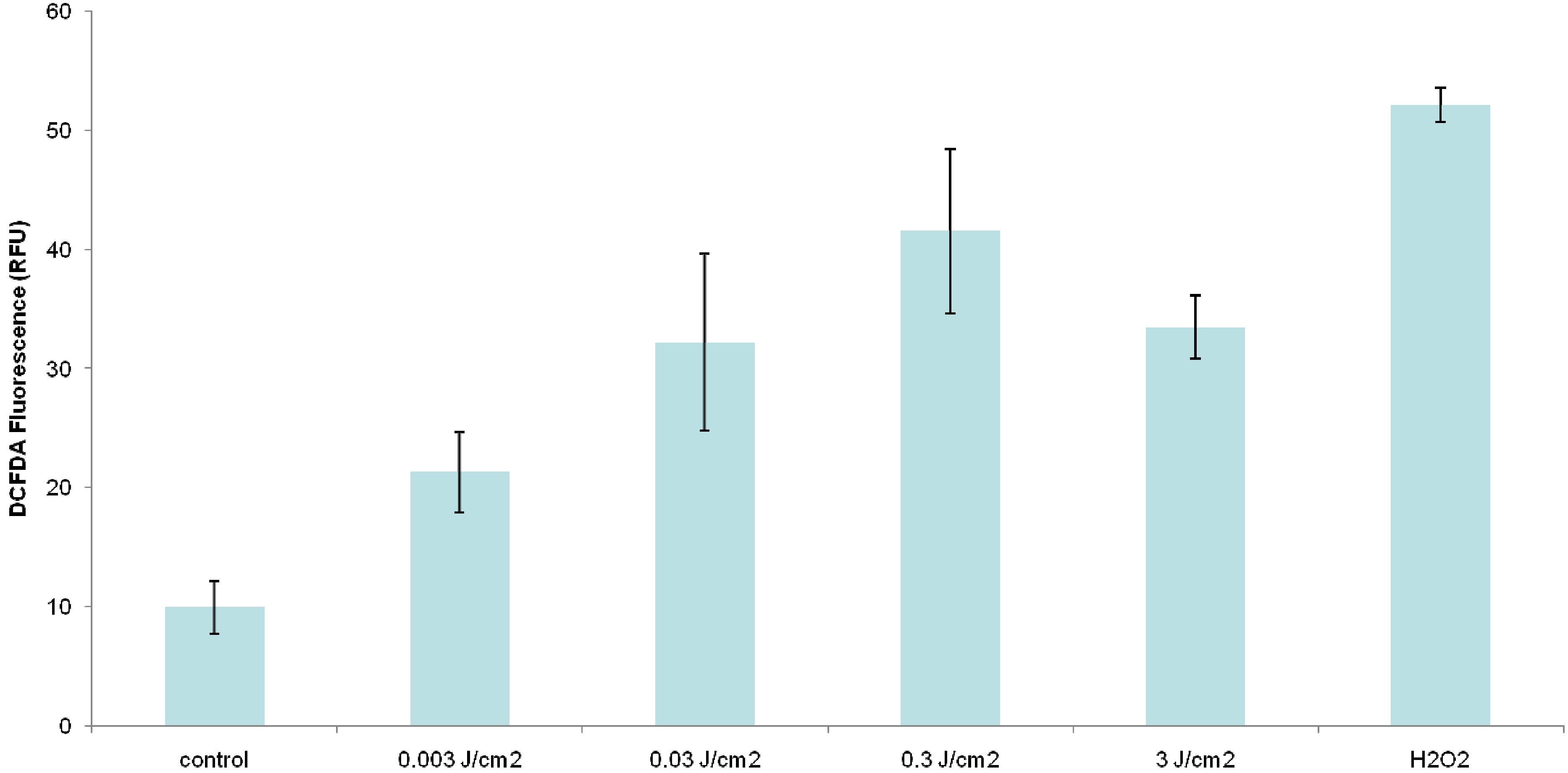






DCFDA
Sensitive to lipid hydroperoxides

Quantification of ROS induced by laser



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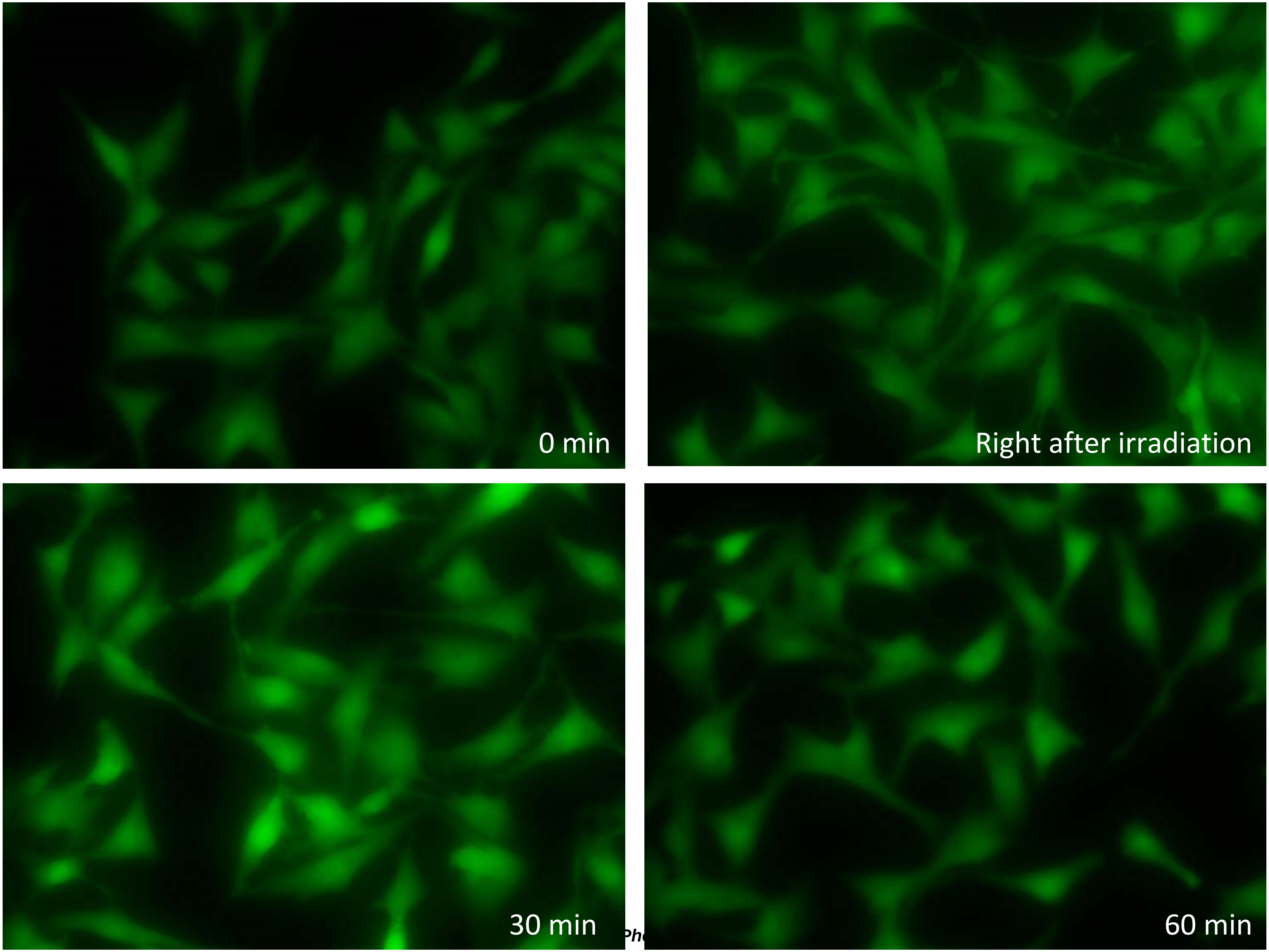
MEF ROS Time Course

MEF ROS Time course

- 1. Irradiate MEF with 810nm Laser
- 2. At each time point, 2x washing with PBS and add the DCFDA probe.
- 3. Incubate the MEF with the probe for 1 hour.
- 4. Microscope Imaging


0 min
Right after irradiation
30 min
60 min

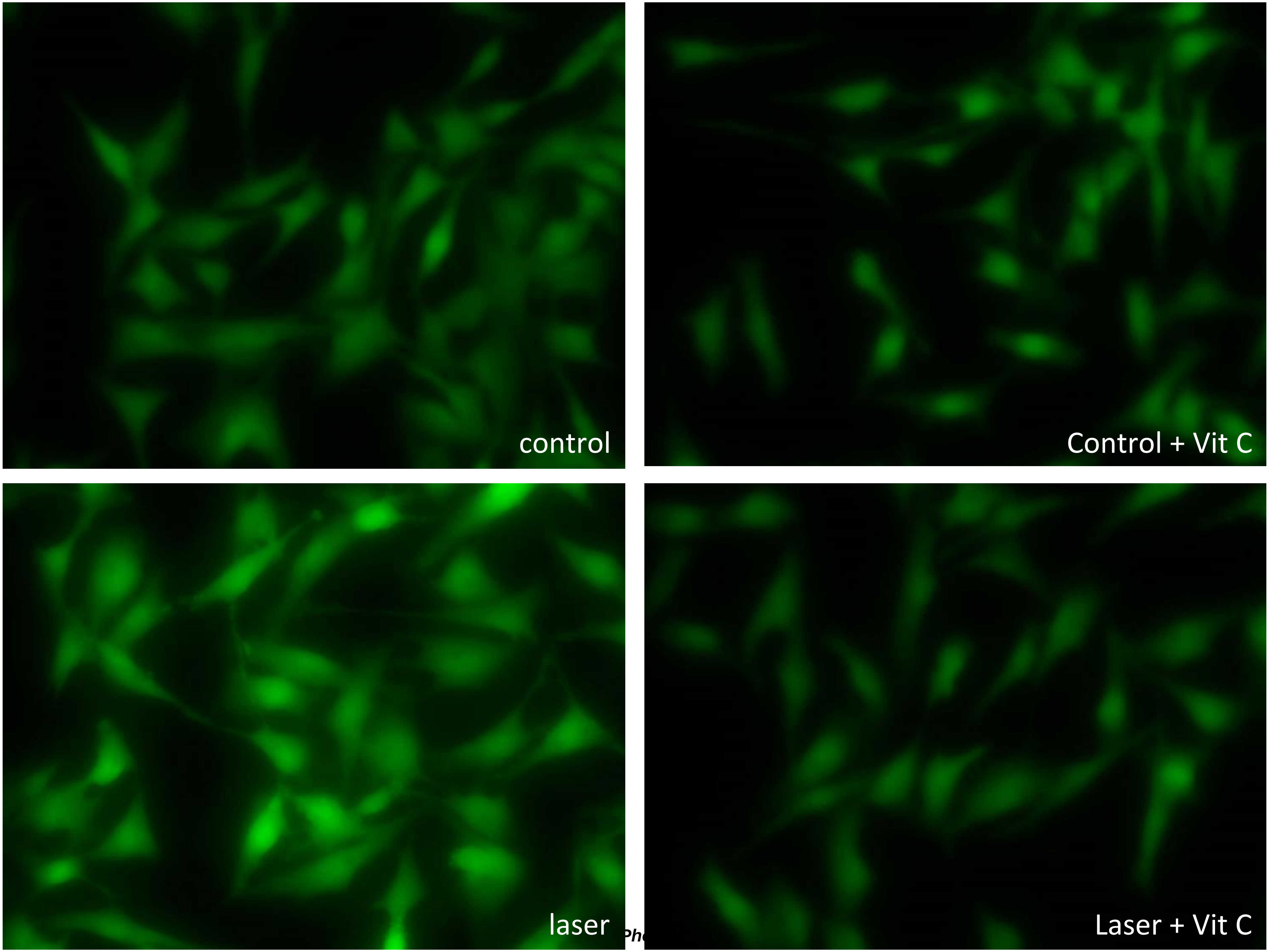
Results

- 1. 810 nm laser promoted ROS production.

