Near-Infrared Photobiomodulation of Living Cells, Tubulin, and Microtubules *In Vitro*

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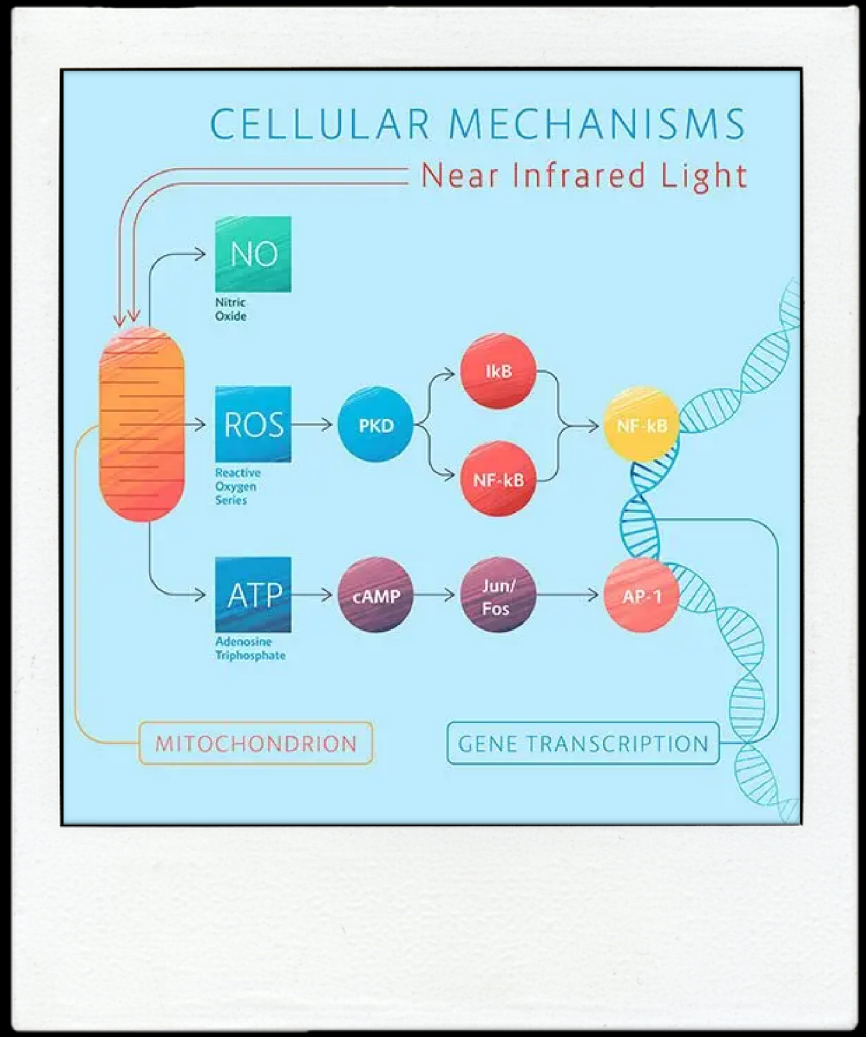
Near-Infrared Photobiomodulation Therapy (PBMT)

Photobiomodulation Therapy (PBMT)

A non-invasive method utilizing non-ionizing sources of low-intensity visible or near-infrared light to stimulate photochemical changes that can induce positive health benefits and treat symptoms associated with various conditions.

- *Accidentally discovered in 1967 by Endre Mester with a low-power ruby laser applied near tumour sites in mice, which resulted in hair growth & wound healing. Radiobiologia, Radiotherapia* **9**, 621–626 (1968); *The American Journal of Surgery* **122**, 532--535 (1971)
- *Originally referred to as low-level light/laser therapy (LLLT)*
- *Effects appear to depend greatly on the parameters & dosimetry used (e.g., spectral irradiance/power density, dose/fluence, wavelength, pulse rate)*



Near-Infrared (NIR) PBM — Clinical Results

Alzheimer's Disease:

- *Significant improvement in patients with mild to moderately severe dementia: better sleep, less anxiety, and increased function were reported, with no negative side effects. Photobiomodulation, Photomedicine, and Laser Surgery* **35**(8), 432–441 (2017)
- *Home PBM treatments were found to improve ADAS-Cog scores by a larger amount than that reported in pharmacological trials w/ donepezil (10 mg/day). Photobiomodulation, Photomedicine, and Laser Surgery* **37**(3), 133–141 (2019)

Parkinson's Disease:

- *In a proof-of-concept study, measures of cognition, mobility, dynamic balance, and fine motor skill were significantly improved ($p < 0.05$) with PBM treatment for 12 weeks. BMC Neurology* **21**, 256 (2021)

Traumatic Brain Injury & Concussion:

- *A single concussion case study found positive changes in behavioural and neuroimaging measures (e.g., increased cerebral perfusion) after 8 weeks of home PBM treatments. Frontiers in Neurology* **11**, 952 (2020)

