Plasma Medicine: Low Temperature Plasma as a Transformational Technology for the Healthcare Field

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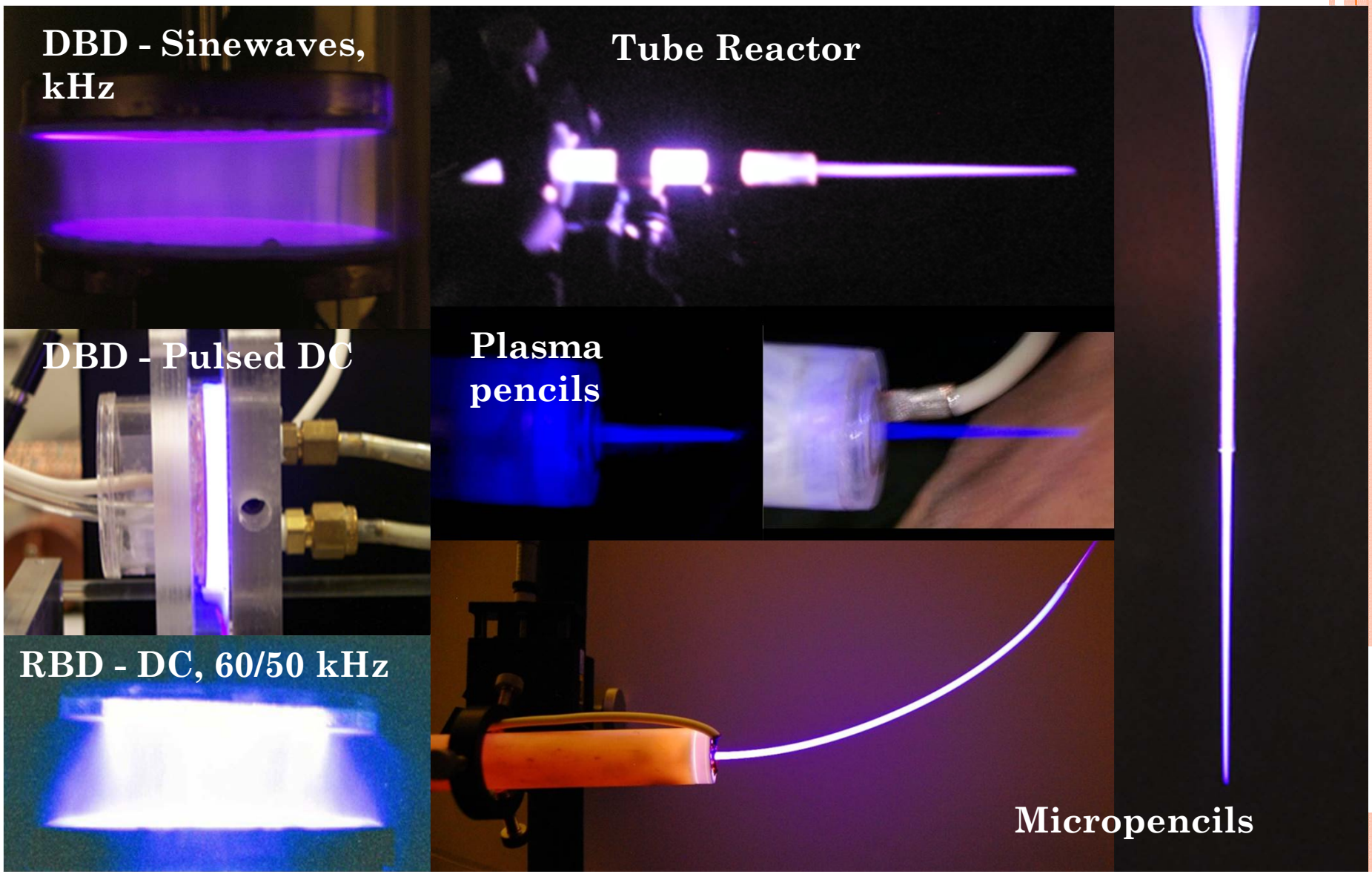
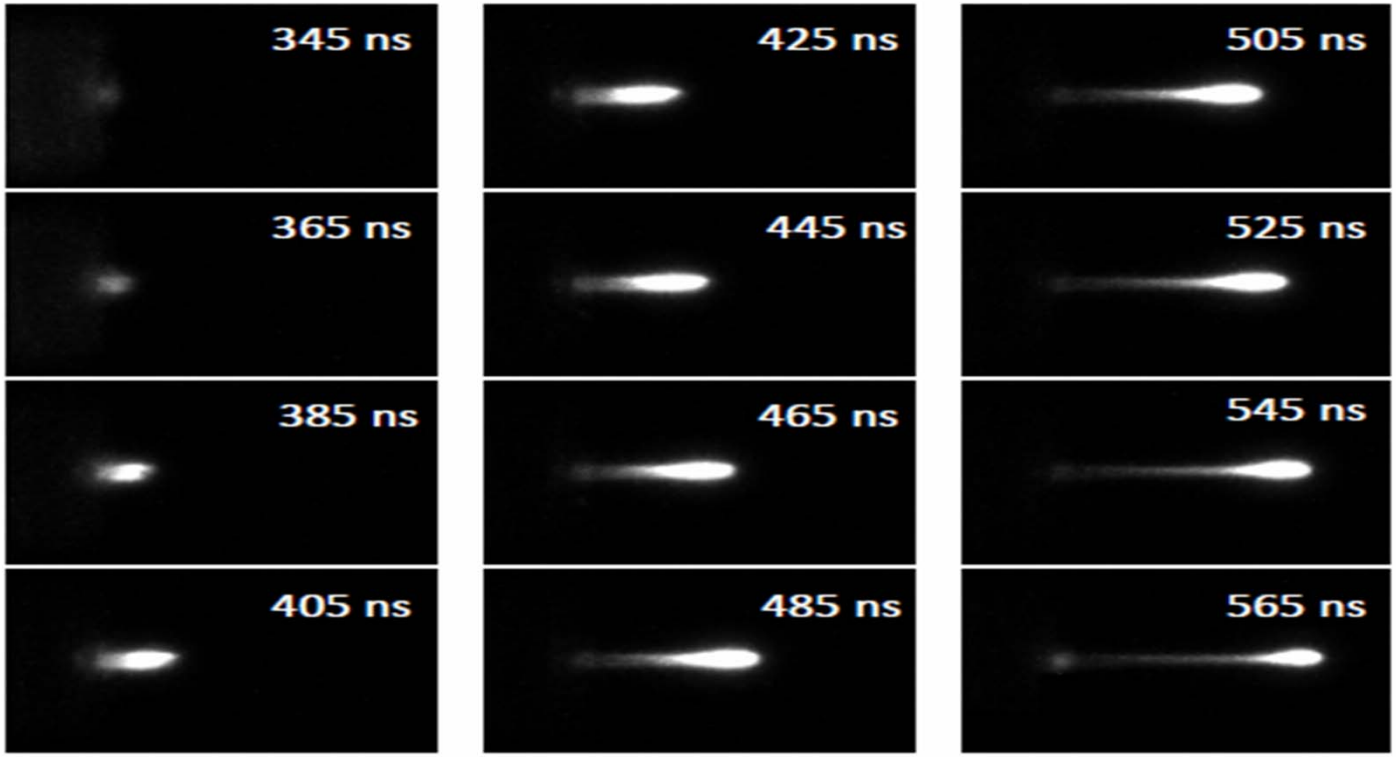
OUTLINE

- **Low temperature plasma sources**
- **Bacteria inactivation**
- **Destruction of amyloid fibrils**
- **Cancer treatment applications**
- **Effects on fibroblasts**
- **Effects on epithelial cells**
- **Subcellular and molecular mechanisms of interactions?**
- **Conclusions**