- 2. Loading the Probe 30 minutes after the irradiation showed the highest fluorescence.

Ascorbic Acid abrogating induced ROS Production?

- 1. Apply 100 µM Ascorbic Acid into the medium and pre-incubated for 30 minutes.
- 2. Irradiate the cells. Since the time course showed the best fluorescent results when loading the probe 30 min after irradiation, cells were incubated for 30 minutes before loading the probe.
- 3. 2x washing with PBS and incubate cells in the loading buffer for 1 hour.
- 4. microscopy imaging

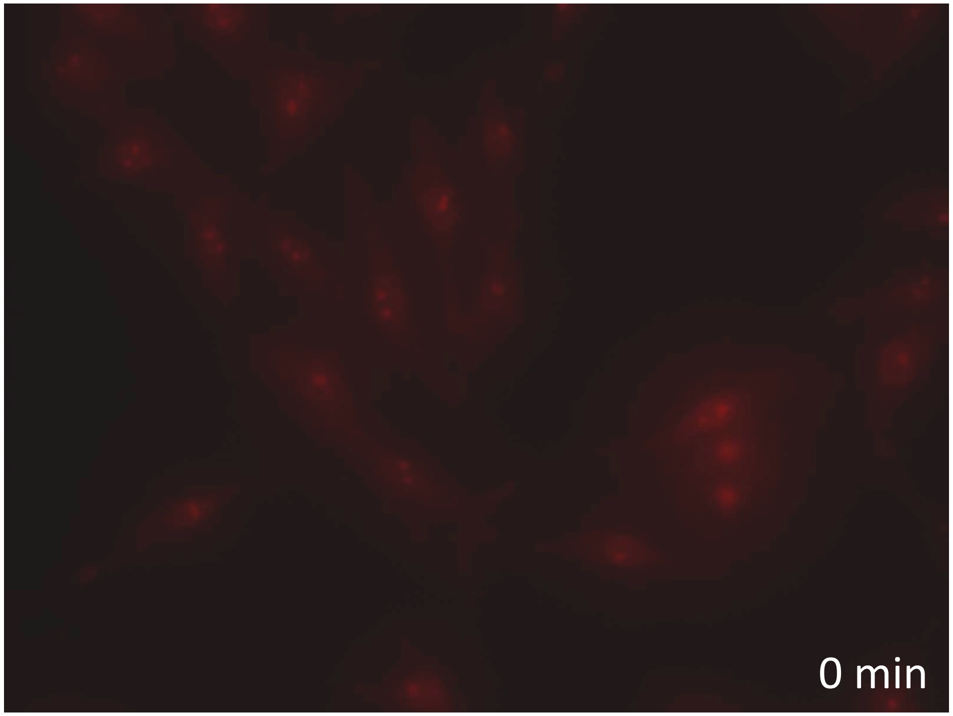

control
Control + Vit C
laser
Laser + Vit C

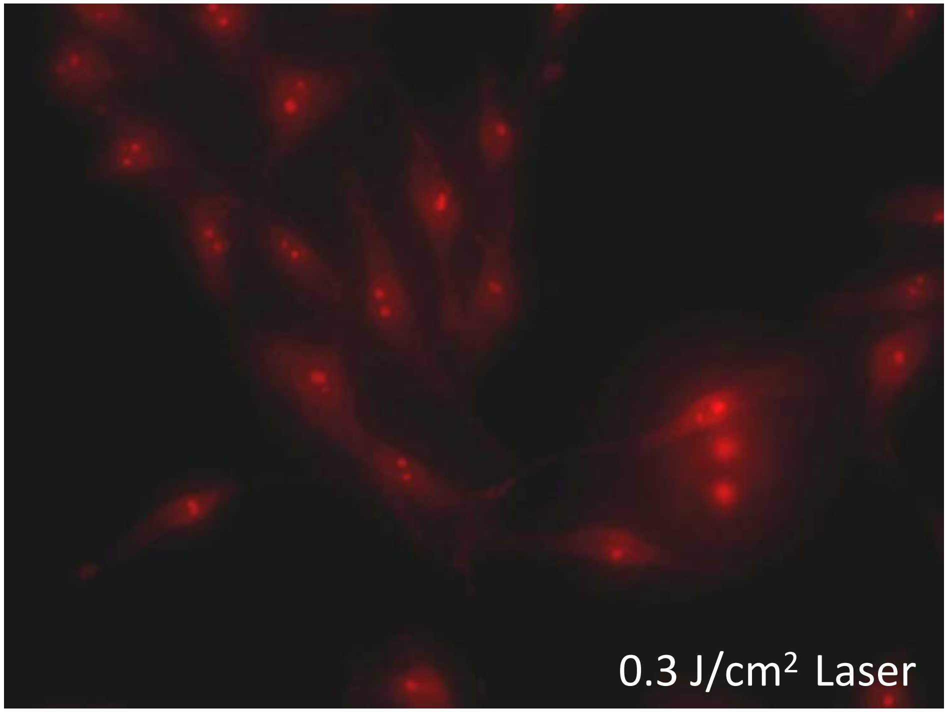
Results

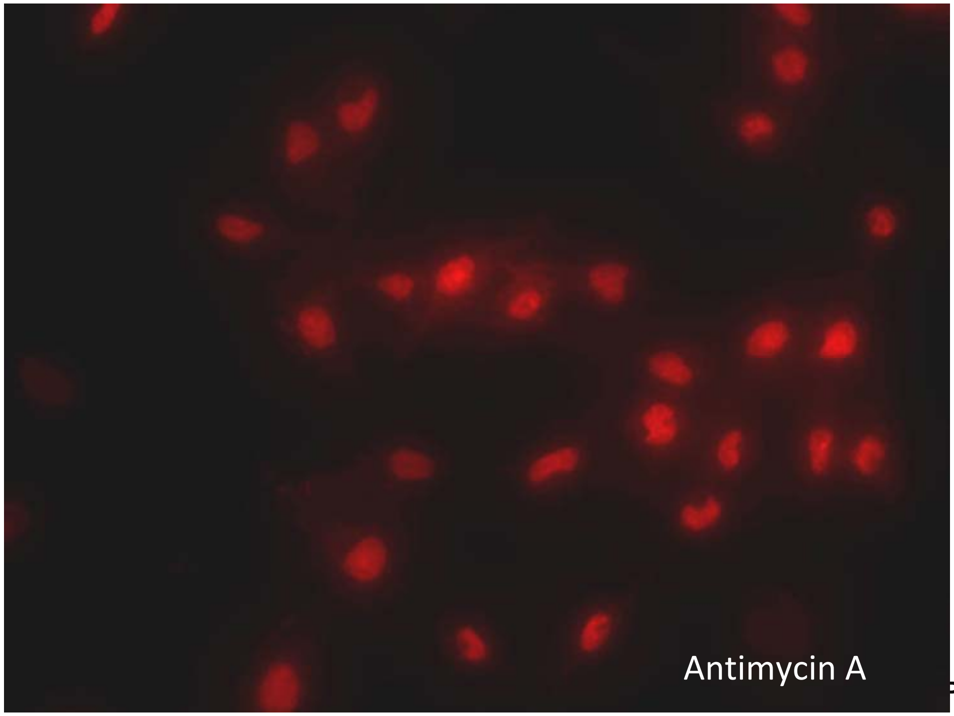
- 1. Ascorbic Acid decreased the DCFDA fluorescence in both control and the laser irradiated cells.

MitoSOX Red Assay
Laser induced superoxide production in the mitochondria.

- 1. Irradiate cells
- 2. 2x washing with PBS and incubated cells in the loading buffer for 1 hour before imaging
- 3. Apply Antimycin A as a positive control