Experimental Device Information

Vielight Neuro Alpha Brain PBM Device

Transcranial-Intranasal NIR PBM

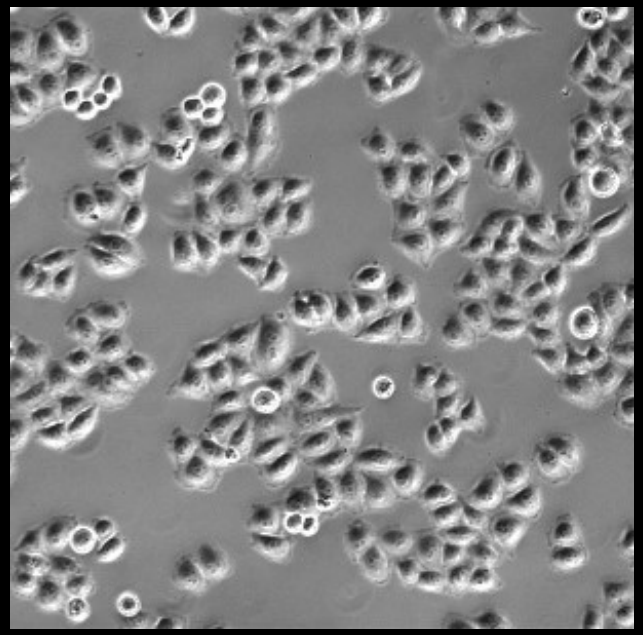
Parameter	Value (Intranasal)	Value (Transcranial)
Light source	810 nm LED × 1	810 nm LED × 4 (3 posterior, 1 anterior)
LED output power	25 mW	100 mW (posterior) and 75 mW (anterior)
LED pulse frequency	10 Hz	10 Hz
Pulse duty cycle	50%	50%
Beam spot-size	~ 1 cm^2	~ 1 cm^2
LED power density	25 mW/cm^2	100 mW/cm^2 (posterior) and 75 mW/cm^2 (anterior)
Application time (default)	20 min	20 min
E_{Net} delivered (per LED)	15 J	60 J (posterior) and 45 J (anterior)
Net energy dose (per LED)	15 J/cm^2	60 J/cm^2 (posterior) and 45 J/cm^2 (anterior)

Table of Device Parameters

- *Proprietary LEDs used produce non-thermal, non-ionizing, and incoherent radiation*
- *LEDs target the DMN (disrupted in Alzheimer's cases)*
- *Low-level NIR photons have been shown to be able to penetrate the skull (both ex vivo & in silico)*

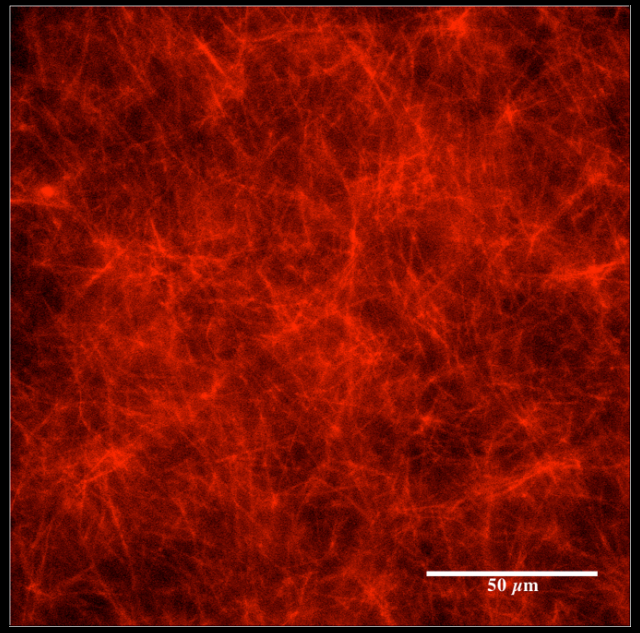
In Vitro Experiments with NIR PBM

Probing the Effects on Cells & Cellular Components



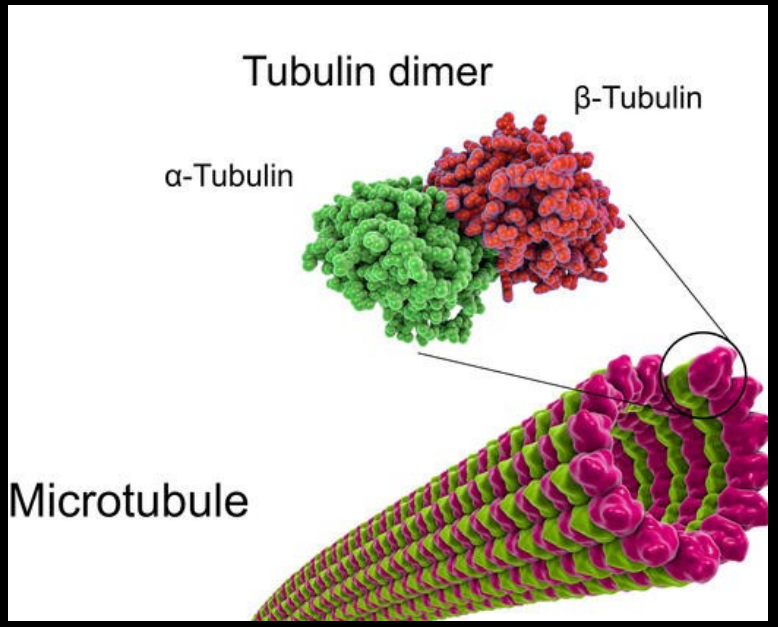
Living Cells (HeLa & U251)

Ionic Conductivity Measurements



Microtubules in Buffer Solutions

Fluorescence Microscopy Analysis



Tubulin in Buffer Solutions

Turbidity Measurements

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NIR PBM—Experiment 1:

Living Cells

Experimental Procedures

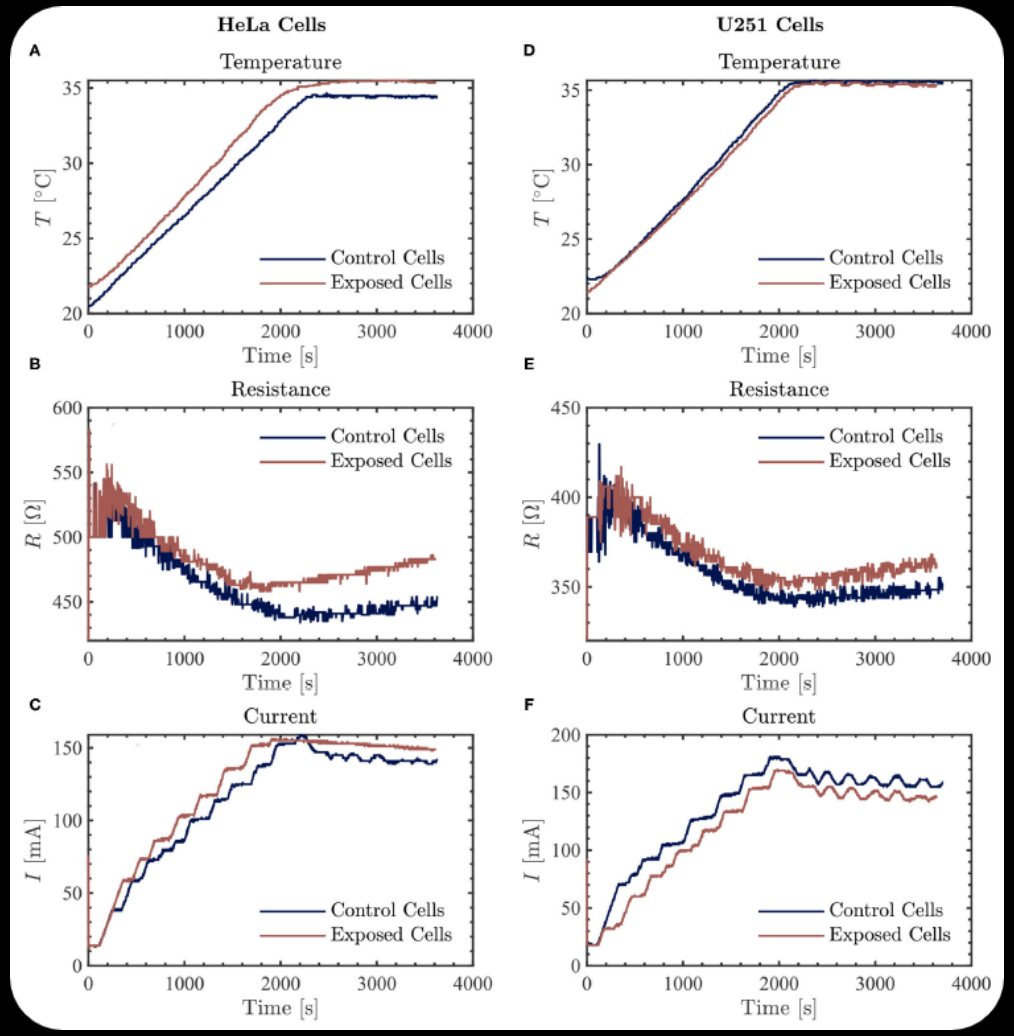
Cell Cultures:

- *HeLa (human cervical cancer cell line) & U251 cells (human glioblastoma cell line)*
- *Cells were cultured in high glucose DMEM, 5% FBS, and antibiotics (penicillin & streptomycin)*
- *All cells were cultured at 37 °C with 5% CO2*

Exposures and Ionic Conductivity Measurements:

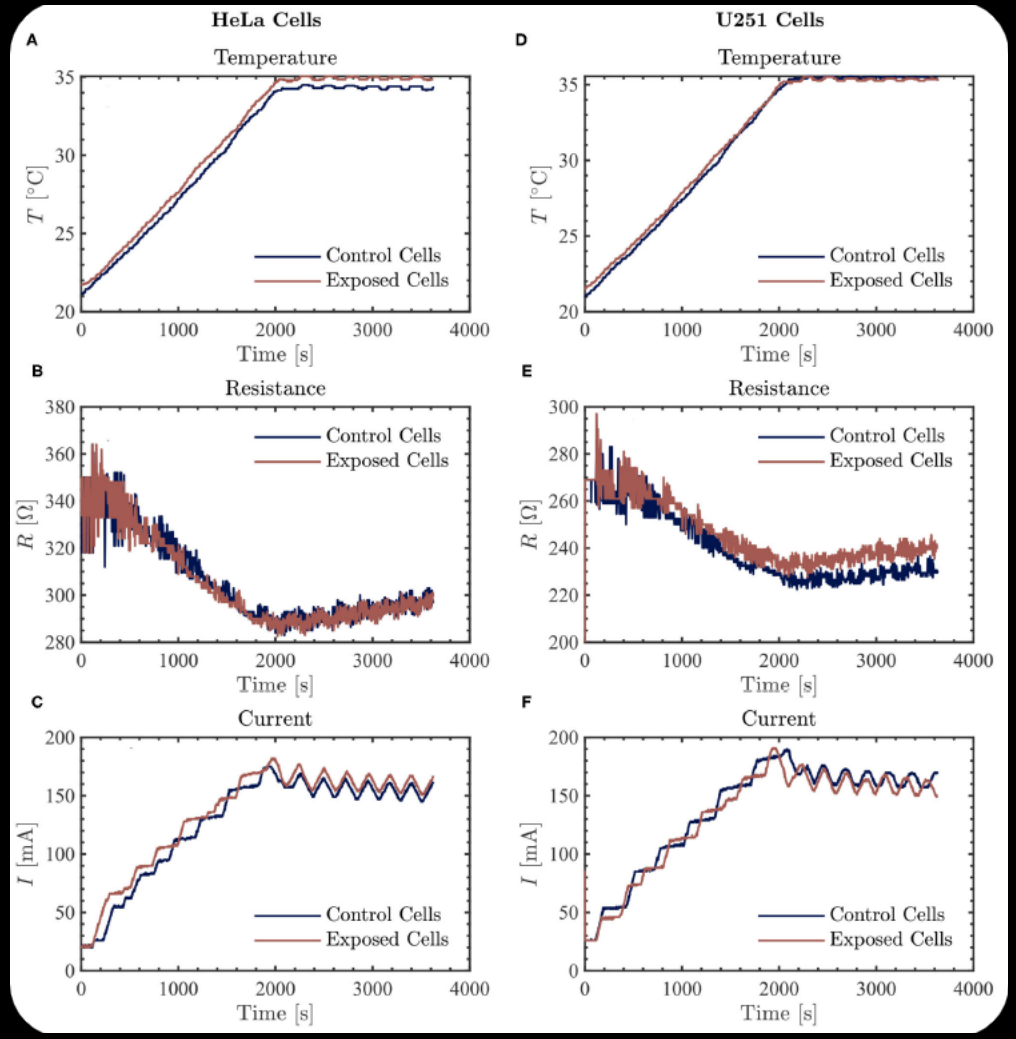
- *On the day of exposure, cells were set up at ~60–80% confluence*
- *We use an additional experimental device, namely, the inovitro live system by Novocure Ltd.*
- *The system generates intermediate-frequency (50–500 kHz) alternating EFs w/ low intensity (so-called, tumour-treating fields or TTFields, known to hinder CC div.)*
- *We investigated the effect of the Vielight LED applicator alongside two different frequencies of TTFields, 50 and 100 kHz*
- *Measurements of T, R, and I are recorded every 3 s by the system*