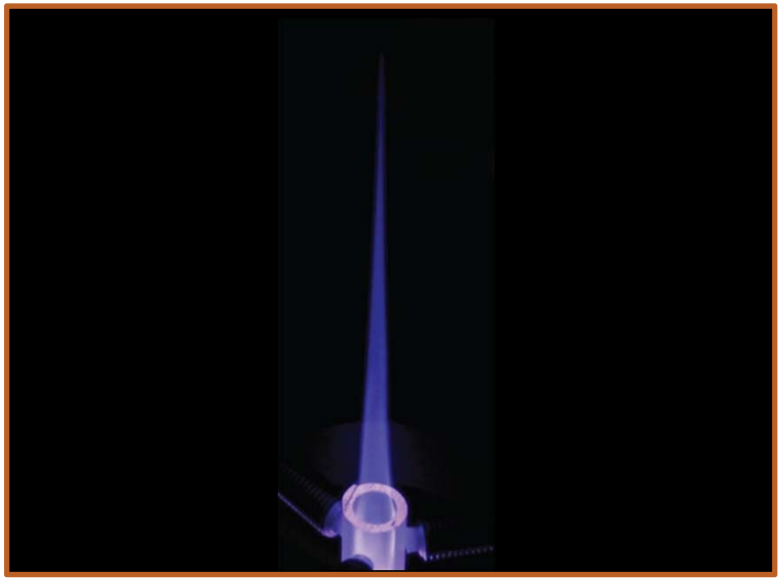
"COLD" PLASMA SOURCES AT THE APTL



Plasma Jets are fast Propagating Plasma Bullets



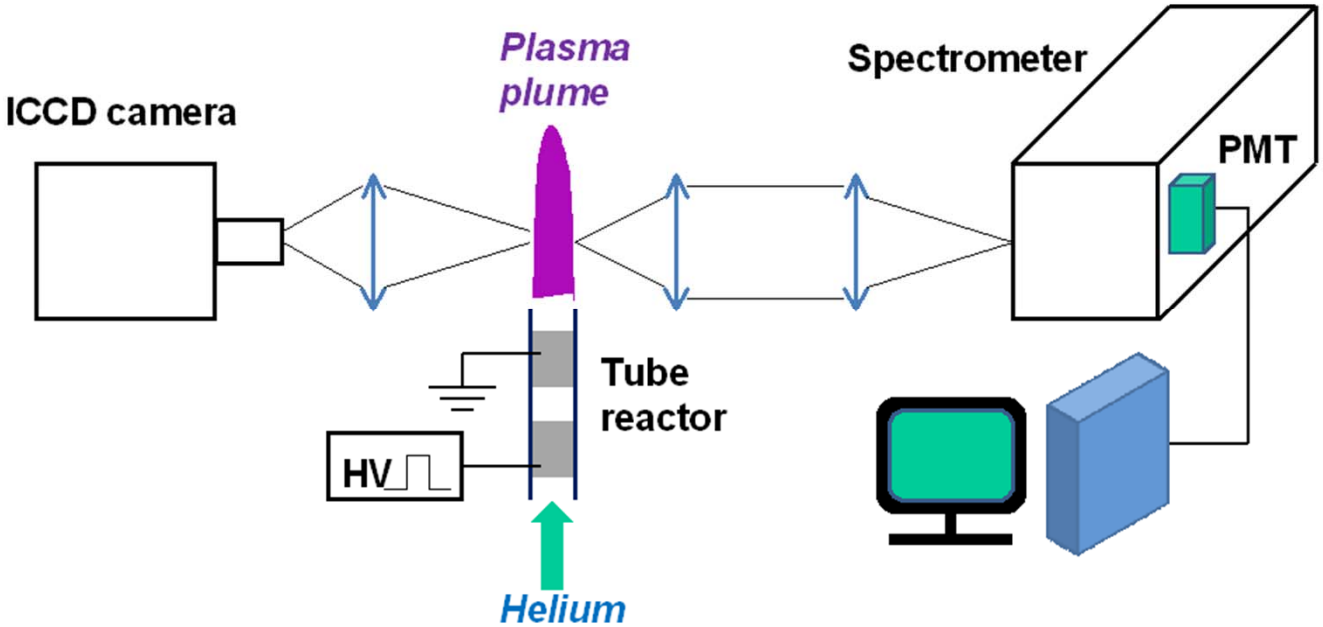
Plasma Bullets



A photograph of the plasma jet

A video of the plasma bullet propagation

EXPERIMENTAL SETUP



Discharge reactor: DBD cylindrical configuration

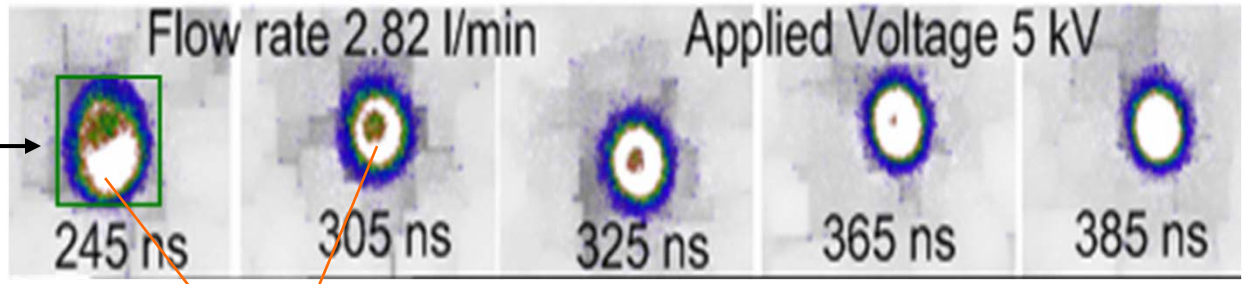
High voltage pulses

- amplitude 5-10 kV
- pulse width: 300 ns to 2 µs
- frequency: up to 10 kHz

He flow rate : 1.4 L/min to 7 L/min

FORMATION OF THE PLASMA BULLET

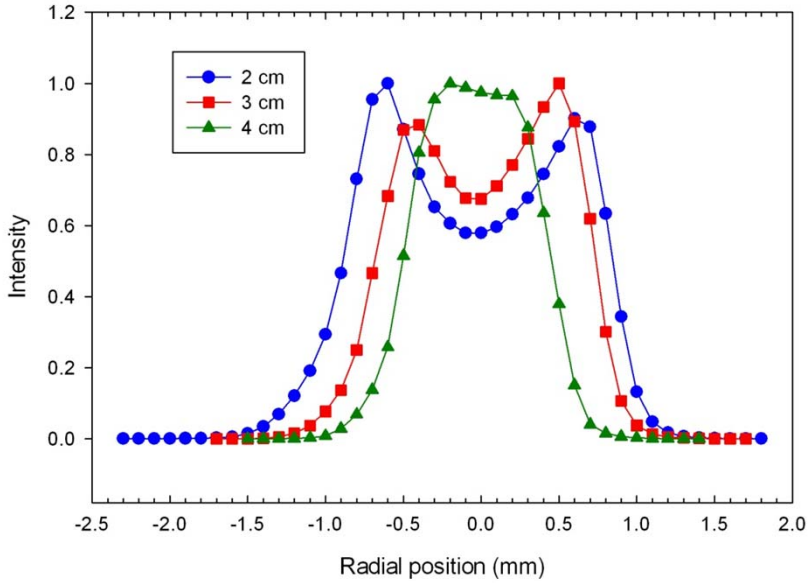
ICCD images of plasma bullet end-on view



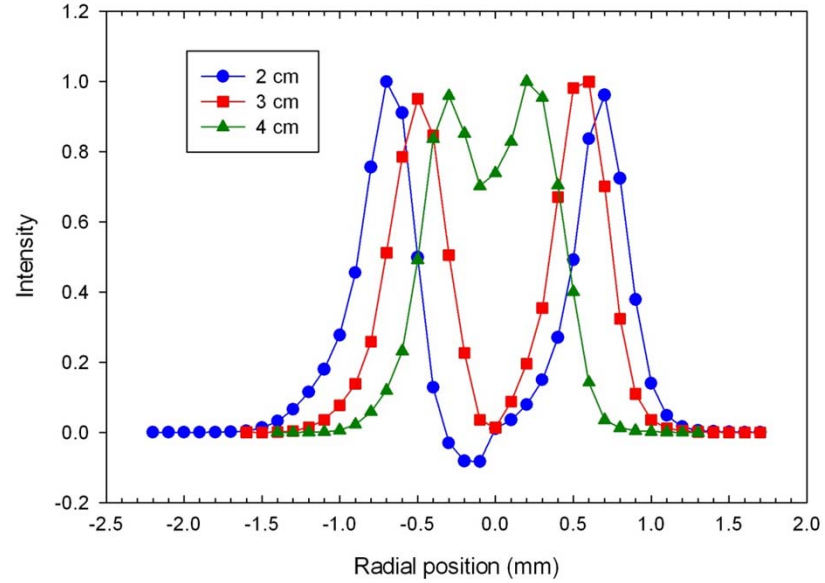
Outlet

White color shows the most intense light emission

OES: Radial profile of the jet for different axial position



Experimental profile



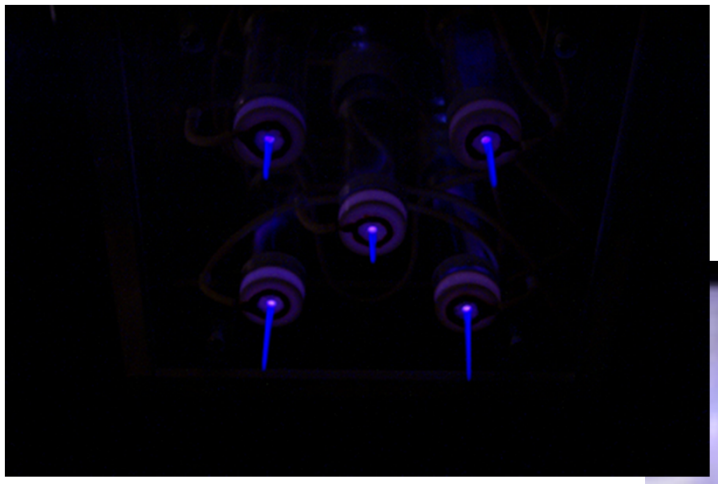
Abel inversion

The bullet is hollow: low emission in the middle of the jet

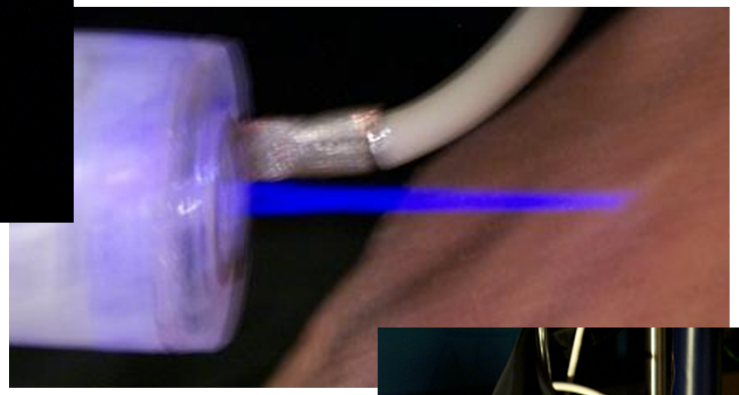
⇒ propagation at the interface between helium channel and ambient air

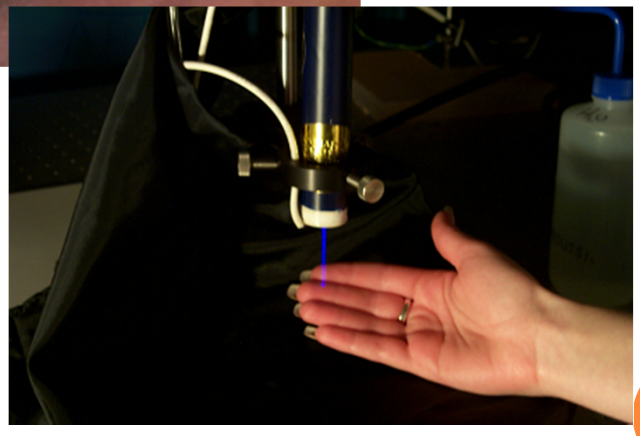
Contraction of the bullet along the axis of propagation