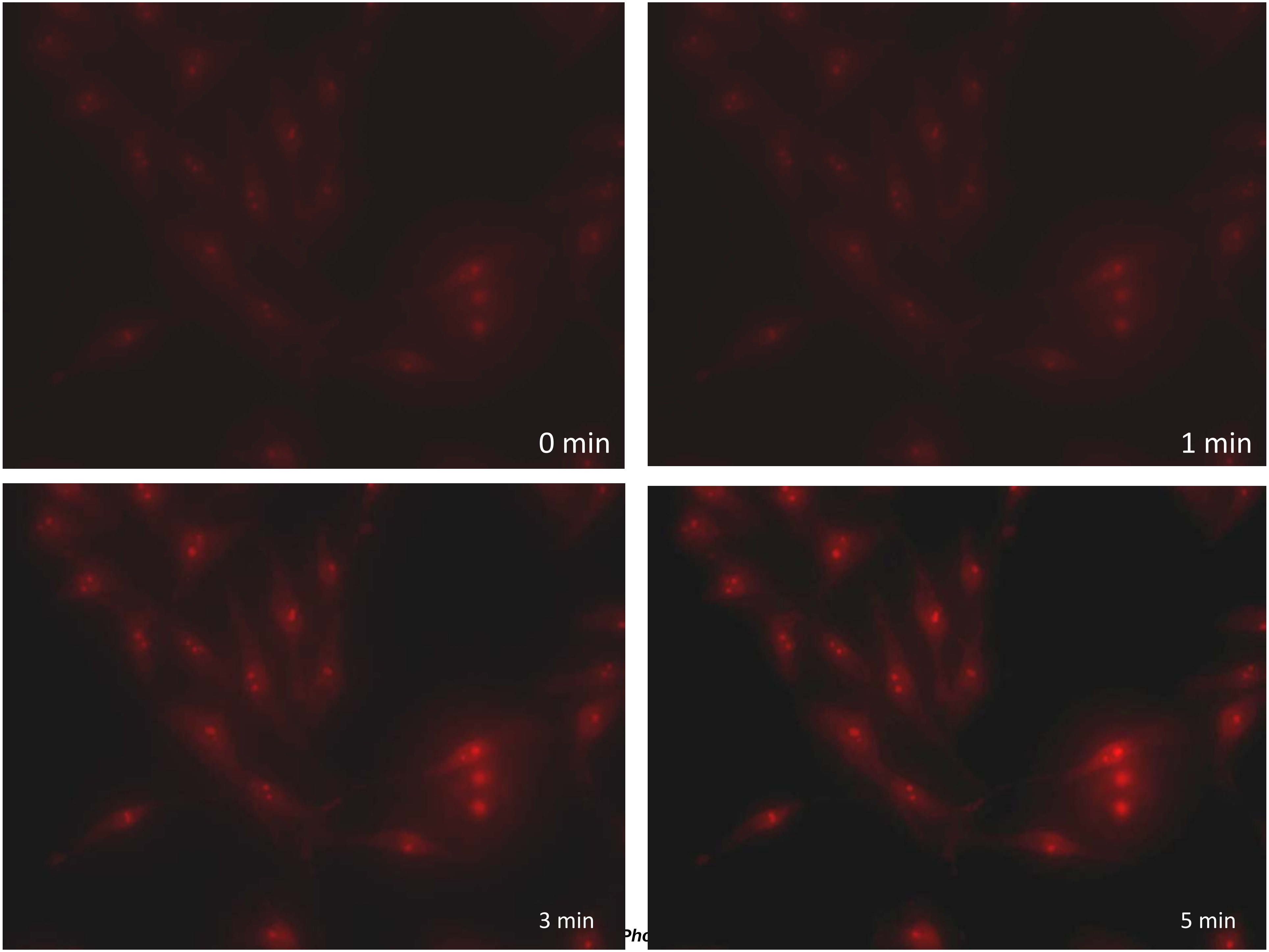




Mitochondrial Superoxide Production
By
MitoSOX Red

Photomedicine. Dose Response Conference 2009

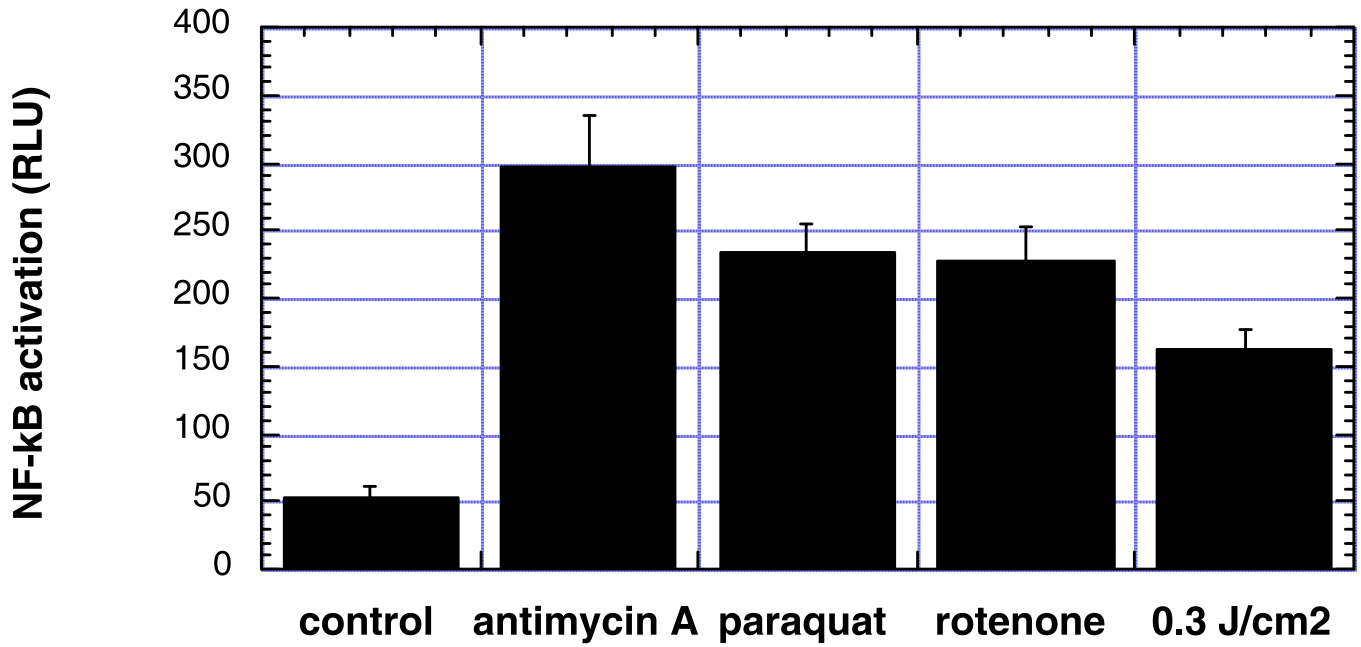
Real Time Fluorescence of Mitochondrial Superoxide by MitoSOX Red


0 min
1 min
3 min
5 min

Results

- Fluorescence in the first minute did not show significant difference from the control.
- Fluorescent difference started to appear from the third minutes
- This test serves an additional evidence supporting mitochondrial superoxide was actually produced by 0.3 J/cm^2 laser during the irradiation.

Do mitochondrial inhibitors increase NF-kB activation?



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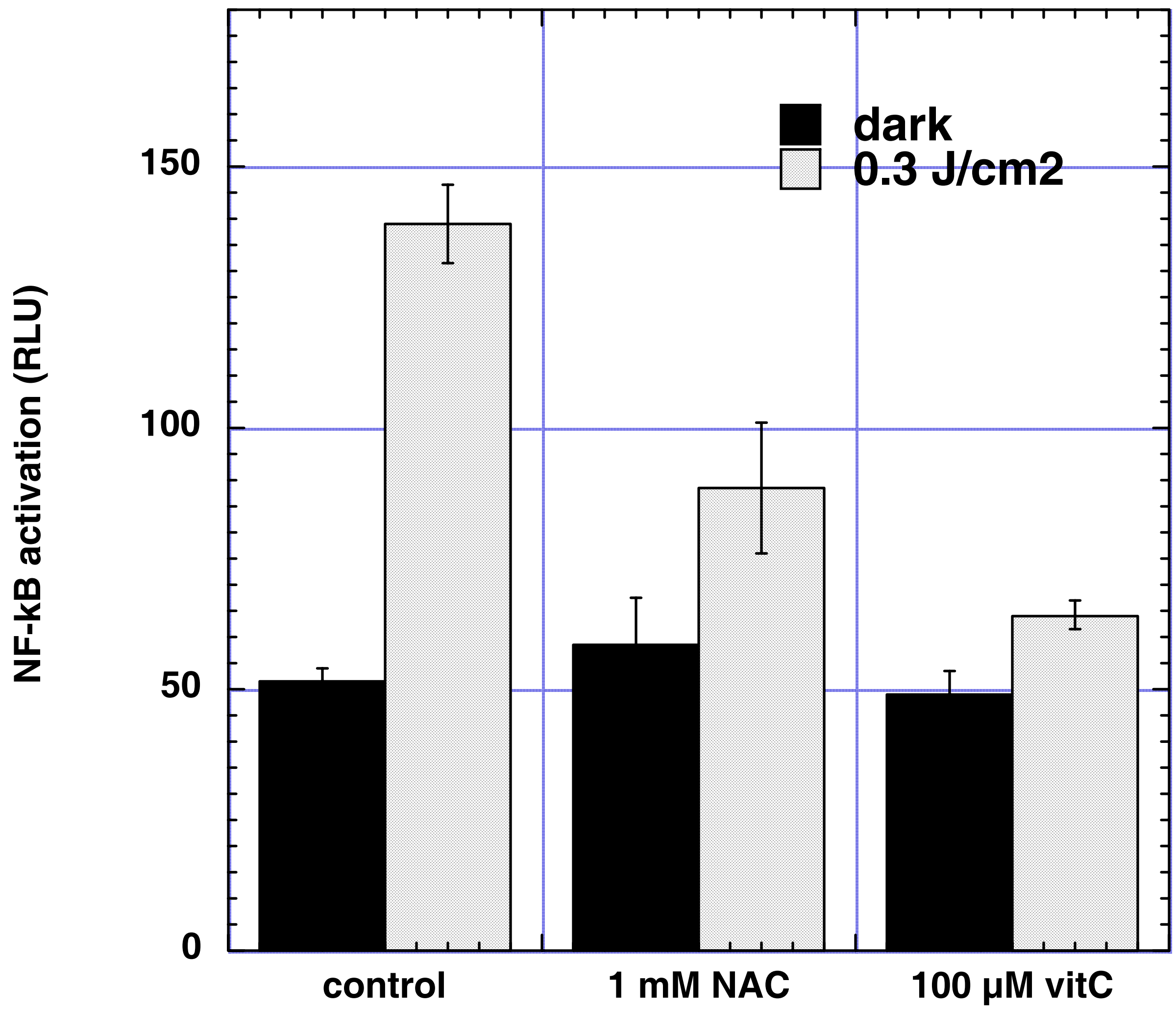
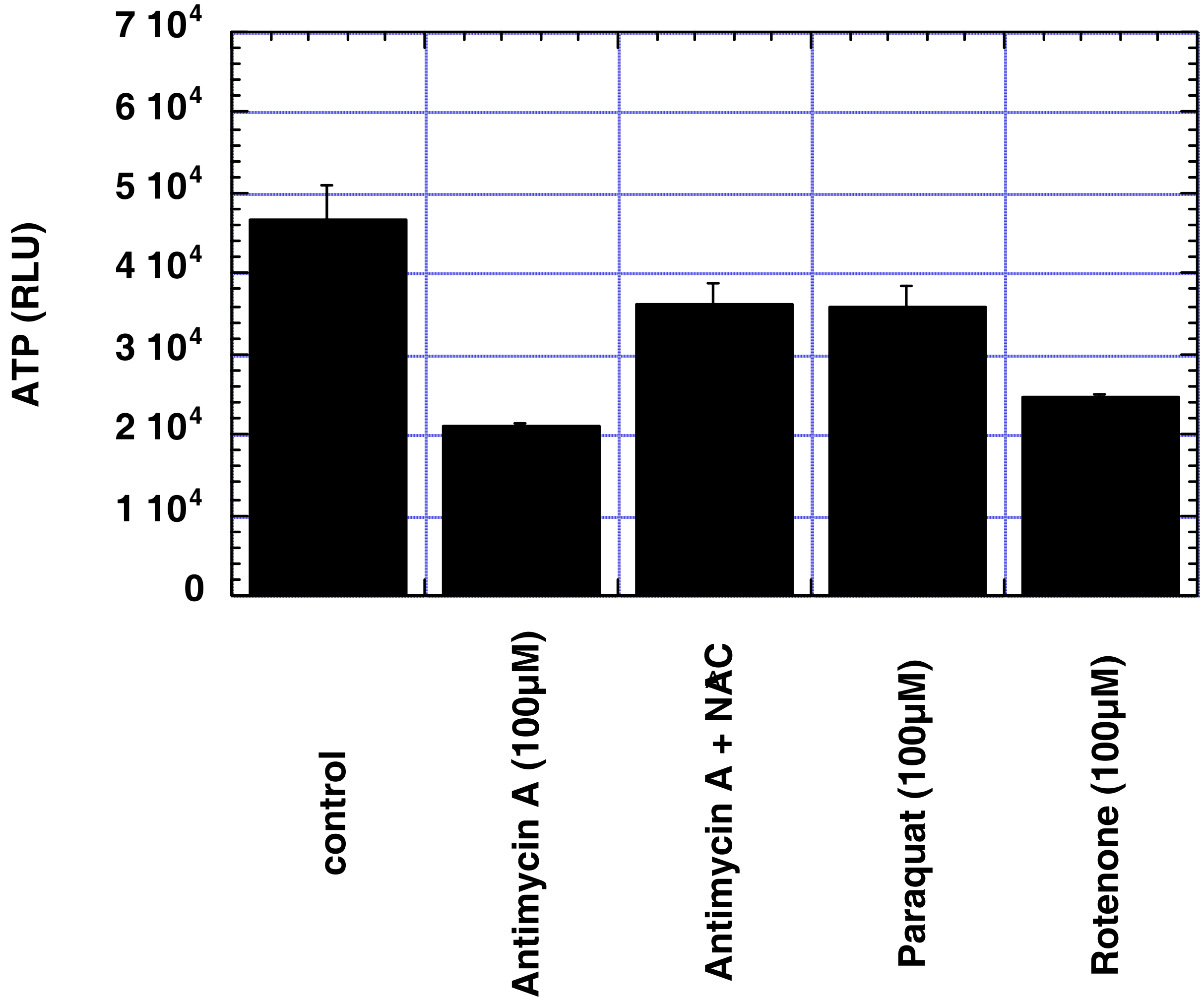
Do antioxidants prevent NF-kB activation?