Conductivity Measurement Results



50 kHz TTFields & NIR PBM

Total exposure time was ~1 h



100 kHz TTFields & NIR PBM

Total exposure time was ~1 h

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NIR PBM—Experiment 2:

Microtubules

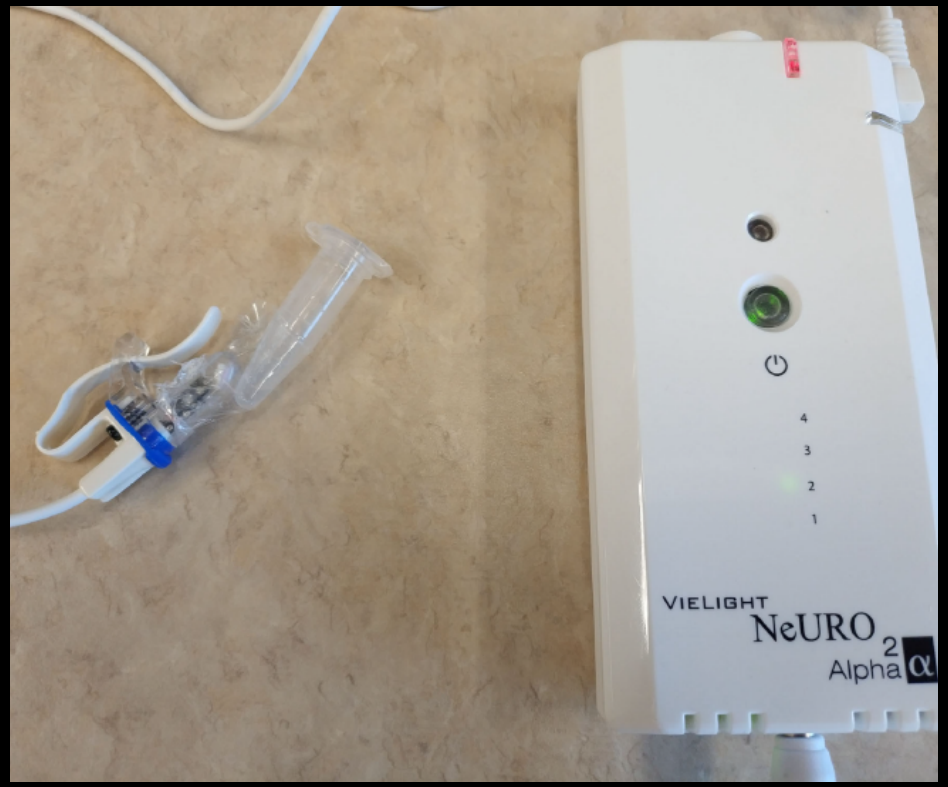
Experimental Procedures

Reconstitution of rhodamine-labeled MTs:

1. *Resuspend tubulin in ice-cold G-PEM buffer to a final tubulin concentration of 4 mg/ml (≃36.4 μM)*
2. *G-PEM buffer was prepared with GTP (100 mM stock) added to cold PEM buffer (aka BRB80) to a final GTP concentration of 1 mM*
3. *Cold G-PEM &* microtubule cushion buffer (BRB80 diluted in 60% [v/v] glycerol), were both added to each labeled tubulin aliquot at a 4 : 1 ratio

PBM Exposures:

1. *Labeled tubulin aliquots were placed in a 37 °C water bath for 45 minutes to polymerize*
2. *After polymerization, the microtubules were stabilized with Taxol, i.e., paclitaxel (either 2 μM, 4 μM, or 20 μM) — a common chemotherapy drug*
3. *MTs were exposed to the intranasal LED applicator of the device for 2 h*



An example of one of the exposures (all performed at room temp)

Fluorescence microscopy was performed on the samples after exposure.