PLASMA PENCIL



A cold plasma plume generated by nanoseconds high voltage pulses

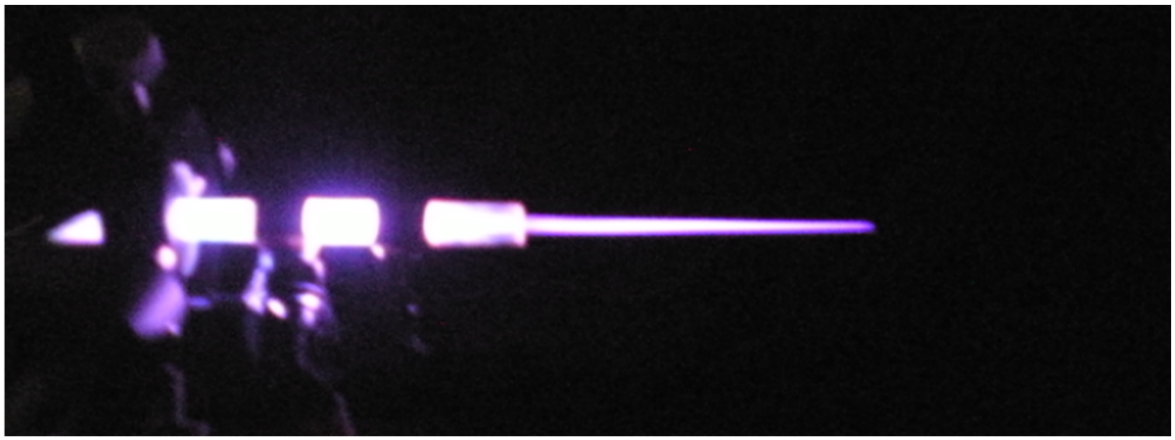


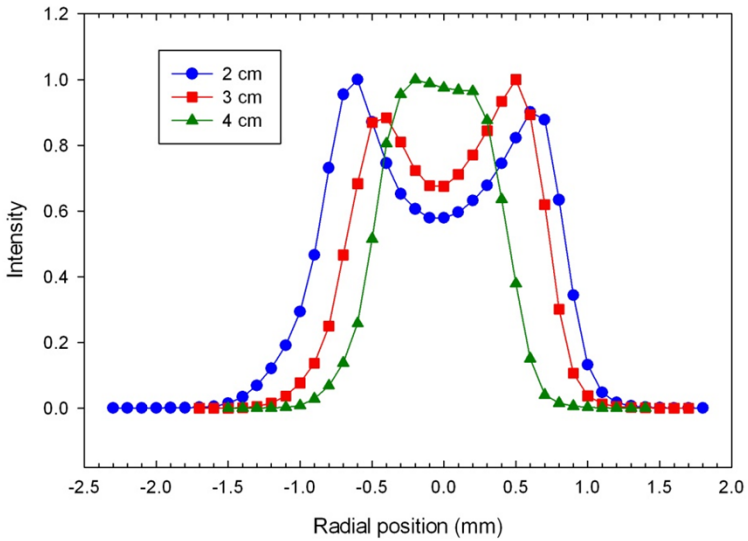


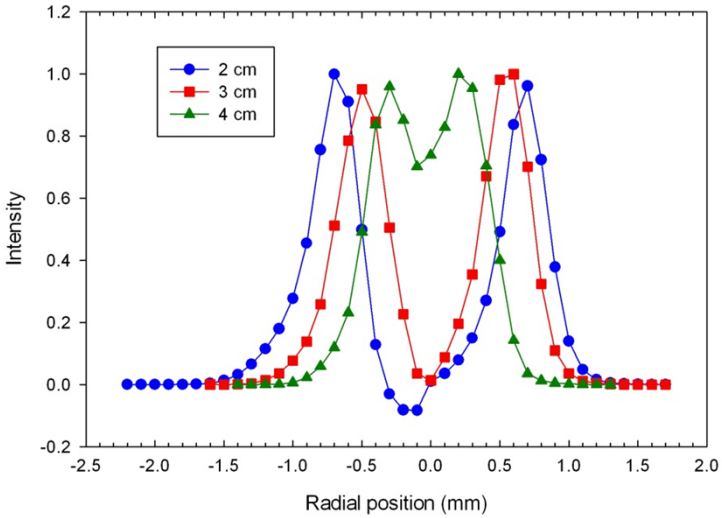
M. Laroussi and X. Lu, "Room Temperature Atmospheric Pressure Plasma Plume for Biomedical Applications", *Applied Physics Letters*, Vol. 87, 2005.