What is effect of mitochondrial inhibitors and antioxidants on ATP?



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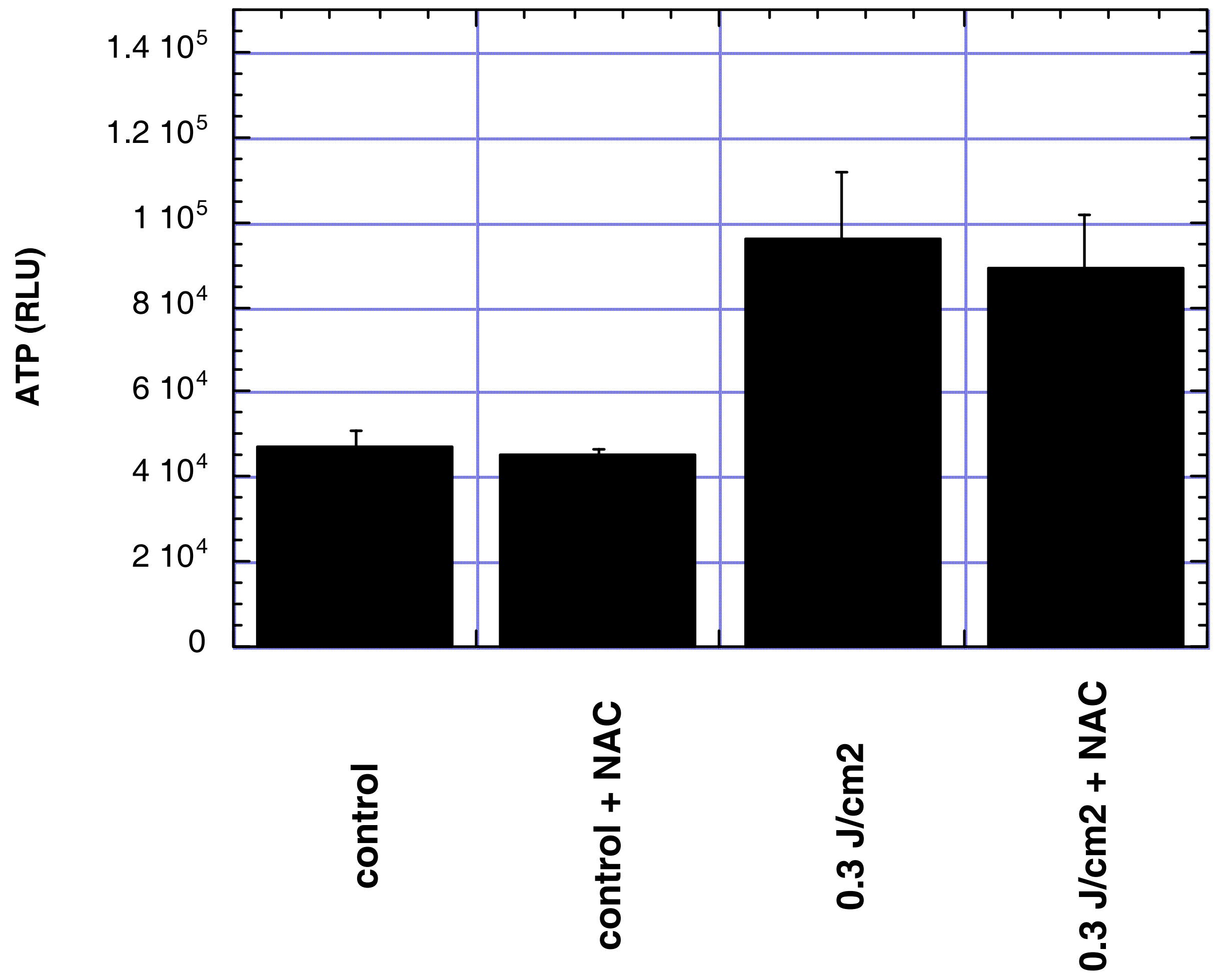
Do antioxidants abrogate laser induced ATP ?



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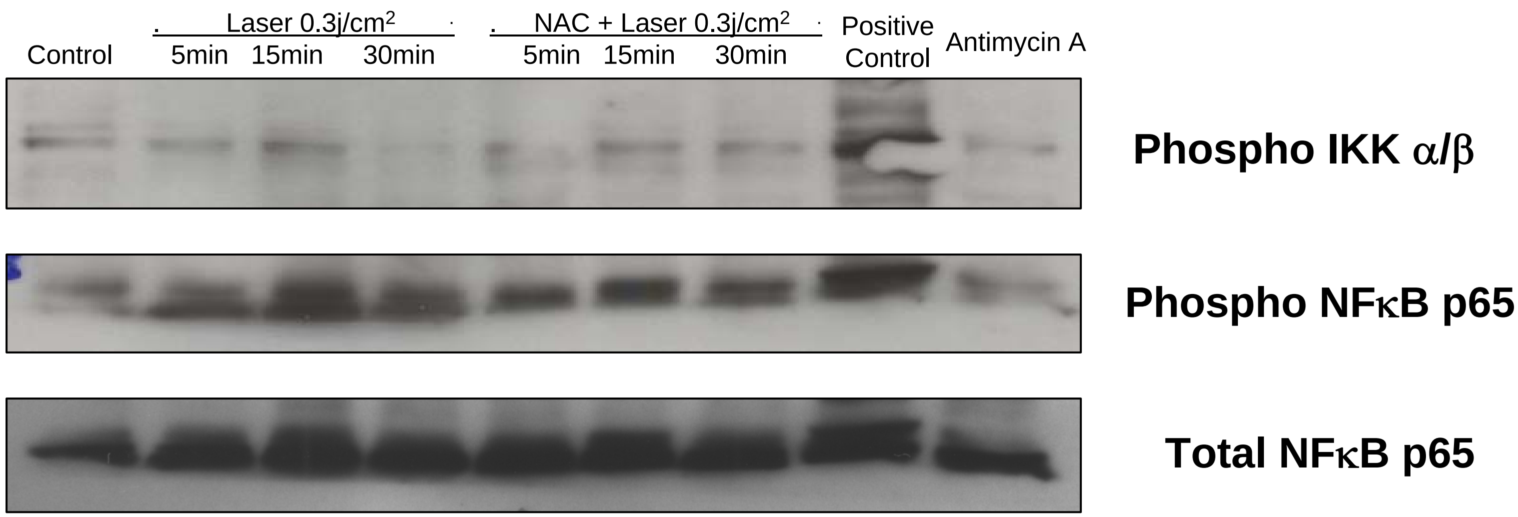
NFκB activation by Lasers



Positive Control: Cells (Primary MEFs) treated with TNFα for 15min

Conclusions: Laser (810nm 0.3j/cm^2) can activate NFκB.

Note: NAC did not neutralize this expression completely suggesting either;

1. incomplete inhibition of ROS
2. alternative (GF) mediated activation of NFκB.