Imaging Results 1

20 µM Taxol:

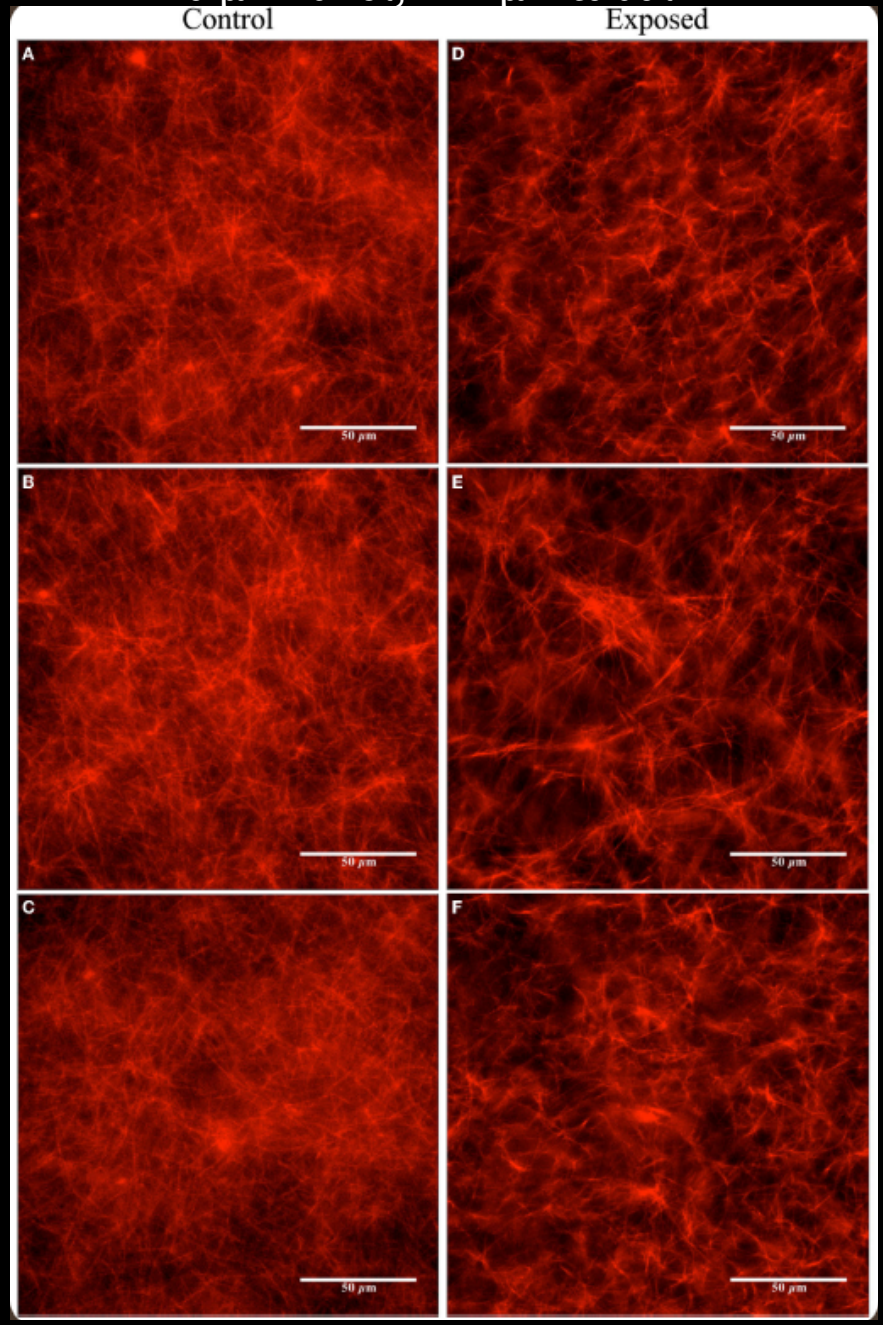
- *A small effect on MT polymerization seems to be present*
- *Notably, a lower total polymer mass appears to be remaining in the PBM-exposed group (N = 1)*

2 µM Taxol:

- *Control microtubules were depolymerizing and unstable at the time of imaging. No conclusions were drawn from these particular experiments.*