M. Laroussi - ODU

Tube Reactor

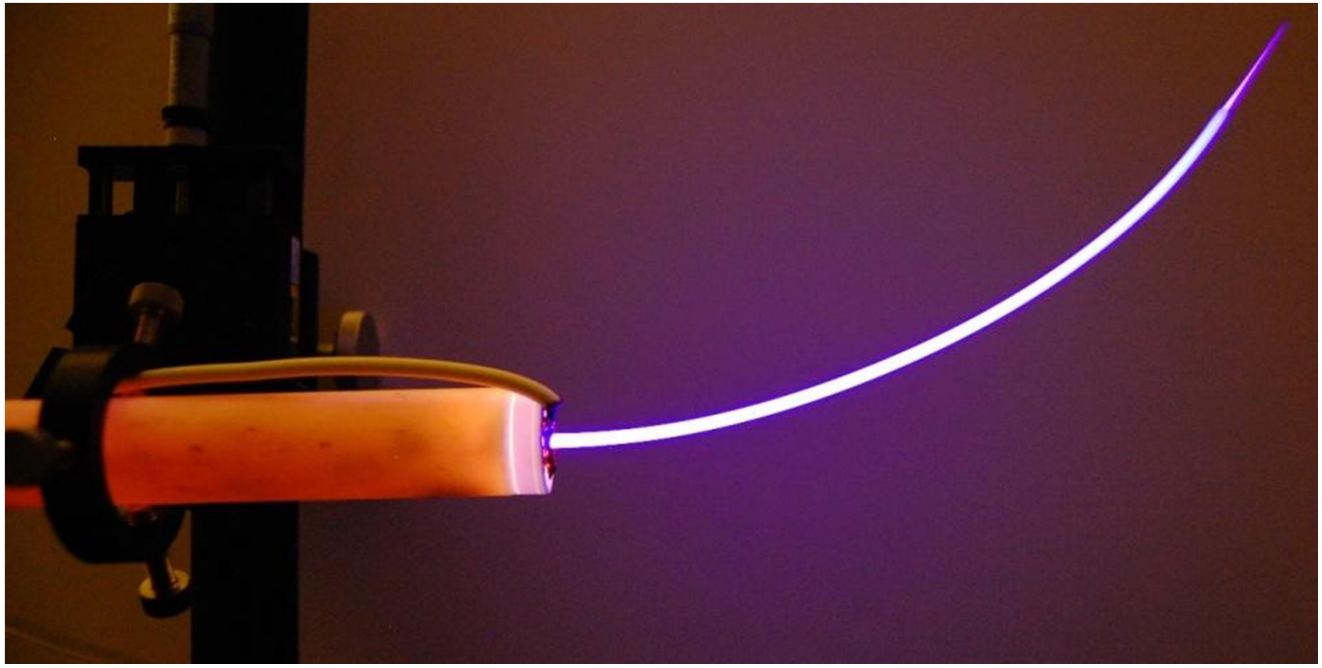


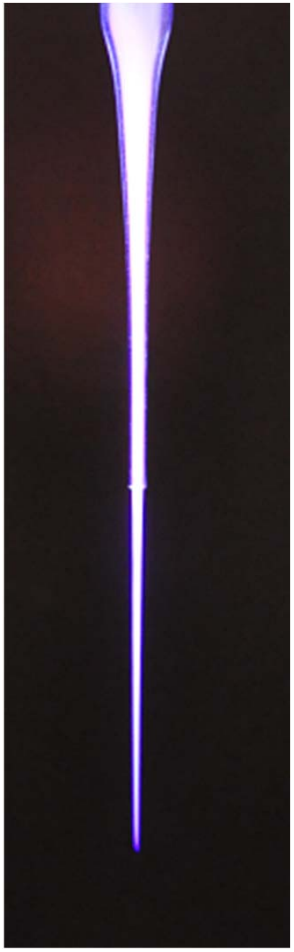




Experimental profile

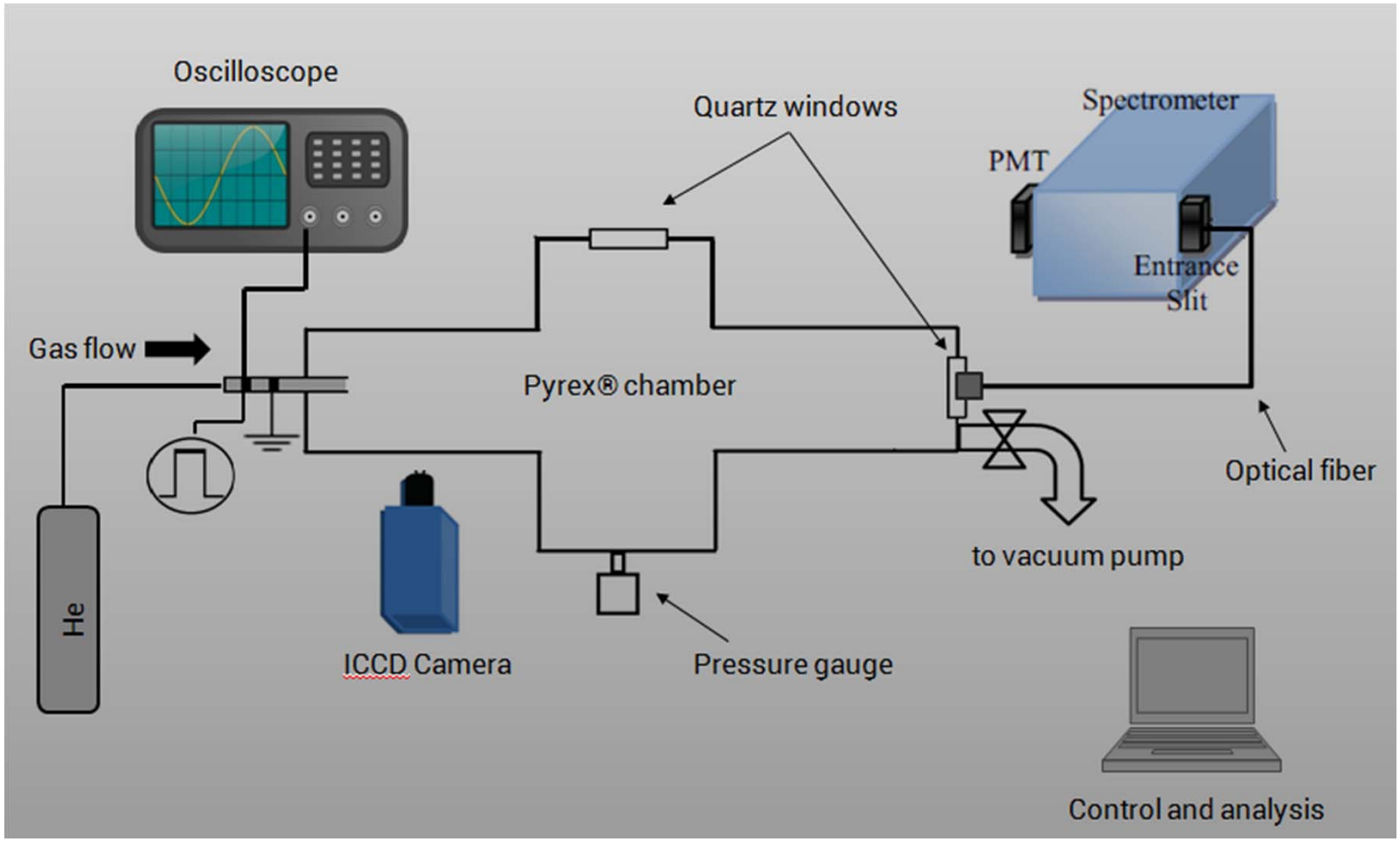
Abel inversion



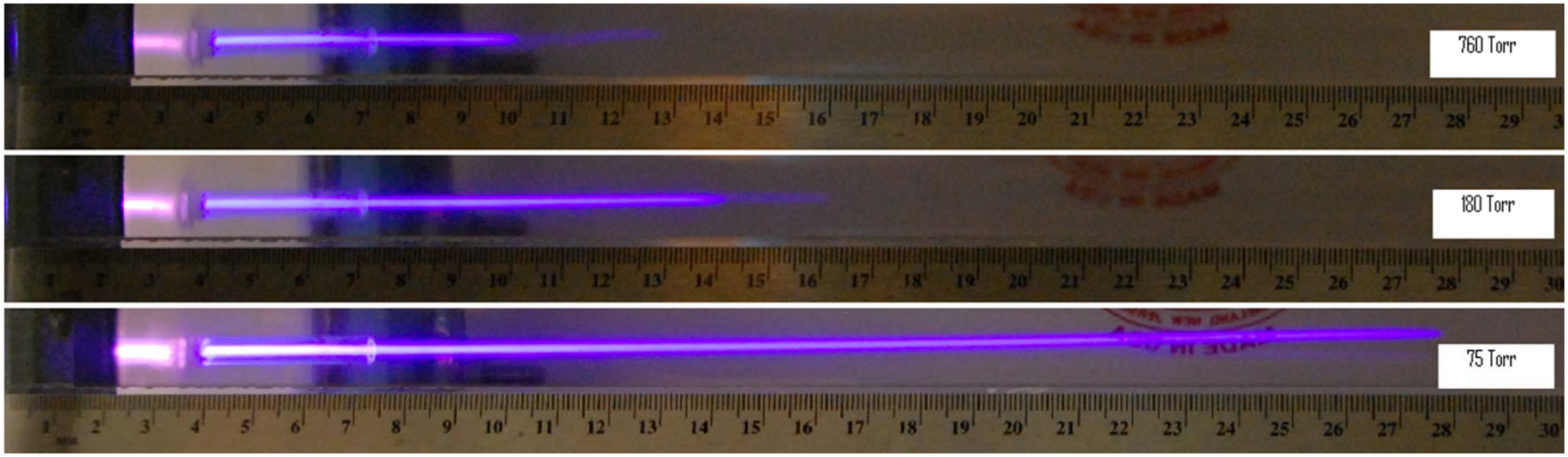


Micropencils

Plasma jet in controlled environment



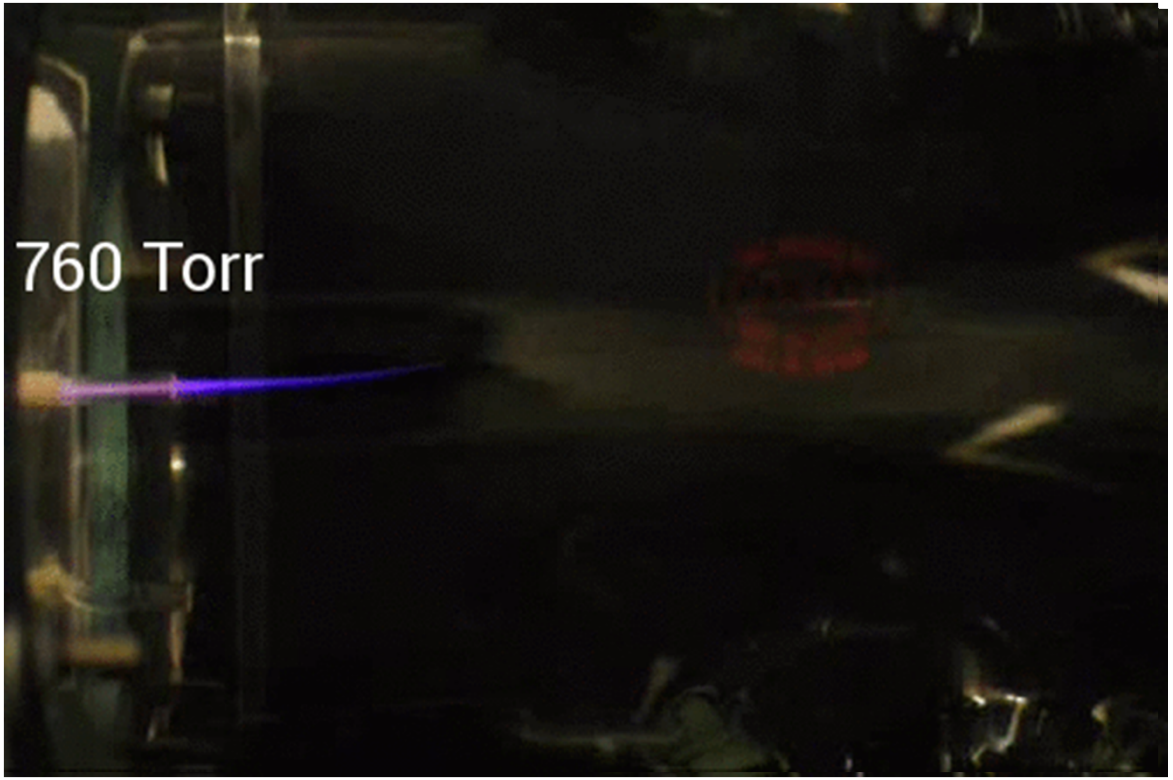
Plasma jets at different pressures



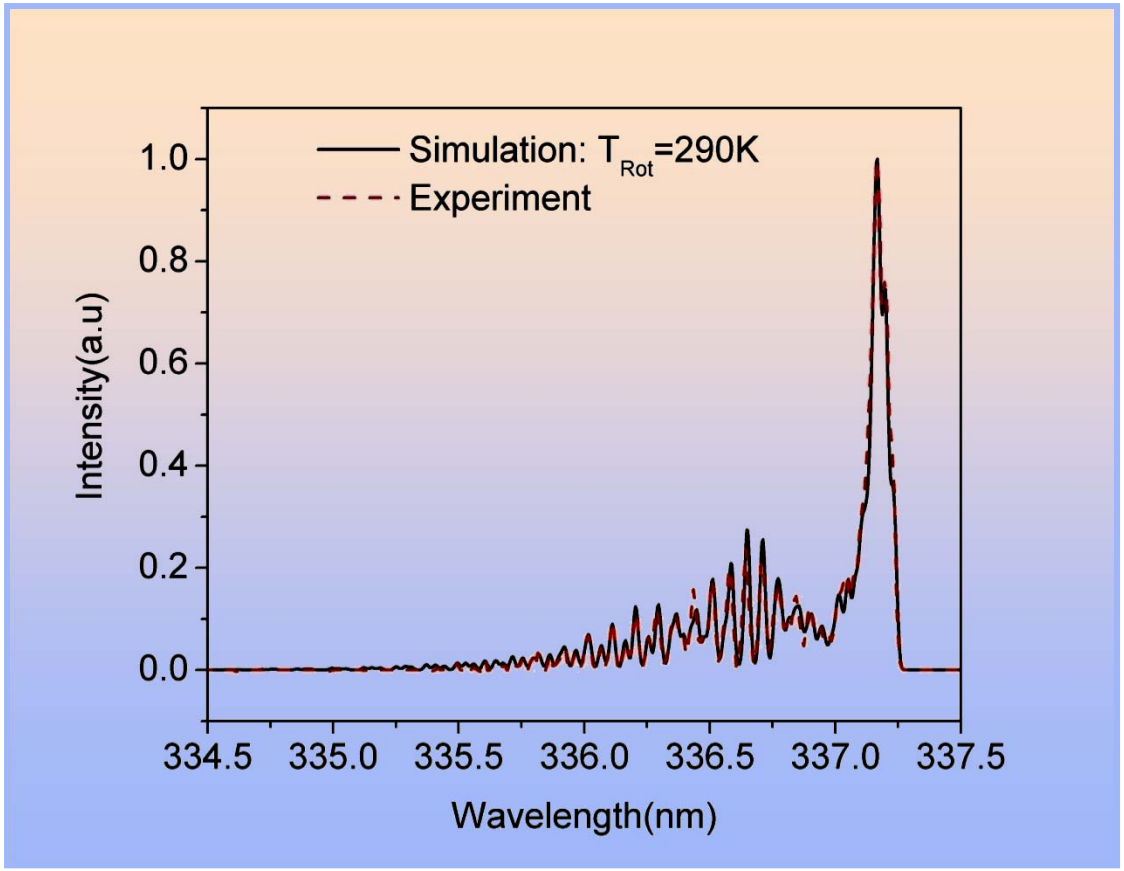
M. Laroussi and M. A. Akman, "Iginition of a Large Volume Plasma by a Plasma Jet", *AIP Advances* **1**, 032138 (2011).

JET & DIFFUSE MODE

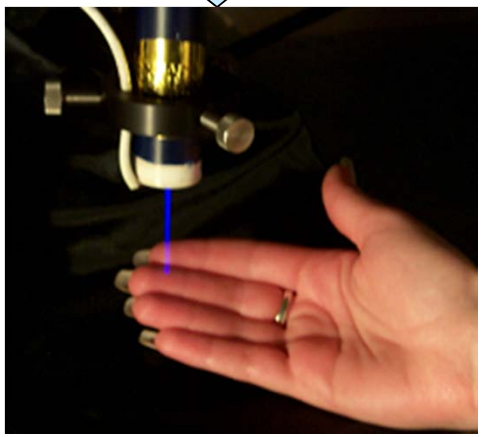
Time-lapse of jet length with reducing pressure



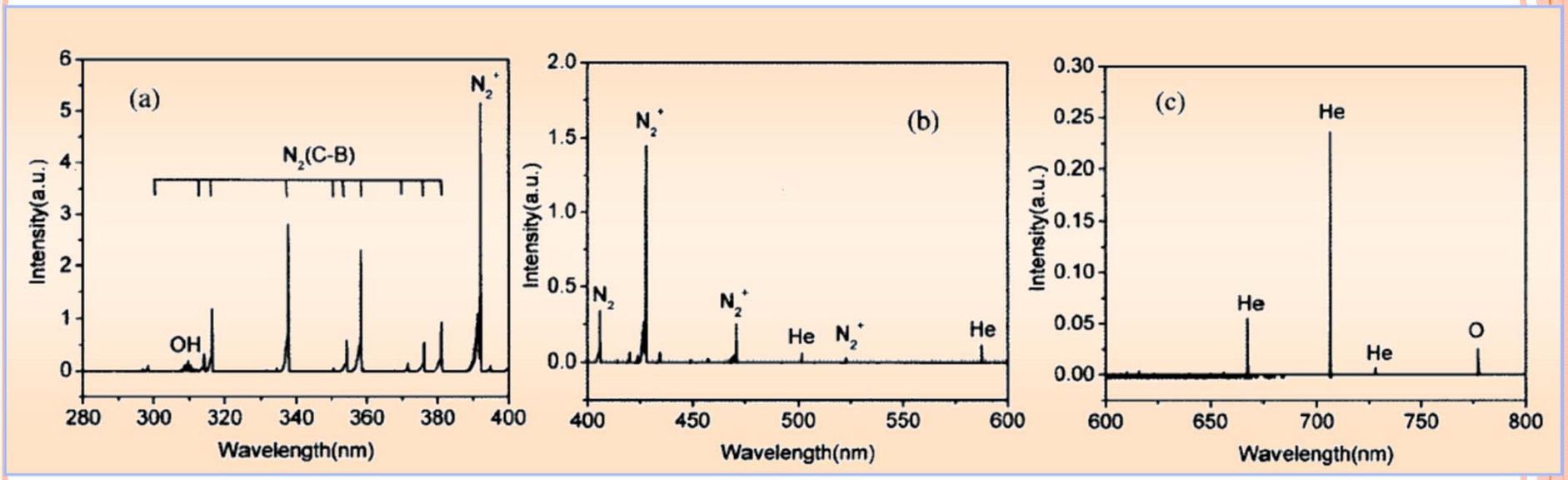
TEMPERATURE MEASUREMENT



Comparing the rotational structures of the N_2 (C – B)