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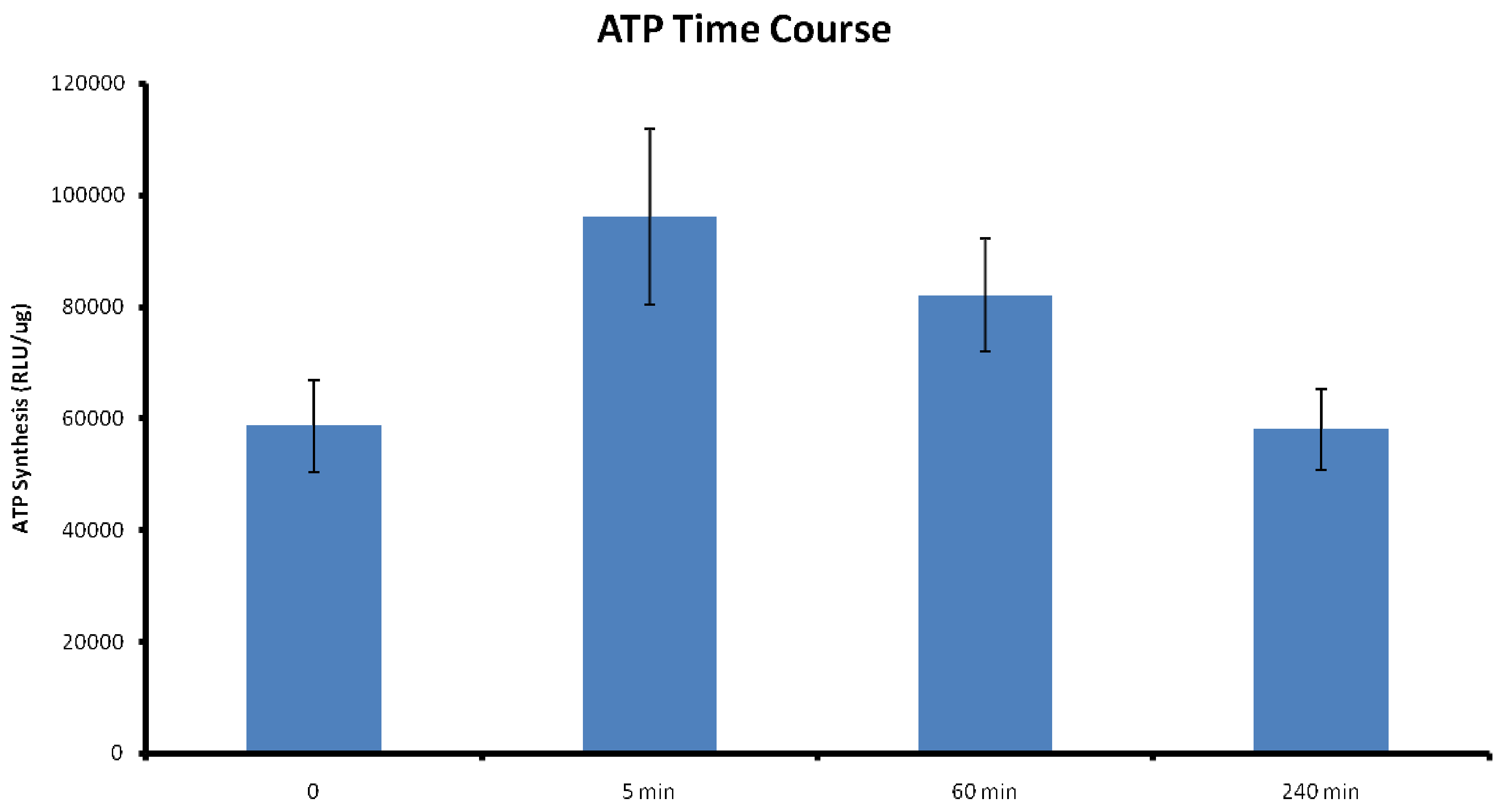
Human Fibroblasts



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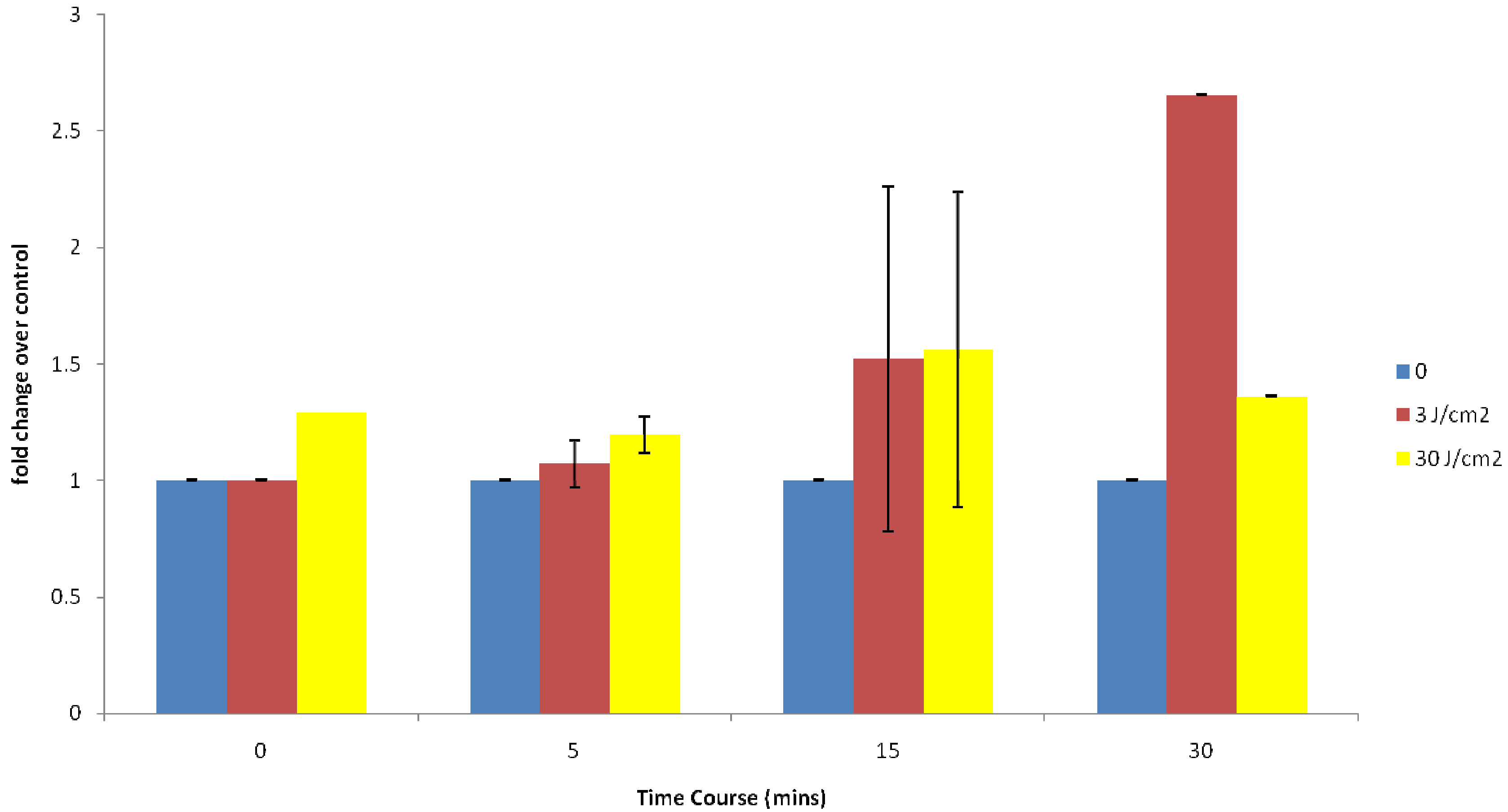
810 LED induced Nitrate Production in HeLa



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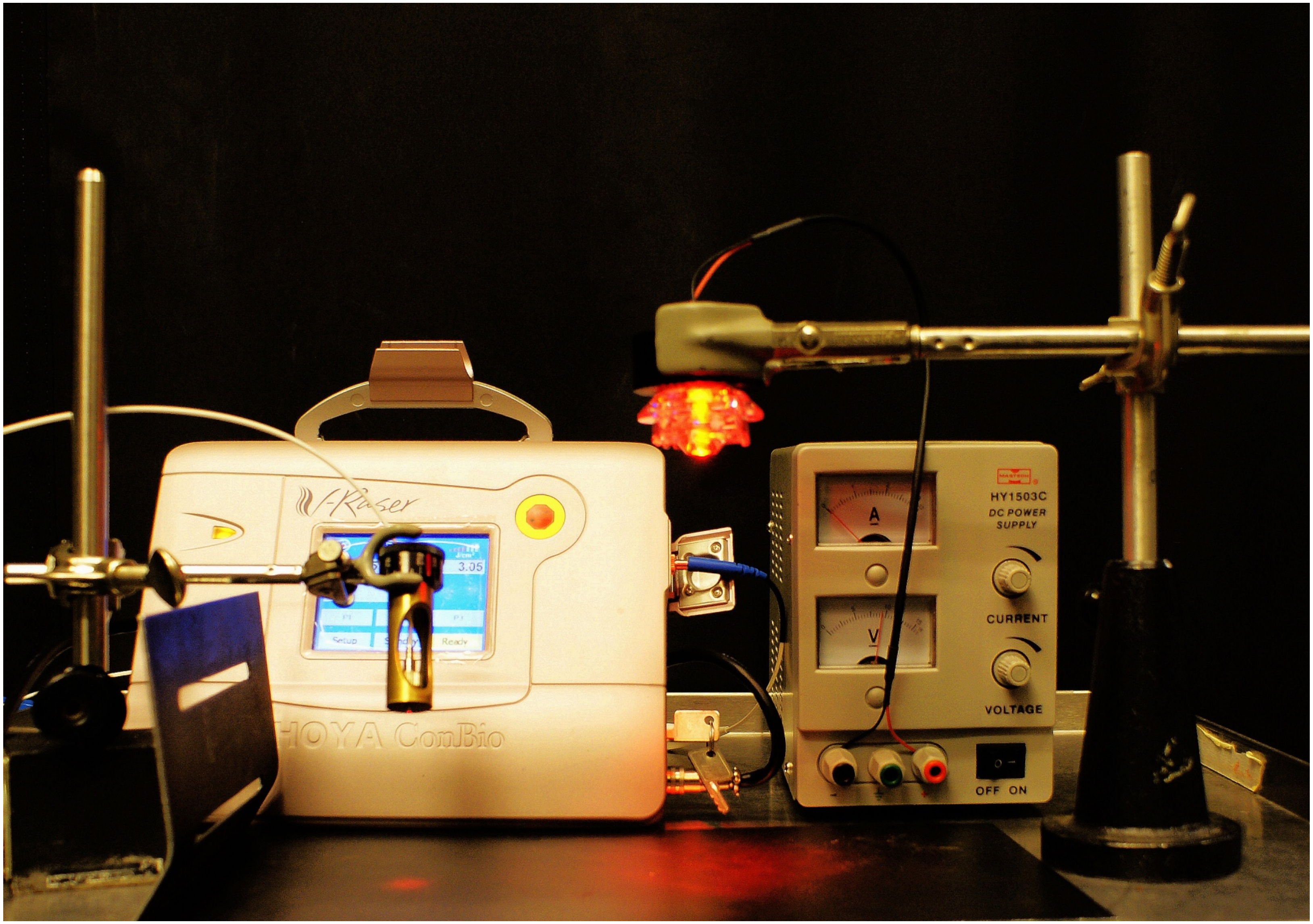
Experiments with HEK293 cells

- Why HEK293?
  - first described in 1977 (Graham et al., J. Gen Virol 1977 Jul;36(1):59-74).
  - Lack of any Toll-like receptors by nature
  - Easy to grow and transfect (transiently and stably)
- Stably transfected TLR9 and NF-kB Luciferase reporter genes into HEK293. (by Marc Lamphier at Eisai.)
- Transiently transfected NF-kB Luciferase reporter
- Irradiated by 810nm LED, 10mW/cm^2 5 min.