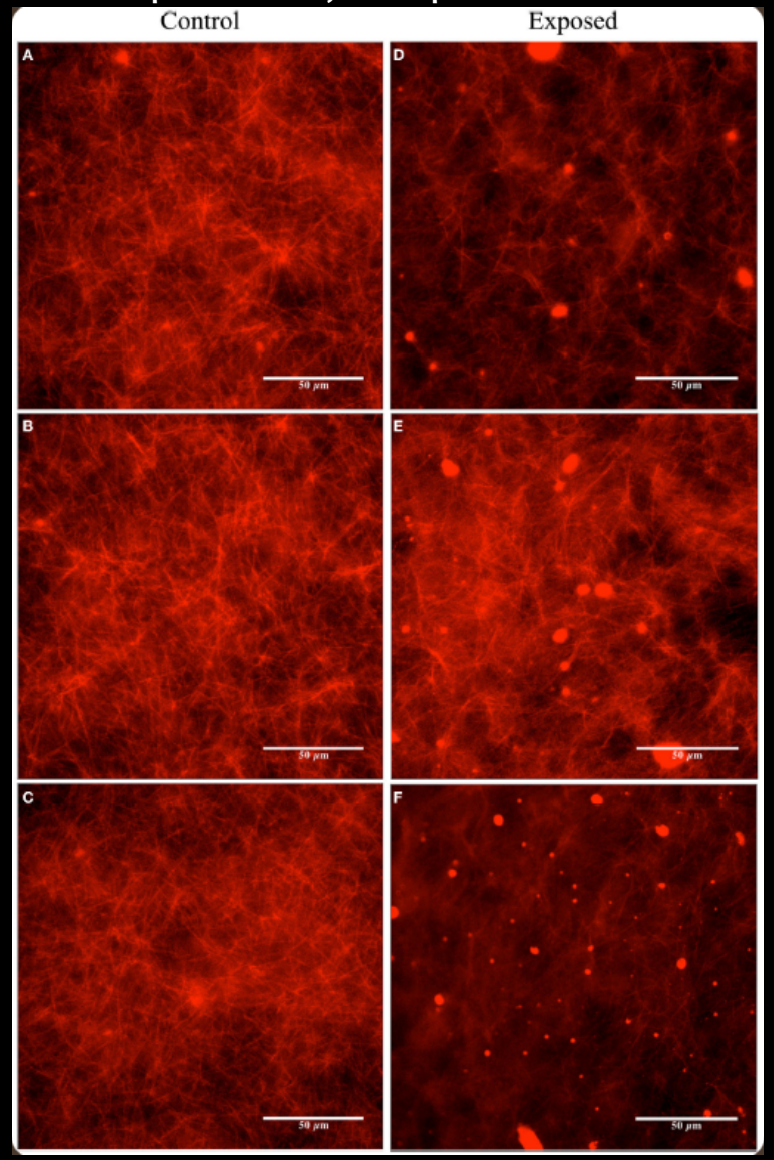
We used a Zeiss Axio Examiner.Z1 fluorescence microscope with a red fluorescent protein (RFP) filter set. Results were imaged with a Hamamatsu C9100 EMCCD camera.

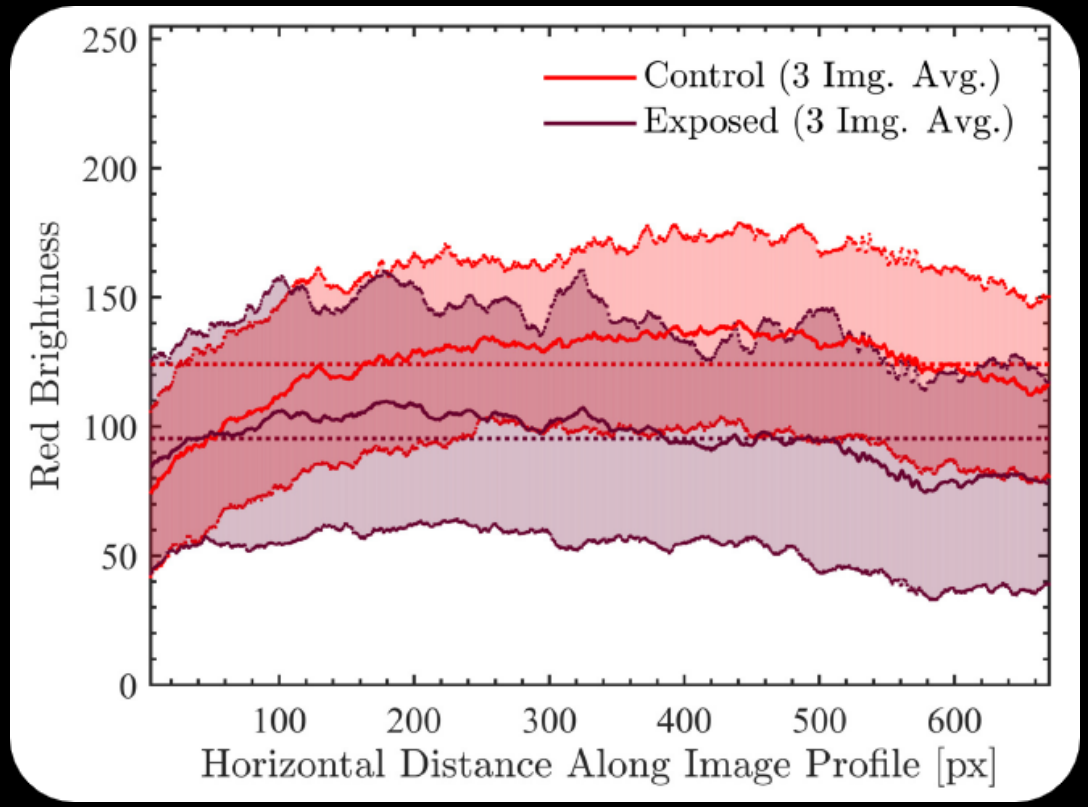
20 µM Taxol, ~ 2 µM tubulin



Imaging Results 2 (4 μM Taxol, $\mathcal{N} = 3$)

4 μM Taxol, ~ 2 μM tubulin





Results of a quick image analysis performed over the red band

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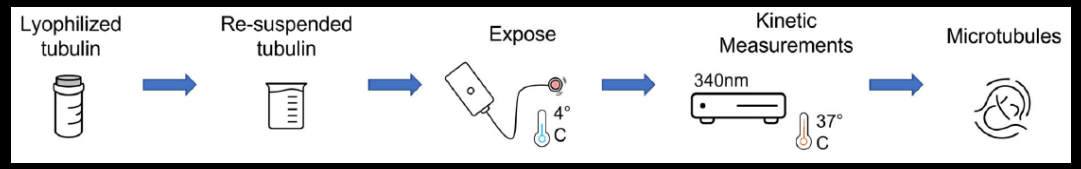
NIR PBM—Experiment 3:

Tubulin

Experimental Procedures

Tubulin Turbidity w/ PBM Exposures (30 min each):