EMISSION SPECTRA



Operating gas: helium

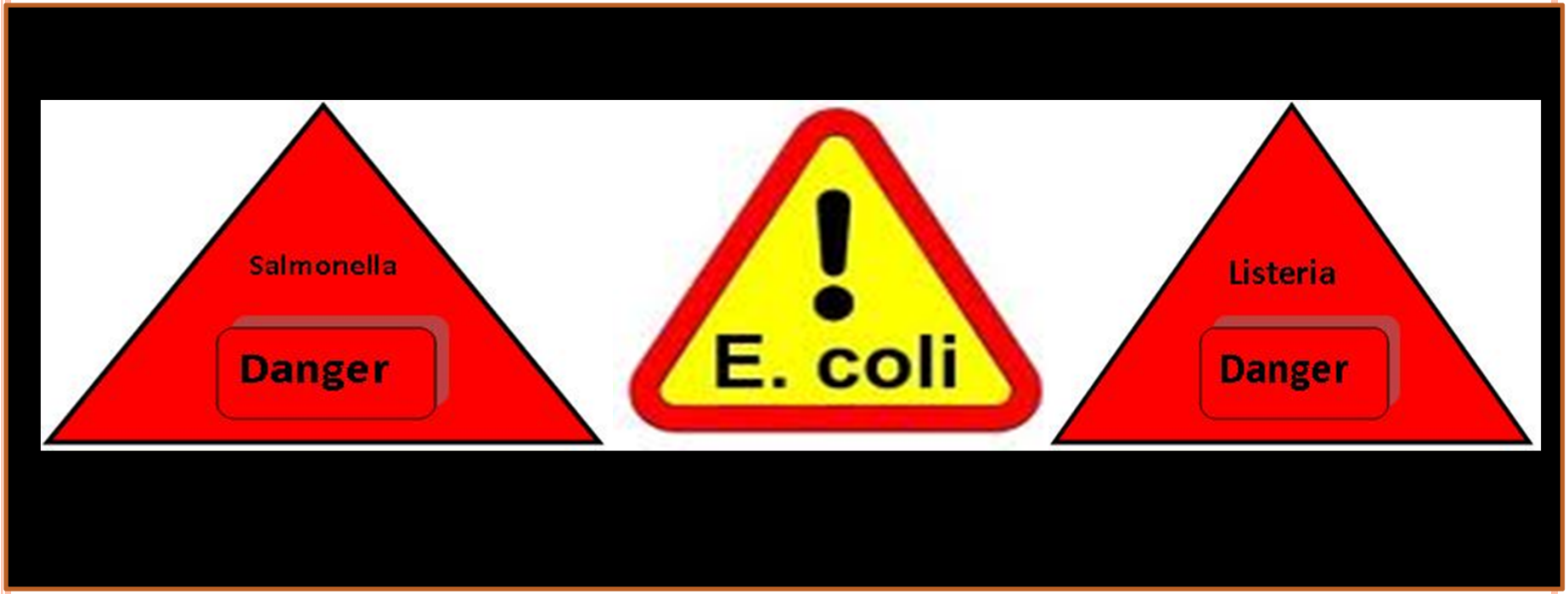
Biomedical Applications

Scientific Objective: Understanding the fundamentals of the interaction of plasma with prokaryotic and eukaryotic cells

Grand Objective: Use plasma to overcome unmet medical/healthcare challenges

Unmet Challenges: Food contamination

EHEC outbreak, *Salmonella* outbreak, *Listeria* outbreak…

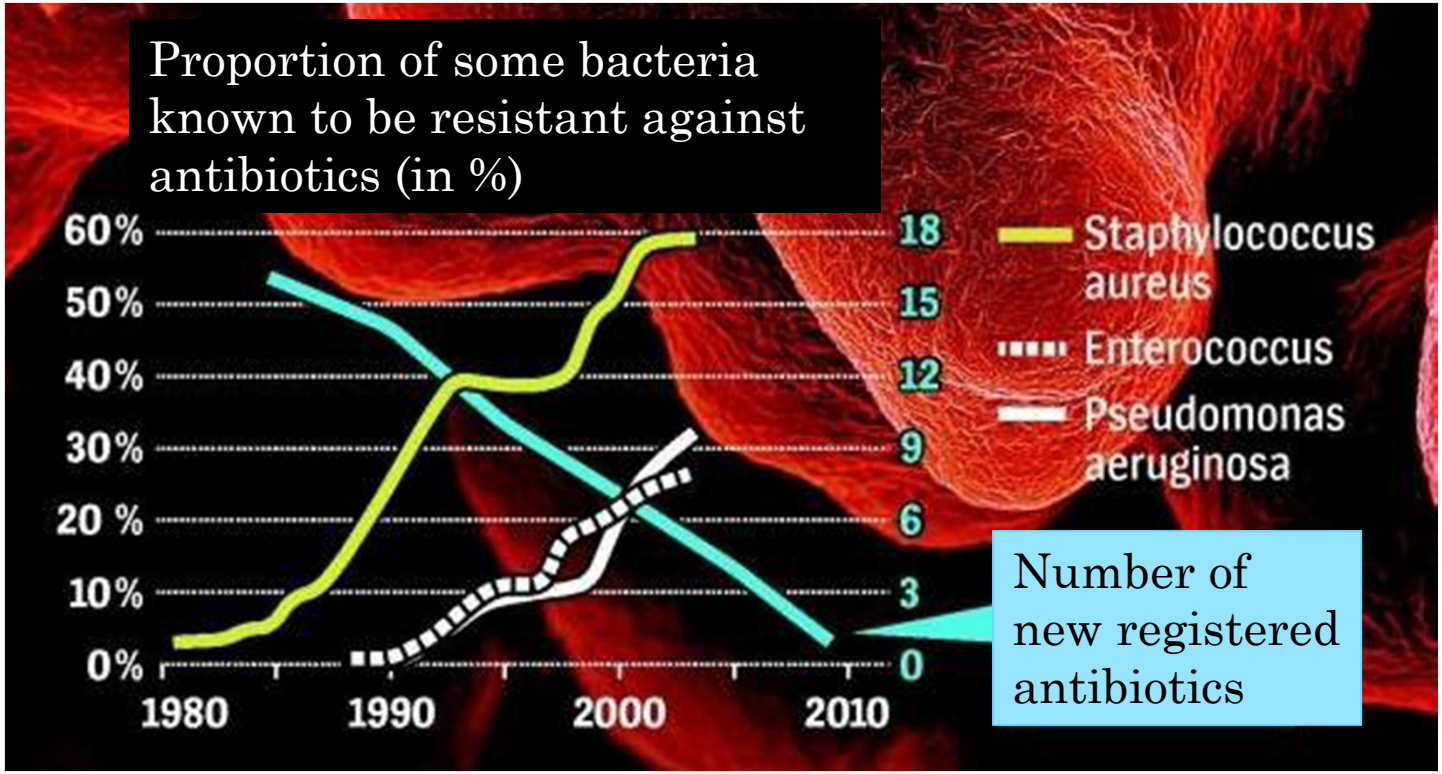


More Unmet Challenges:

Drug resistant bacteria: The ability of bacteria to become more resistant to antibiotics.
Example:
- MRSA: Main cause of nosocomial infections. From 1999 to 2005 MRSA related **hospitalizations more than doubled**.
- MRSA infection kills about **19,000** patients per year in the US alone. Higher deaths in other countries.

Chronic wounds: Wounds that persist, never heal or do not heal in an acceptable period of time. Example: Diabetic ulcers. **Thousands of amputations per year** in the US due to chronic wounds.

A Real and Present Crisis!!



A Real and Present Crisis!!

Eight country study finds high rates of drug resistance

* Resistant strains more costly to treat, often more fatal
* Almost 9 million people a year get TB, 1.4 million die
(Adds expert comment on drug costs, vaccines)

By Kate Kelland
LONDON, Aug 30 (Reuters) - Scientists have found an alarming number of cases of the lung disease tuberculosis in Africa, Asia, Europe and Latin America that are resistant to up to four powerful antibiotic drugs.

http://in.reuters.com/article/2012/08/30/tuberculosis-drug-resistance-idINL6E8JU1VD20120830

A Real and Present Crisis!!

J Antimicrob Chemother 2011; **66**: 1939–1940
doi:10.1093/jac/dkr261 Advance Access publication 23 June 2011

Journal of Antimicrobial Chemotherapy

The urgent need for new antibacterial agents

Richard Wise* on behalf of the BSAC Working Party on The Urgent Need: Regenerating Antibacterial Drug Discovery and Development†

British Society for Antimicrobial Chemotherapy, Griffin House, 53 Regent Place, Birmingham B1 3NJ, UK

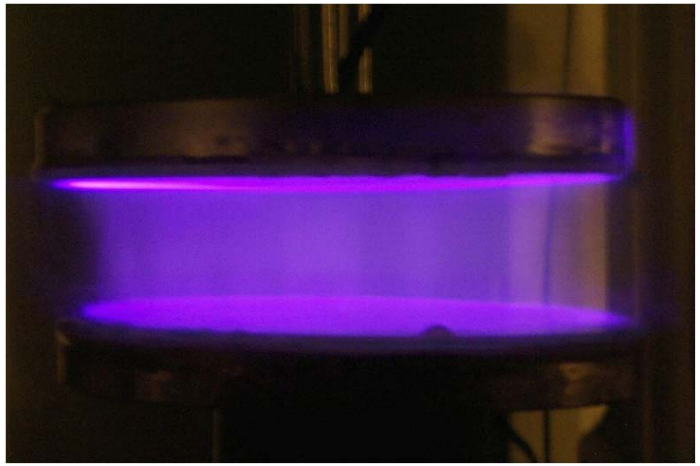
*E-mail: rjwise2@aol.com
†Members are listed in the Acknowledgements section.