980-nm CW laser   810-nm and 970-nm LED array



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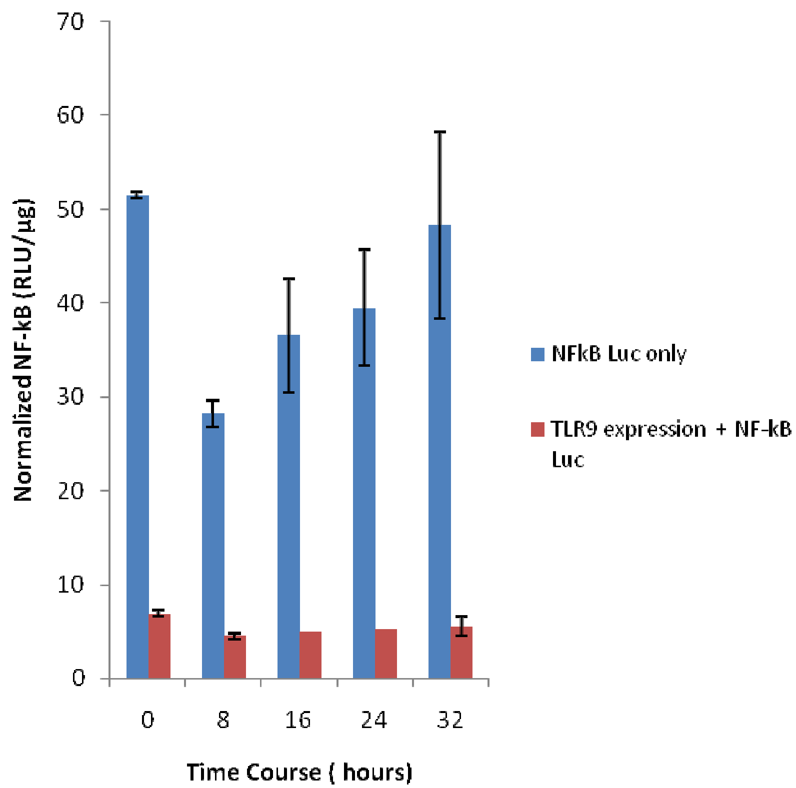
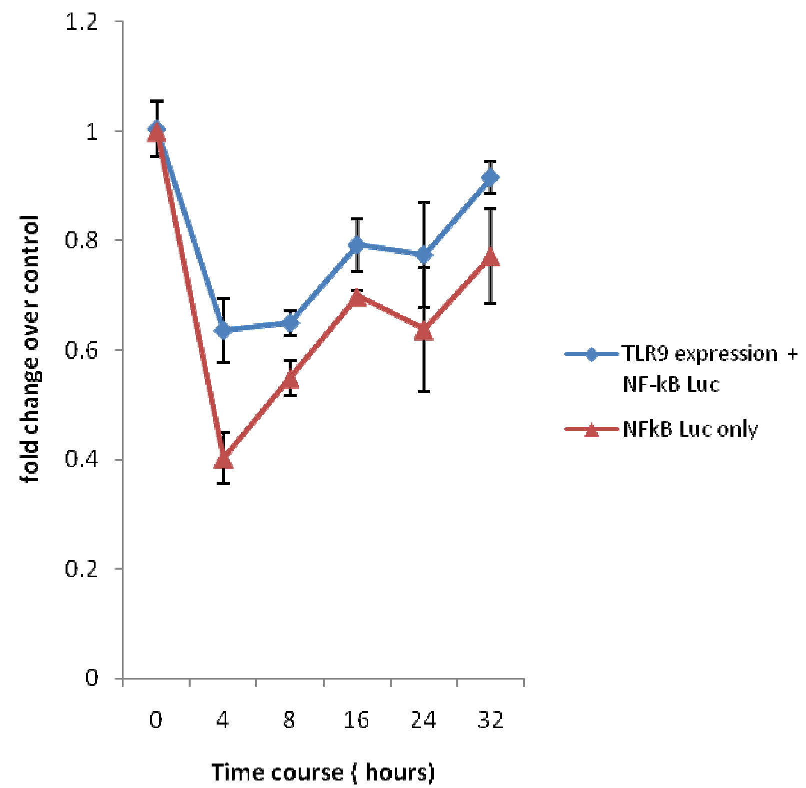
810 Laser induced NF-kB inhibition in HEK293





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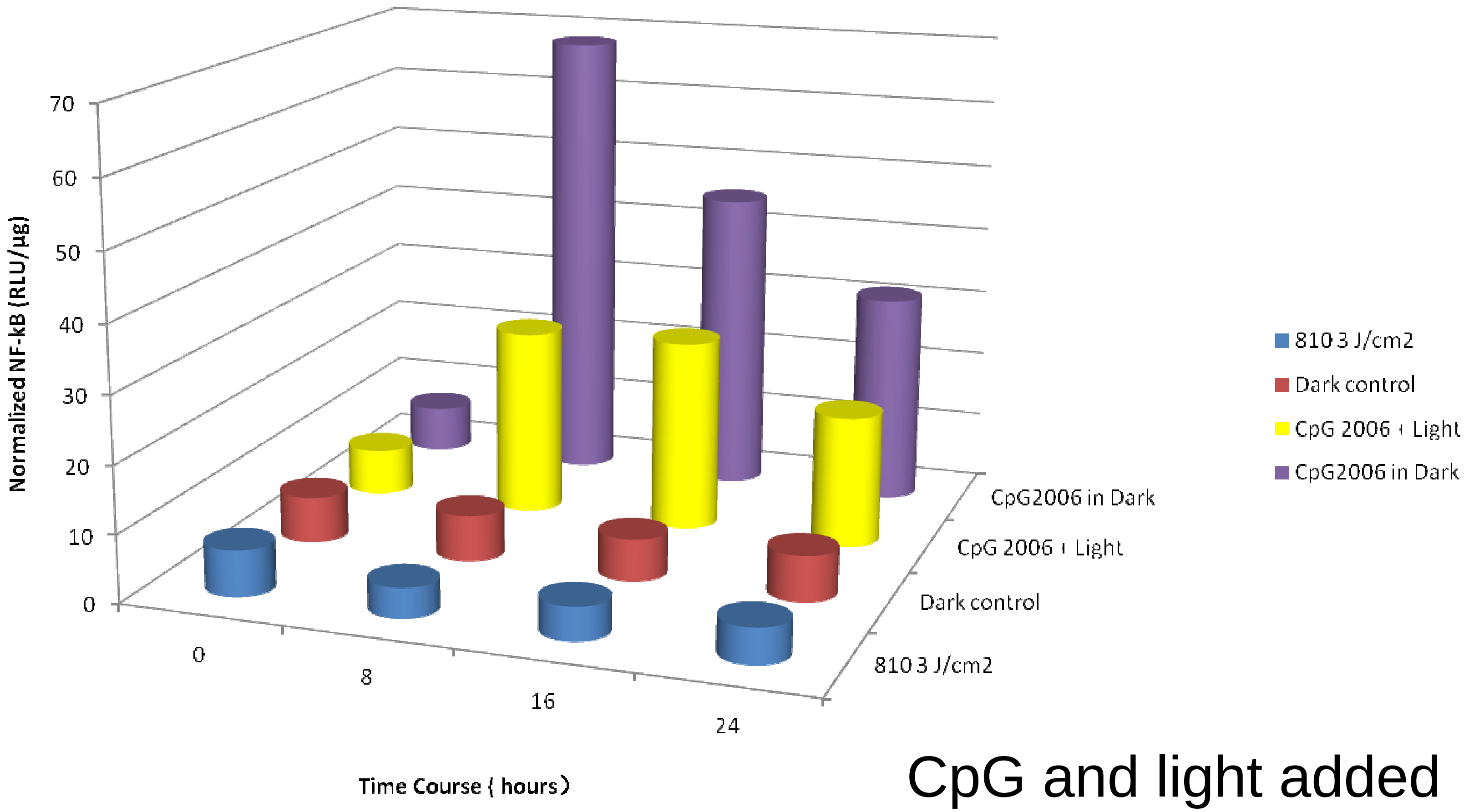
Light effect on TLR9 signaling



CpG and light added at same time

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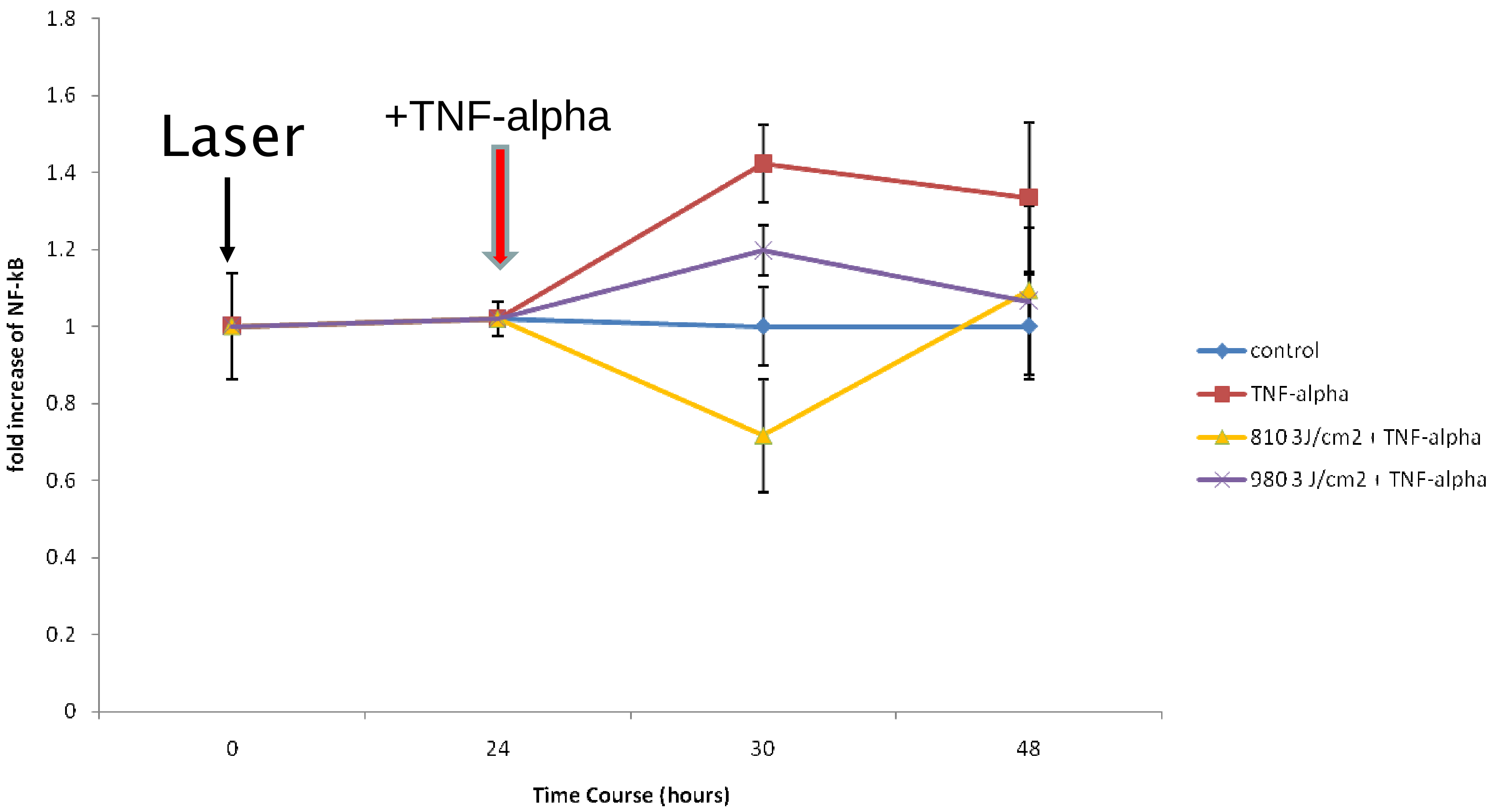
Laser pre-illumination abrogates effects of TNF-alpha 24 h later