Two different tubulin concentrations were studied: 2.5 & 5 mg/ml

Turbidity Measurements (Absorbance at 340 nm):

Parameter	Value
t_{total}	2400 s
t_{int}	30 s
N_{reads}	81
Plate type	96 well standard (clear bottom)
Well height/depth	14.6 mm
λ_{abs}	340 nm
Shake before	Yes, 5 s orbital, medium
Shake between	Yes, 5 s orbital, medium

SpectraMax iD5 Multi-Mode Microplate Reader

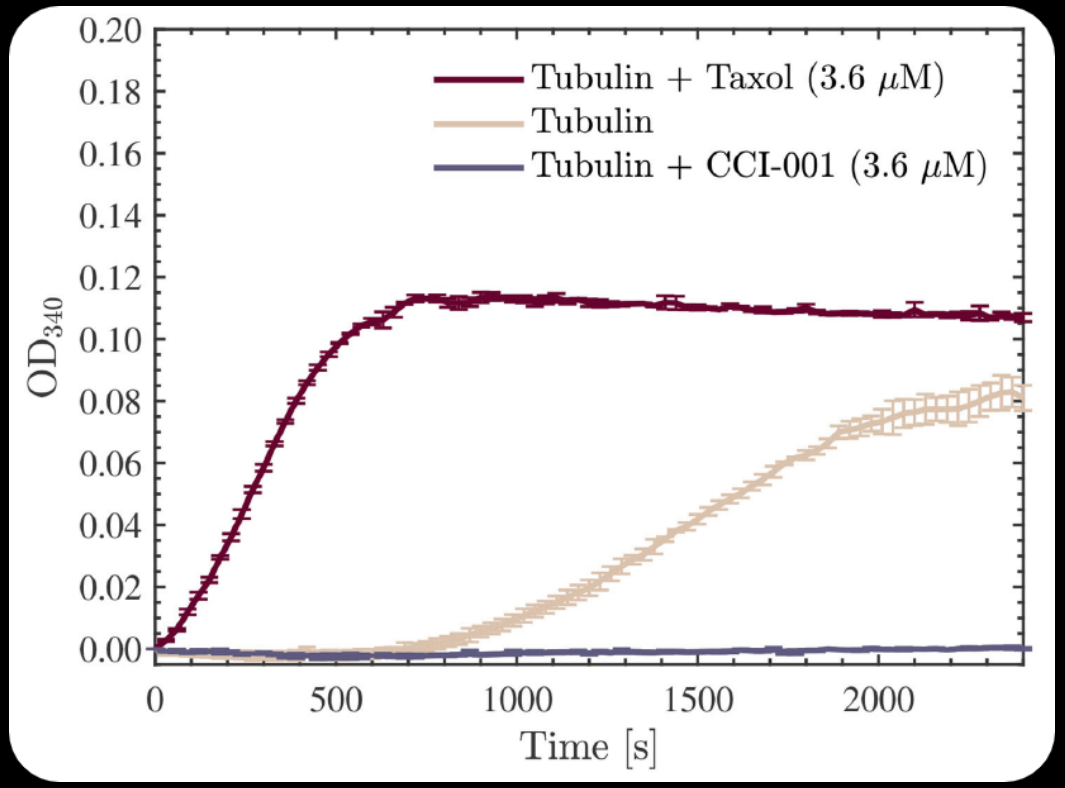
Measurements were performed with ~100 μl of each sample per well.

Validation of Turbidity Protocol and Methodology

Prior to performing any exposures, we tested our equipment and protocol.

Three scenarios were compared:

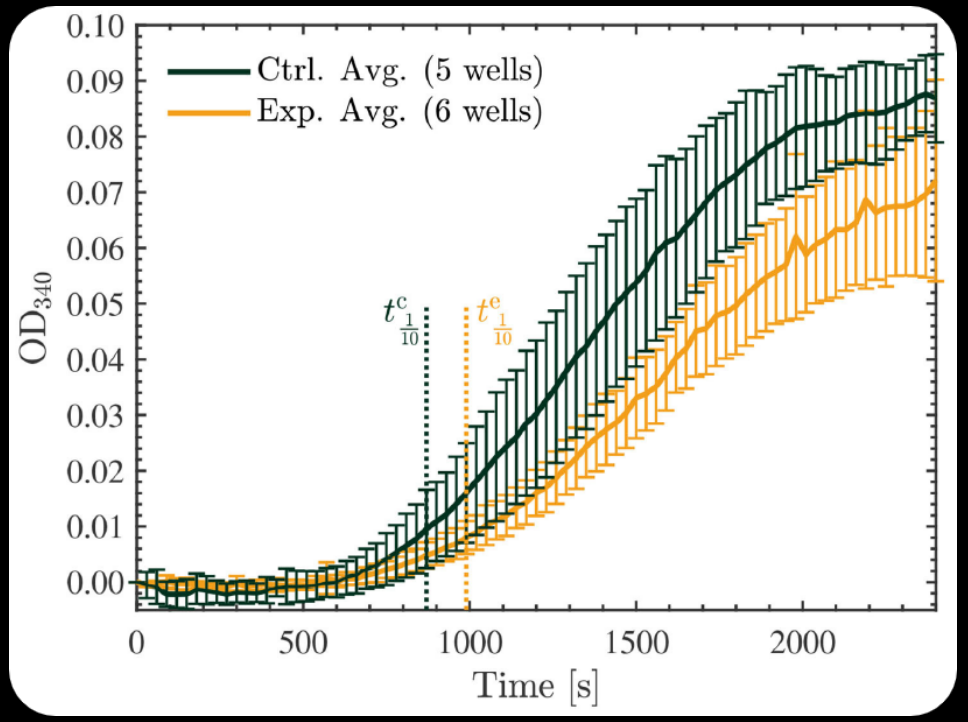
- *Tubulin Only*
- *Tubulin + Taxol (encourages & supports MT polymerization)*
- *Tubulin + CCI-001 (cytotoxic, inhibits β-tubulin polymerization)*