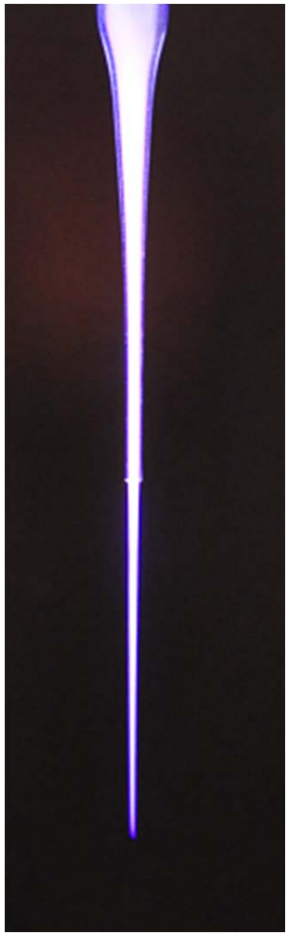
The crisis of no new antibiotics—what is the way forward?

Laura J V Piddock

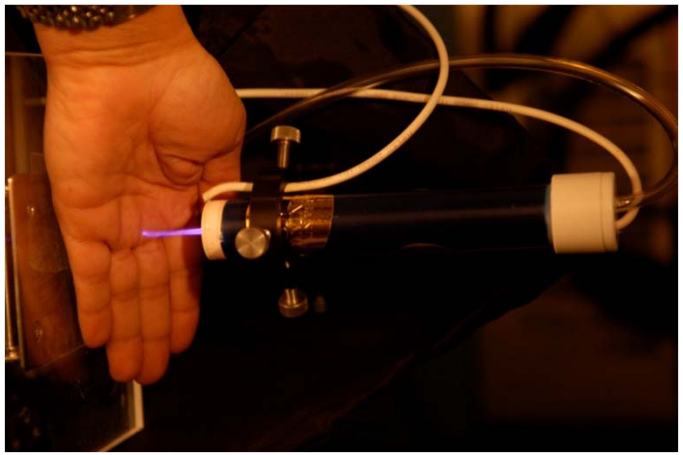
Lancet Infect Dis 2012;
12: 249–53

Slide courtesy G. Isbary



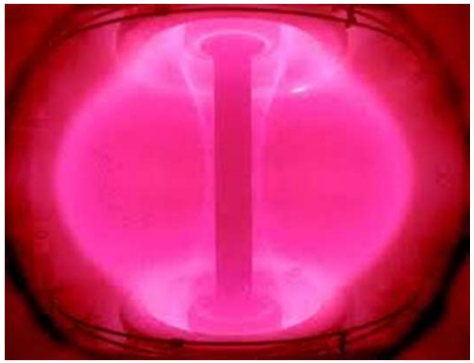


Can low temperature plasma meet all or some of these challenges?

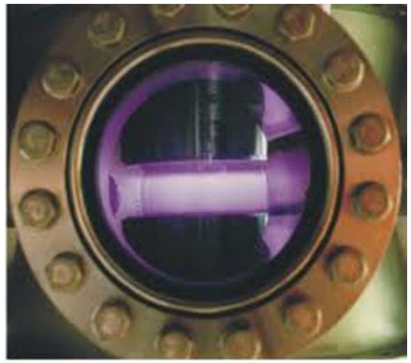


Getting a "cold" plasma out: Biologically "compatible" plasmas

Useful plasmas but unsuitable for biomedical applications



Tokamak

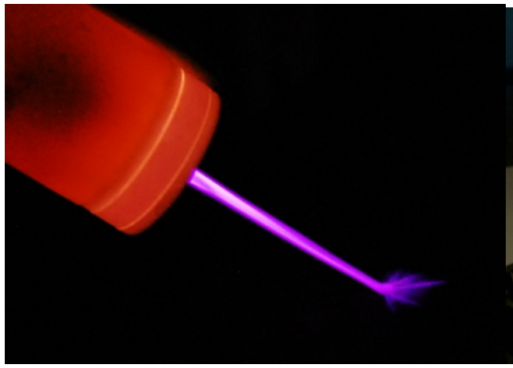


Low Pressure plasma

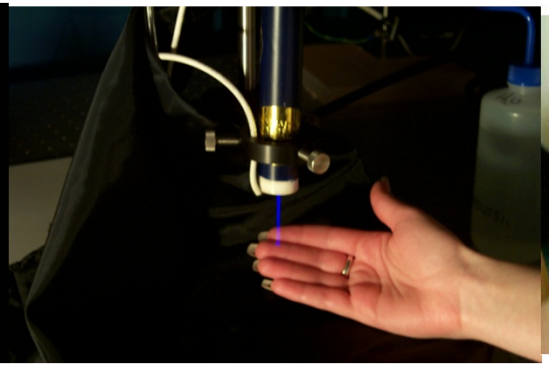
Plasma torch

Plasma cutter

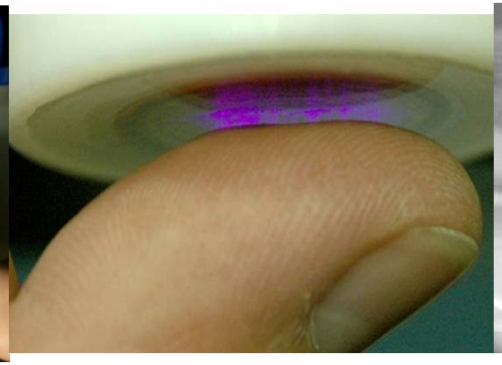
Suitable for biomedical applications



Plasma pencil - ODU



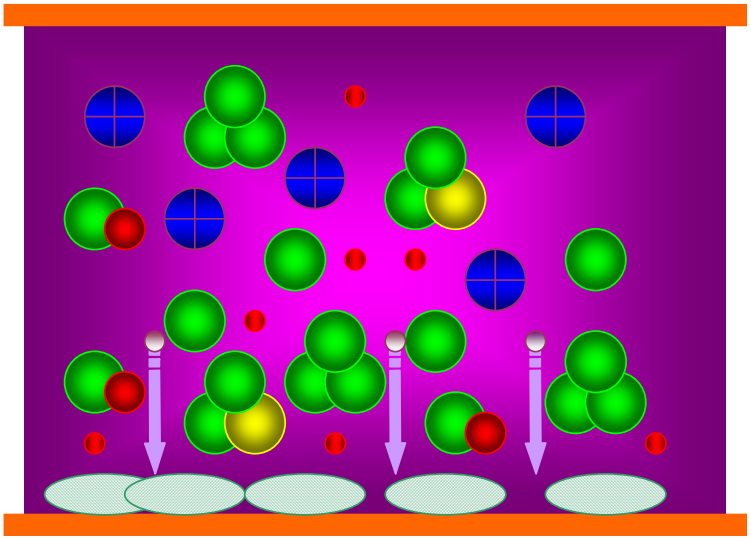
Plasma pencil - ODU



FE plasma - Drexel

Plasma needle – Eindhoven Univ

Active Agents in Plasmas



- **Temperature: thermal effects**
- **UV radiation: especially between 200 nm and 300 nm**
- **Charged particles: electrons and ions**
- **Reactive species: oxygen-based, nitrogen-based, radicals, non-radicals**

$$e + N_2 \longrightarrow N + N + e$$

$$e + N_2 \longrightarrow N_2^+ + 2e$$

$$e + O_2 \longrightarrow O + O + e$$

$$N_2 + O_2 \longrightarrow 2NO$$

$$O + O_2 + M \longrightarrow O_3 + M$$

$$O_3 + NO \longrightarrow NO_2 + O_2 + h\nu$$

$$e + O_2 \longrightarrow O_2\,(^1\Delta) + e$$

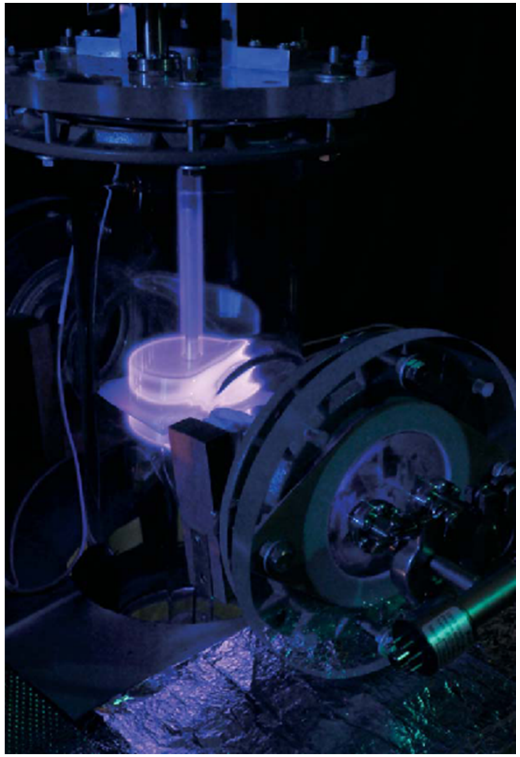
$$e + O_2 \longrightarrow O^- + O$$

$$e + H_2O \longrightarrow OH + H + e$$

$$e + H_2O \longrightarrow OH^- + H$$

$$4NO + O_2 + 2H_2O \longrightarrow 4HNO_2$$

These are but a few examples of possible reactions that take place in a non-equilibrium air plasma



Reactive Oxygen Species (ROS):

O, O_2^-, O_3, OH, H_2O_2,.....

ROS effects on cell membrane: Peroxidation of lipids, oxidation of proteins, DNA strands, …

Reactive Nitrogen Species (RNS):

N, NO, NO_2, NO_2^-, NO_3^-, N_2O_5,....