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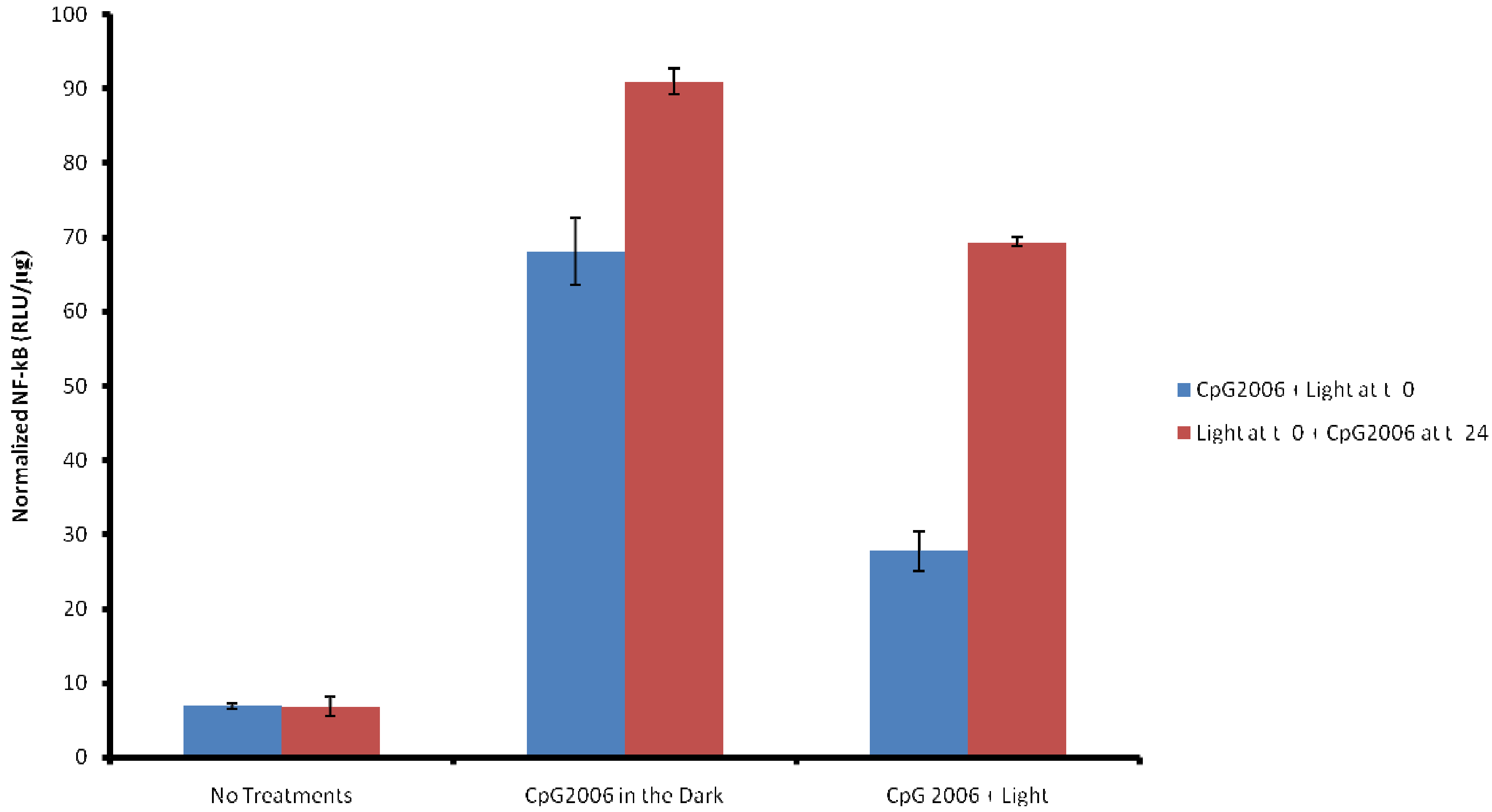
Light effect on TLR9 Signaling



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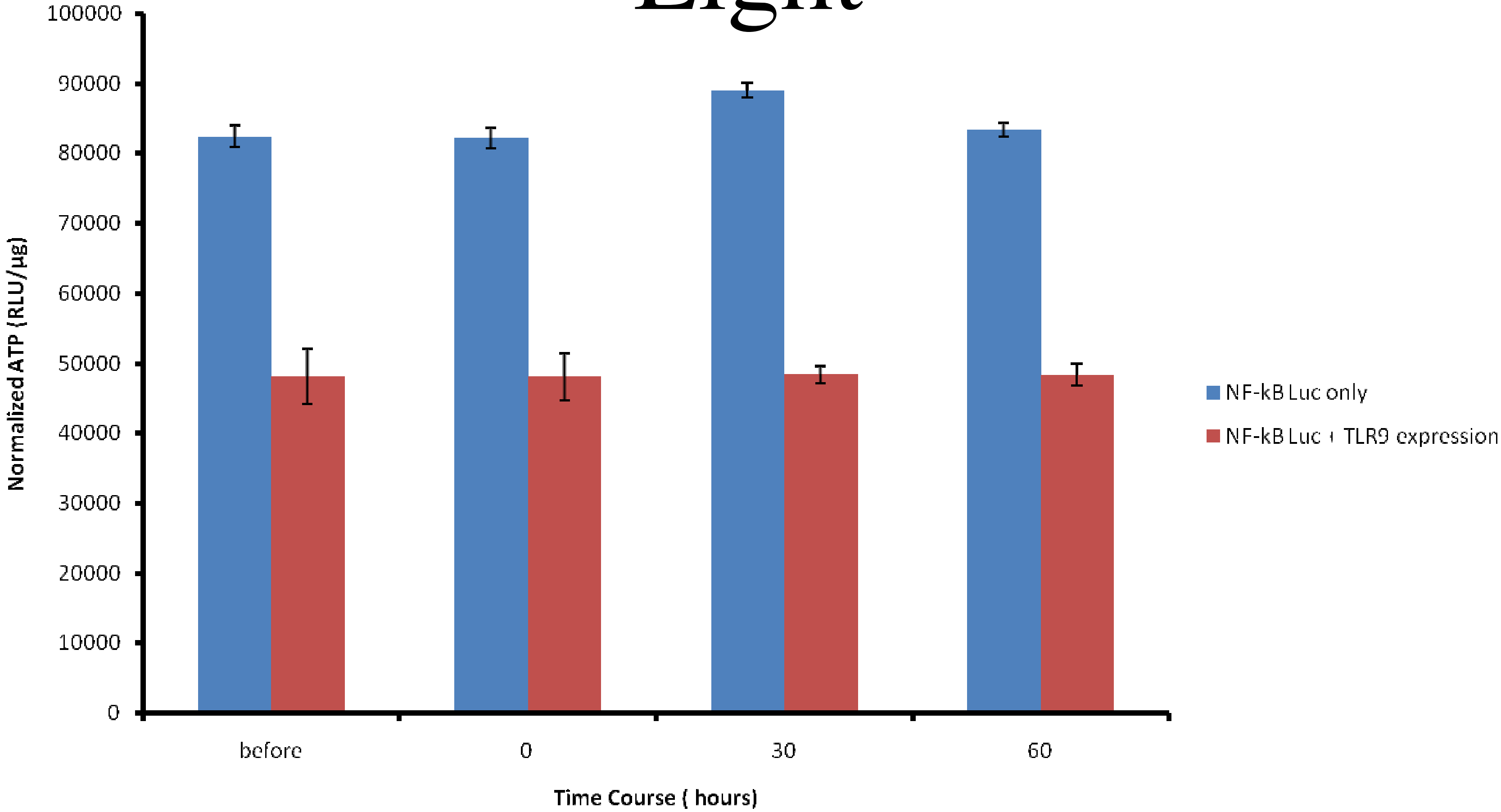
ATP change by 810nm 3J/cm^2 Light



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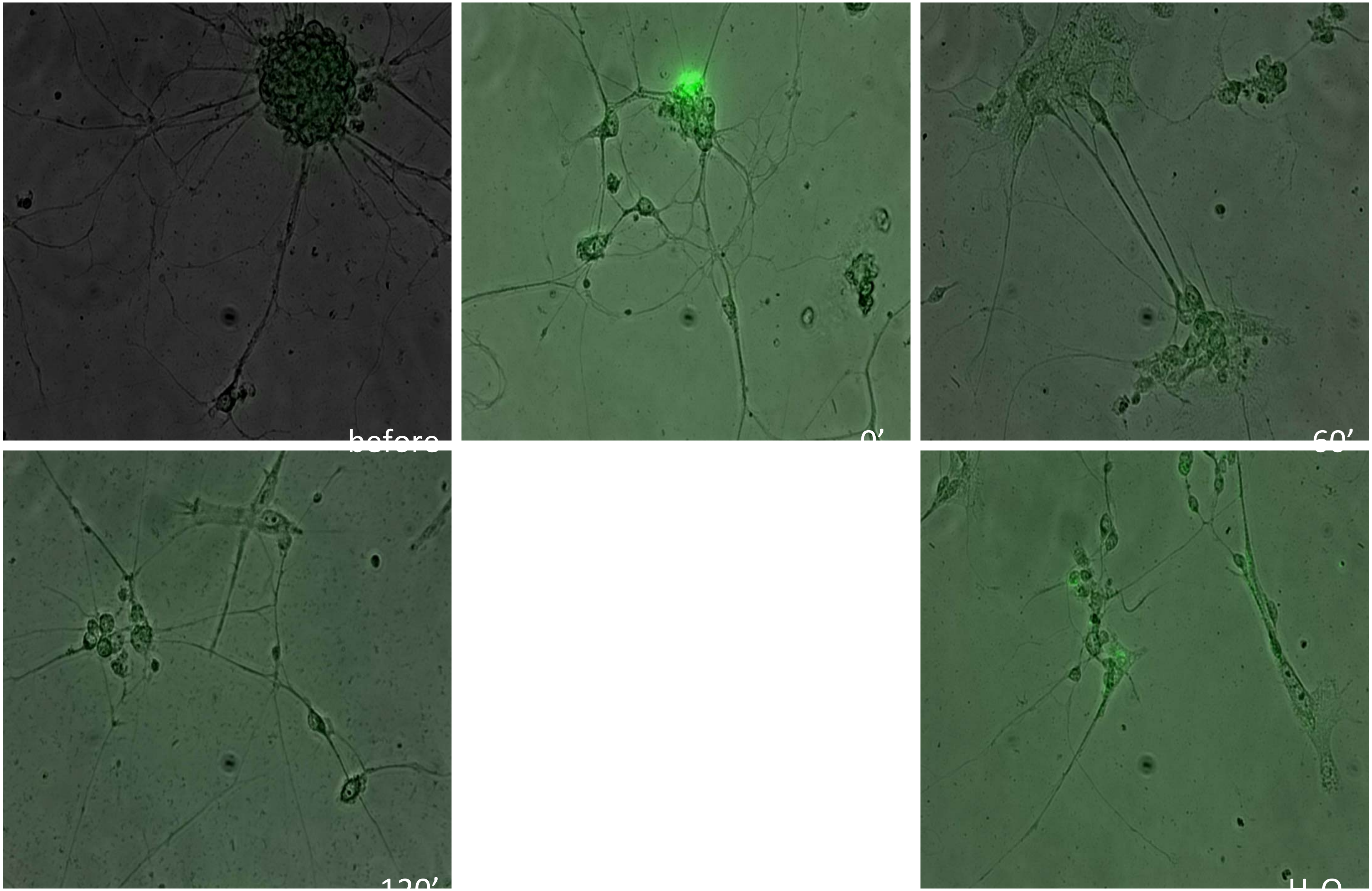
ROS in Murine Embryonic Neurons