Results of turbidity measurements performed on 22.7 μM tubulin (*N* = 1)

Curves shown are the average of 3 wells measured separately

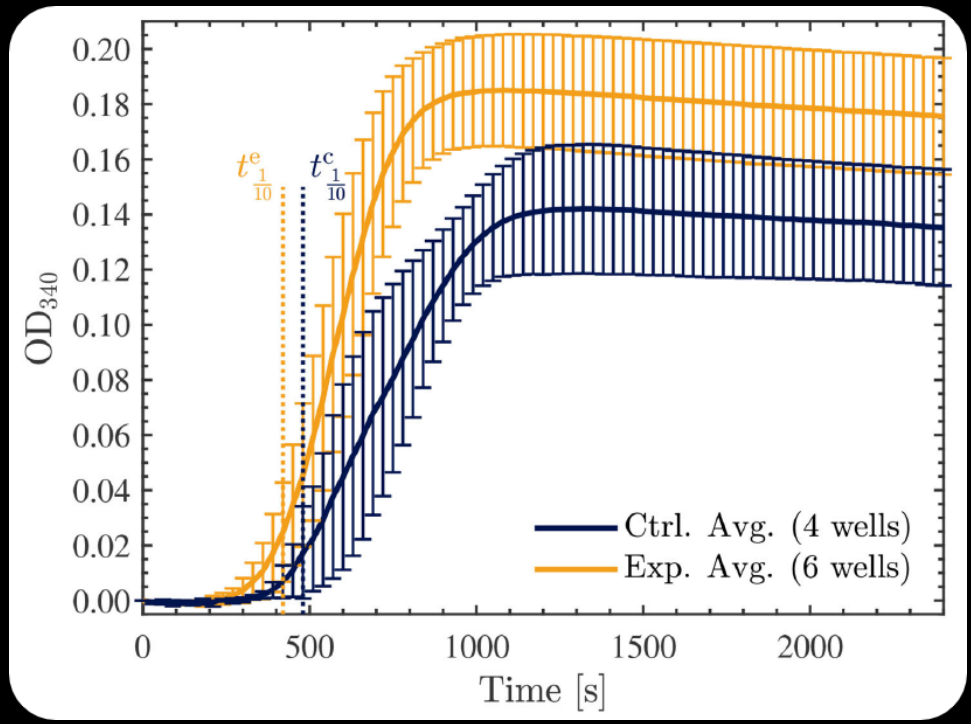
Turbidity Results for PBM-Exposed Tubulin



22.7 µM tubulin

PBM-Exposed vs. Unexposed

Tenth times calculated for the control and exposed curves were 870 s and 990 s, respectively. Maximal slopes were 5.0 ± 0.1 & 3.8 ± 0.1 mOD/min.



45.5 µM tubulin

PBM-Exposed vs. Unexposed

Tenth times calculated for the control and exposed curves were 480 s and 420 s, respectively. Maximal slopes were 17.6 ± 0.5 & 33.2 ± 0.8 mOD/min.

Hypothesis

Smoluchowski Equation: $$K = \frac{k}{V} = \frac{4\pi\,(D_1 + D_2)\,(R_1 + R_2)}{V}$$

Describes the simultaneous coagulation of particles involved in processes such as polymerization. *Phys. Z.* **17**, 557–571 & 585–599 (1916)

- *K = coagulation kernel, k = macroscopic reaction rate*
- *Diffusion coefficients governed by:* $$D = \frac{k_B T}{f} = \frac{k_B T}{6\pi R\eta}$$

Hydration shell increases under NIR irradiation:

- *Proteins in solution interact with the solute, creating what is known as a 'hydration shell' in their immediate vicinity. PNAS* **104**(52), 20749–20752 (2007)
- *THz absorption spectra studies of protein solutes (validated against MD simulations) show that the dynamic hydration shell around proteins can extend from ~14–22 Å, corresponding to at least five layers of water molecules. Methods* **52**(1), 74–83 (2010)
- *This increased effective R causes D to decrease, thereby making it harder for the tubulin dimers to coalesce.*

Currently being tested with dynamic light scattering (DLS) experiments.

An Additional Consideration:

NIR PBM is affecting the tubulin and MTs at a molecular and/or structural level (e.g., H-bonds could be affected, or even secondary structures)

Preliminary results w/ Raman Spectroscopy demonstrate an effect on secondary structures (α-helices & β-sheets)

Conclusions & Future Directions

Living Cells

An **increased *R*** in both cell lines at 50 kHz and in U251 cells only at 100 kHz: suggests an **inhibitory effect**, that in the case of HeLa cells, was balanced with an increased current.

Microtubules

Irradiation with NIR PBM appears to lead to significant **depolymerization of microtubules, i.e. MT dynamics are upregulated, facilitating cytoplasmic remodelling.**

Tubulin

Tubulin concentrations representative of the cellular concentration that were exposed to NIR PBM showed a **measurable decrease polymerization rates and total polymer mass**.

Future Directions: (1) *in vitro* experiments that study **the effects of different PBM pulse frequencies**,
(2) in vitro experiments that study **the effects in an environment that more accurately resembles the cellular environment**

Thank you!

Questions?

Backup Slides

GTP Exposure