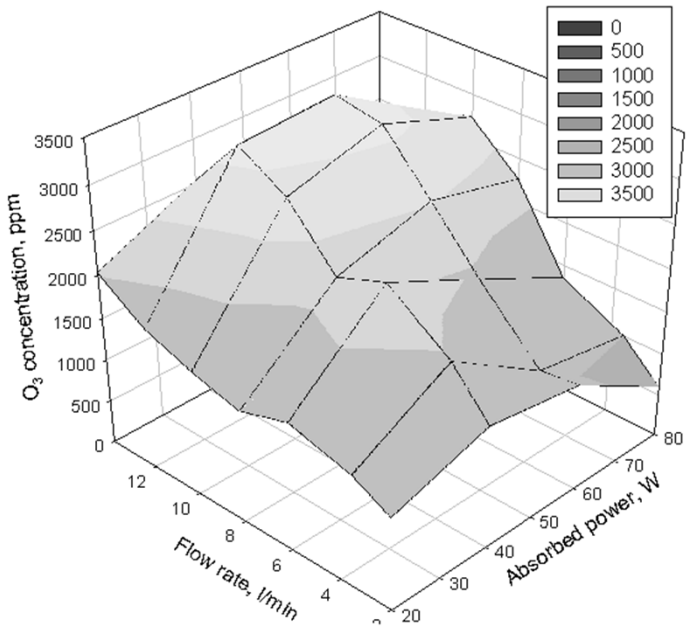
RNS effects on biological cells: Cell signaling

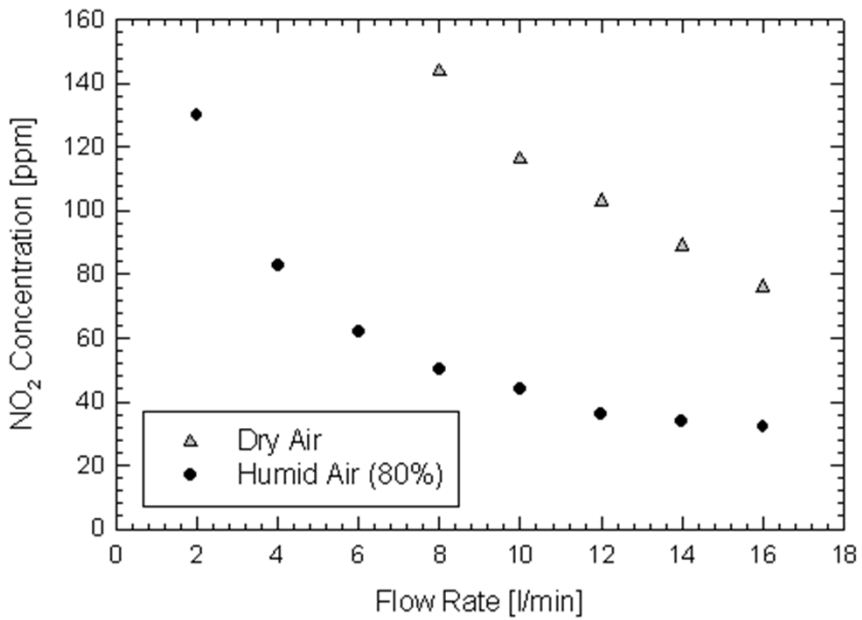
Plasma generates a cocktail of chemical agents with biological implications.

CHEMICAL & PHYSICAL EFFECTS OF THE REACTIVE SPECIES

- **Peroxidation of the lipid bilayer (fatty acids): OH, H_2O_2**
- **Oxidation of the proteins (chain of aminoacids) that play the role of gateways between the exterior and interior of the cells: O, H_2O_2, O_2^-**
- **DNA oxidation: O, H_2O_2**
- **Cell respiration: O_3**
- **Etching/erosion of biomaterials: O, O_2^***

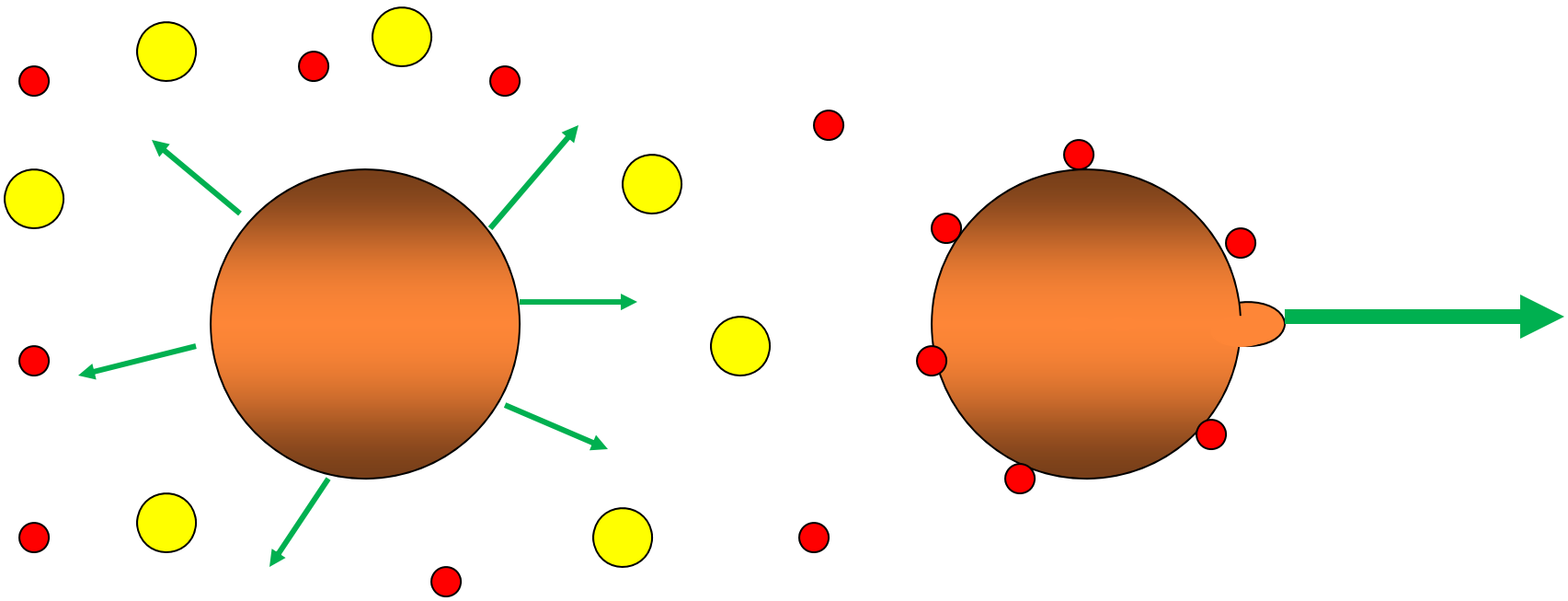
Plasmas produce Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS): O, O_2^-, O_3, NO, NO_2, OH, H_2O_2, …





Air Plasma

Charged particles effects: Electrostatic Pressure



Electrostatic tension: $F_e \sim \Phi^2/a^2$, a is the radius and Φ is the surface potential

$$\Phi = f\,(\,T_e/\,T_i,\ m_i/m_e\,)$$

ELECTROSTATIC CELL DISRUPTION

Electrostatic disruption occurs when $F_e > F_t$, where F_t is the tensile strength of the outer structure.

Mendis et al. derived the condition for disruption:

$$\Phi > 0.2\,(r.\Delta)^{1/2}.\,F_t^{1/2}$$

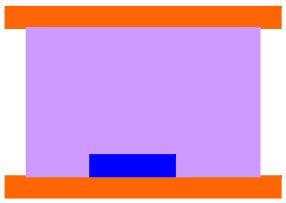
where r is the "bump" radius of curvature, and Δ is the thickness of the outer membrane.

→ **Disruption is more likely to occur at surface irregularities**

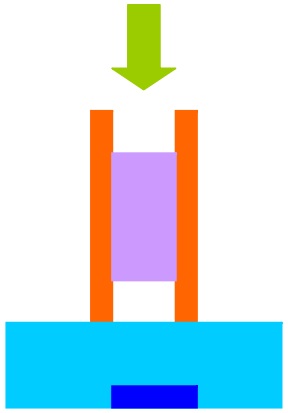
D. A. Mendis, M. Rosenberg, and F. Azam, *IEEE Trans. Plasma Sci.* **28**, 1304, (2000).

M. Laroussi, D. A. Mendis, and M. Rosenberg, "Plasma Interaction with Microbes", *New Journal of Physics*, Vol. 5, pp. 41.1-41.10, (2003).

Direct Vs. Remote Exposure

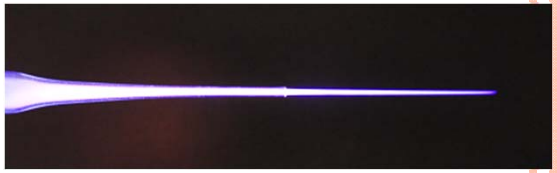


Direct exposure



Remote exposure

Timeline of our Research Activities

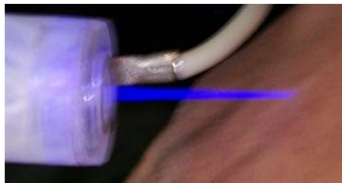


Dental applications 2007

Destruction of amyloids Parkinson, APL 2010

1st Publication TPS, 1996

Plasma Pencil/bullets APL, 2005; JAP, 2006



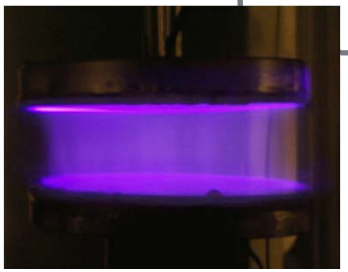
1st Experiments: Inactivation of bacteria 1994

Morphological study & Biochemical pathways TPS 1999 & APL, 2002

Spore survivability TPS, 2006

Cytotoxicity study Wound healing TPS, 2009

Present & Future work: Cancer studies



Sterilization of Contaminated Matter with an Atmospheric Pressure Plasma

Mounir Laroussi, *Member, IEEE*

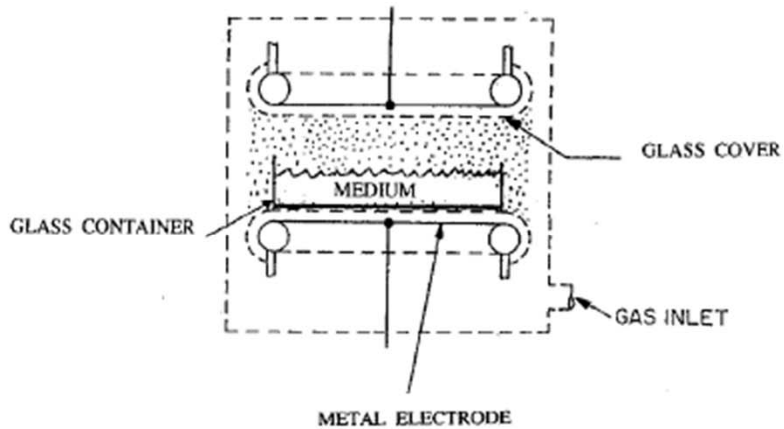
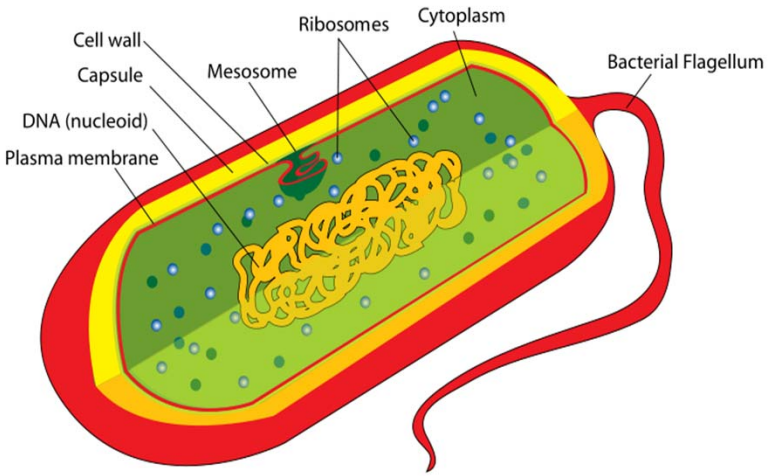


Fig. 6. Contaminated medium being treated by plasma.