DCDHF-DA



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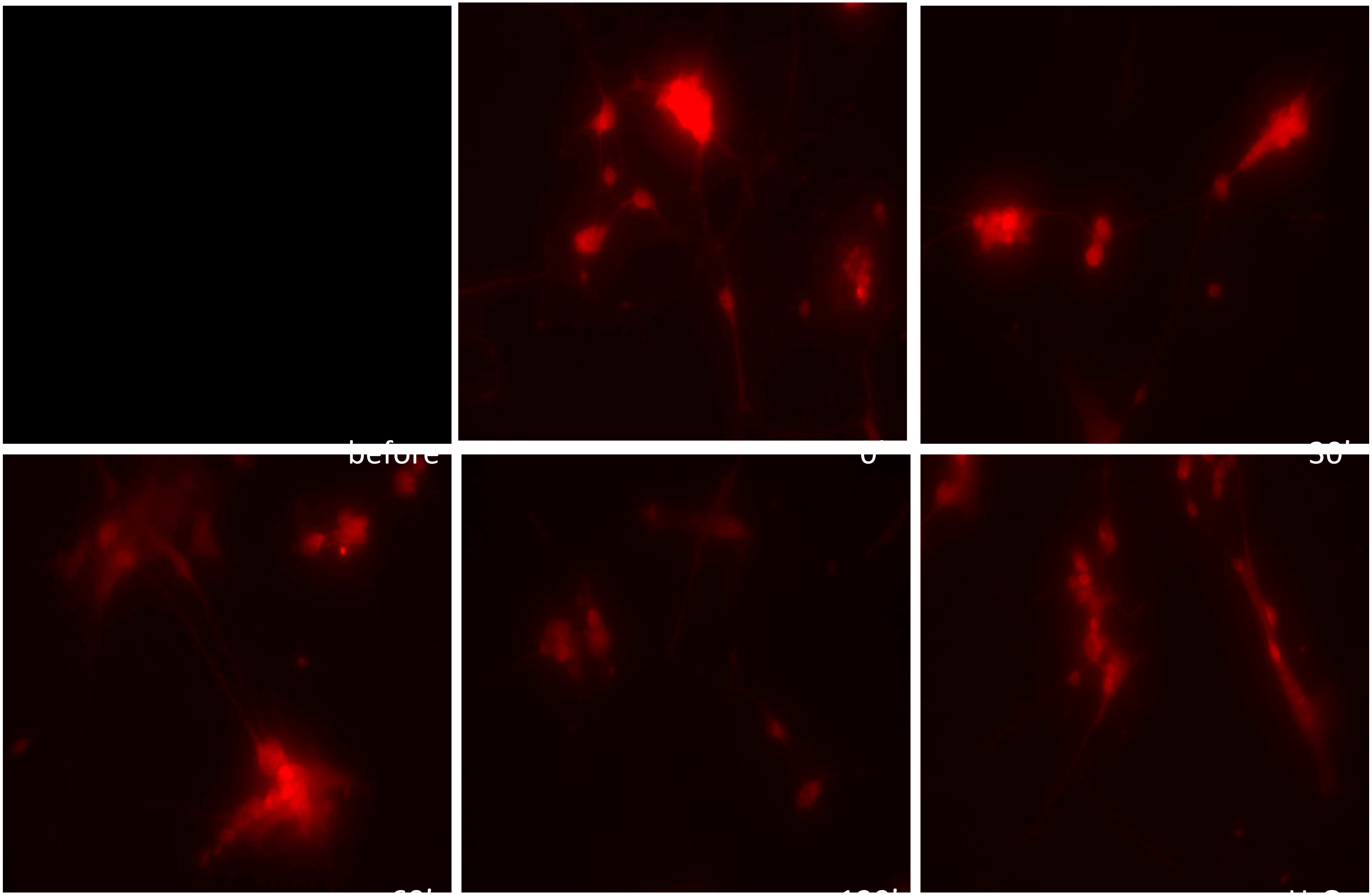
MitoSox Red



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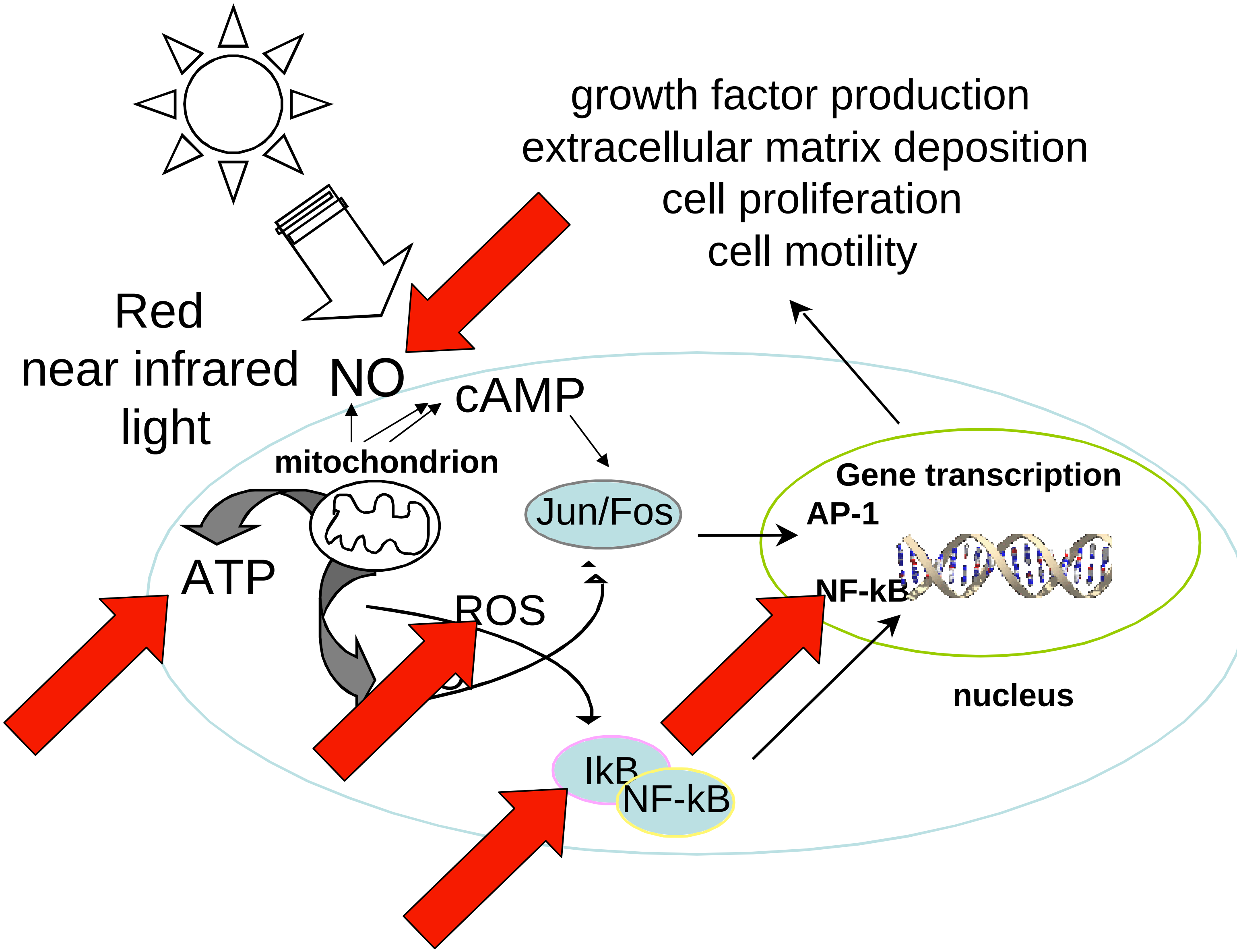
Conclusions I

1 810-nm laser increases cellular ATP
2 810-nm laser activates NF-kB
3 810-nm produced mitochondrial ROS
4 Mitochondrial inhibitors and (H_2O_2) activate NF-kB without increasing ATP
5 Mitochondrial inhibitors produce ROS
6 Antioxidants abrogate laser and mitochondrial inhibitor-induced NF-kB activation but have no effect on ATP
7 Preliminary evidence of NO production 15 and 30 min post-light
8 Different cell types (HEK293 cells and neurons) may behave differently

Mechanisms of LLLT



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What is the mechanism for biphasic dose response?

ROS have been shown many times to stimulate at low doses but to be harmful at high doses

NO (and peroxynitrite) may also have biphasic response: stimulate at low dose and inhibit at high dose

Protective transcription factors may be induced at low dose (NF-kB) and additional different harmful pathways activated at high dose

Future work

Test other cell types: neurons, leukocytes, epithelial cells

Test other wavelengths and non-coherent light sources

Investigate other redox sensitive transcription factors (AP1)

Repeat experiments (bioluminescence imaging, effect of antioxidants) in vivo

Study mouse model of traumatic brain injury

Acknowledgments

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Palomar