TABLE I
VARIOUS STERILIZATION METHODS AND THEIR CHARACTERISTICS

since this paper is not aimed at a readership of biologists, but at plasma scientists, it is enough, as a preliminary step, to show that the glow discharge at atmospheric pressure is a powerful sterilization agent. More "biology" oriented work is underway and will be published in an appropriate journal at a later time. Some of the questions to be answered are: which plasma regime is the most effective (uniform of filamentary)? What is the minimum plasma power density sufficient to kill unwanted microorganisms? Which physical process plays the dominant sterilization role? What biological and chemical processes induce the death of the cells? Which type of gas is more desirable for a particular application? Some of these questions will be more appropriately answered by biologists after more work is done on this method. However, it appears that plasma-aided sterilization requires short exposure times, is very practical

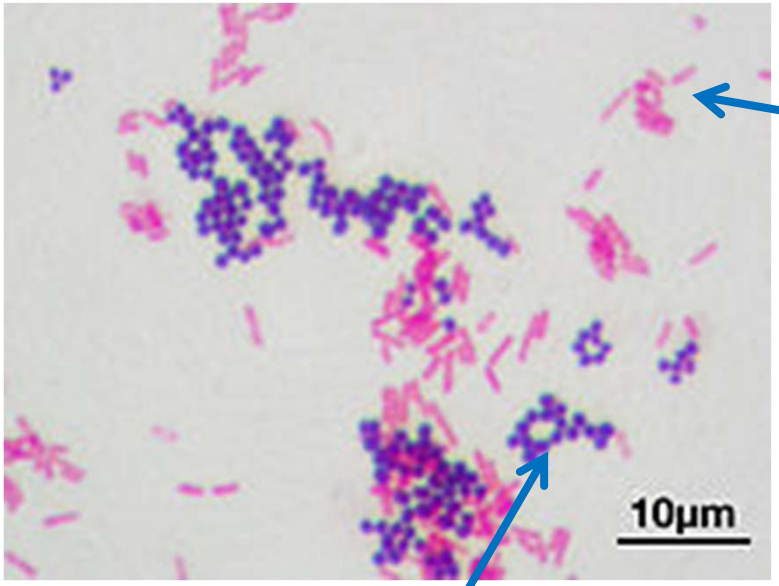
BACTERIA INACTIVATION



BACTERIA TYPES

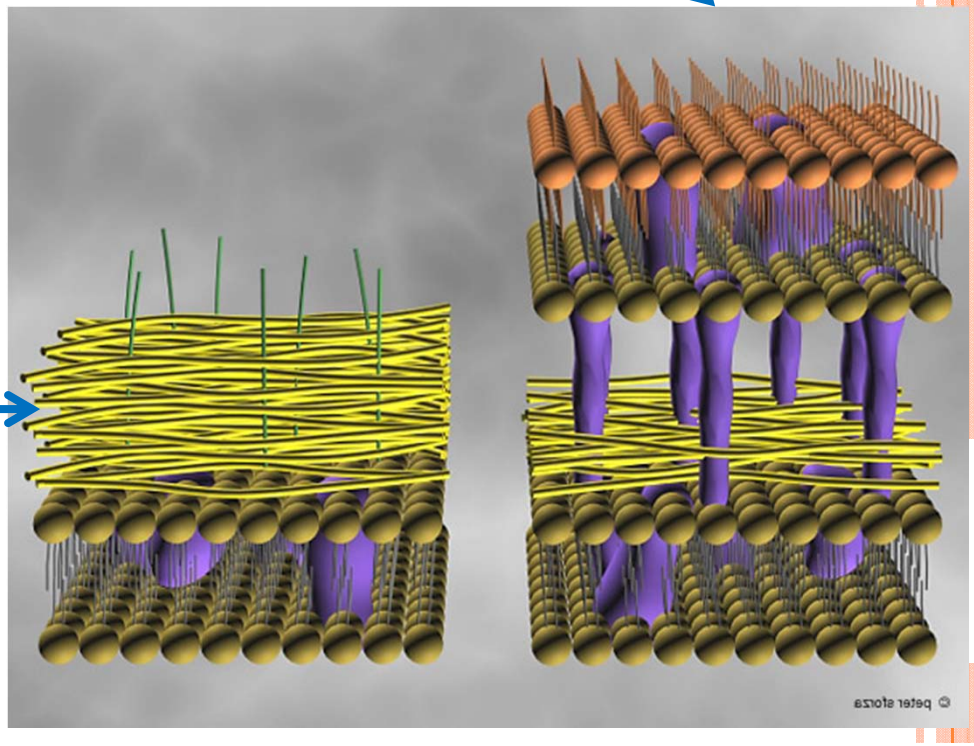
Gram negative	***Gram positive***
Escherichia coli	***Staphylococcus aureus***
Vibrio cholerae	***Micrococcus luteus***
Klebsiella pneumoniae	***Bacillus subtilis***
Acinetobacter calcoace	***Bacillus megaterium***
Shigella sonnei	***Streptococcus salivarius***
Enterobacter aerogenes	***Streptococcus agal***
P vulgaris	***Candida albicans***
C. freundis	***Enterobacter faecalis***
Enterobacter cloacae	***Staphylococcus epidermis***
S thyphimurium	***Streptococcus pneumoniae***
Serratia marcescens	***Streptococcus pyogenes***

Morphology of bacteria – Gram Stain

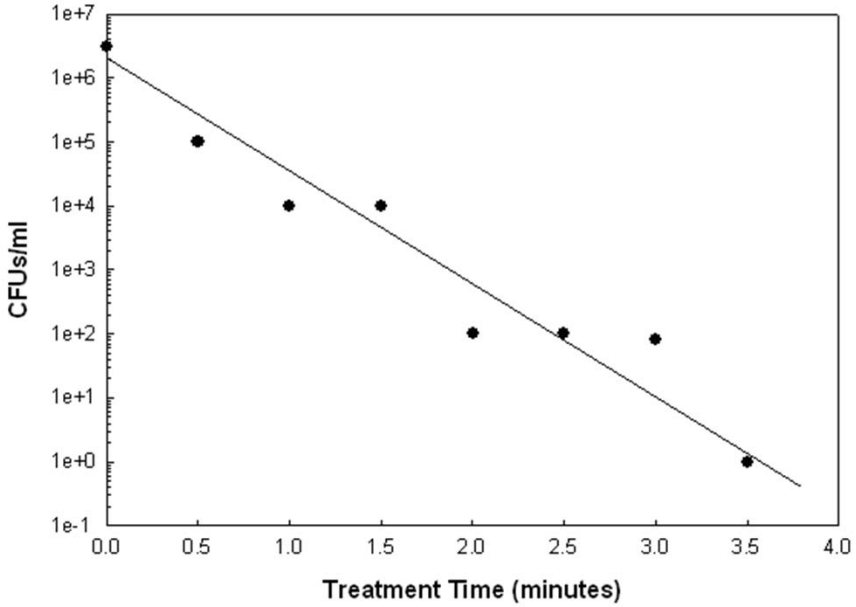


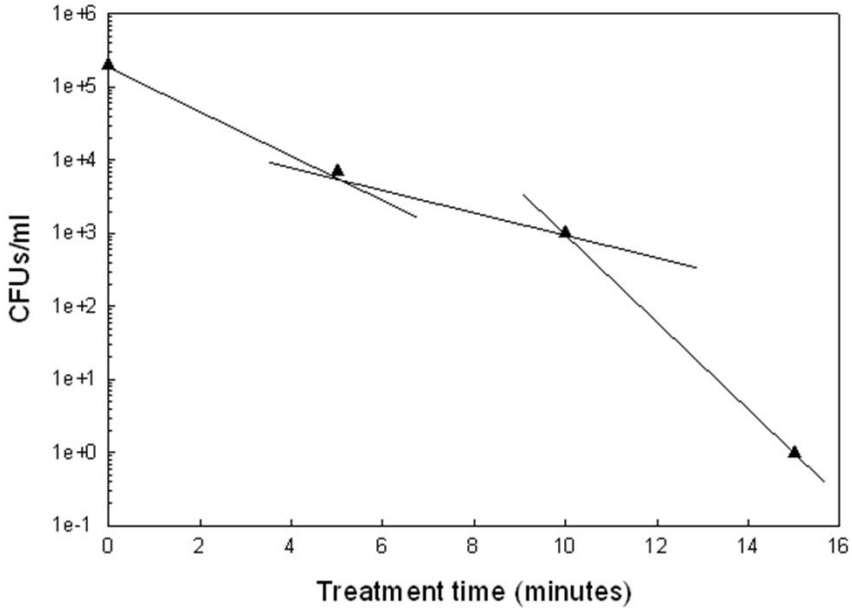
Gram neg. (-) = Pink/Red
- ***E. coli***
- **OM, CM**
- **LPS**

Gram pos. (+) = Purple/Blue
- ***B. subtilis***
- **Thick PG layer**

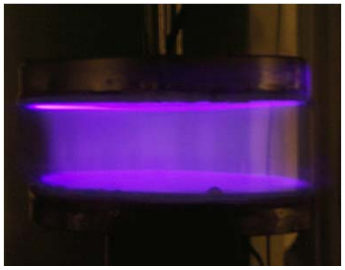


The Beginning (Mid to Late 1990's)



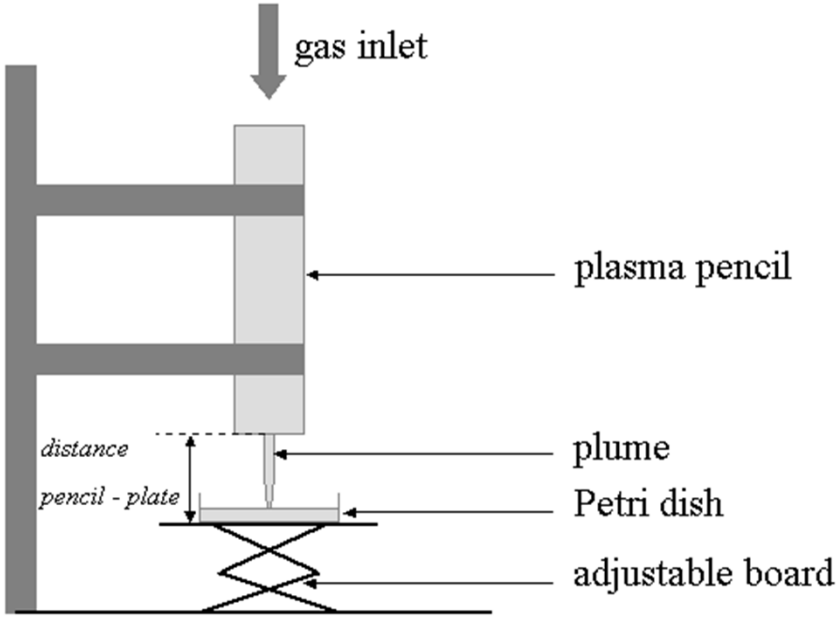


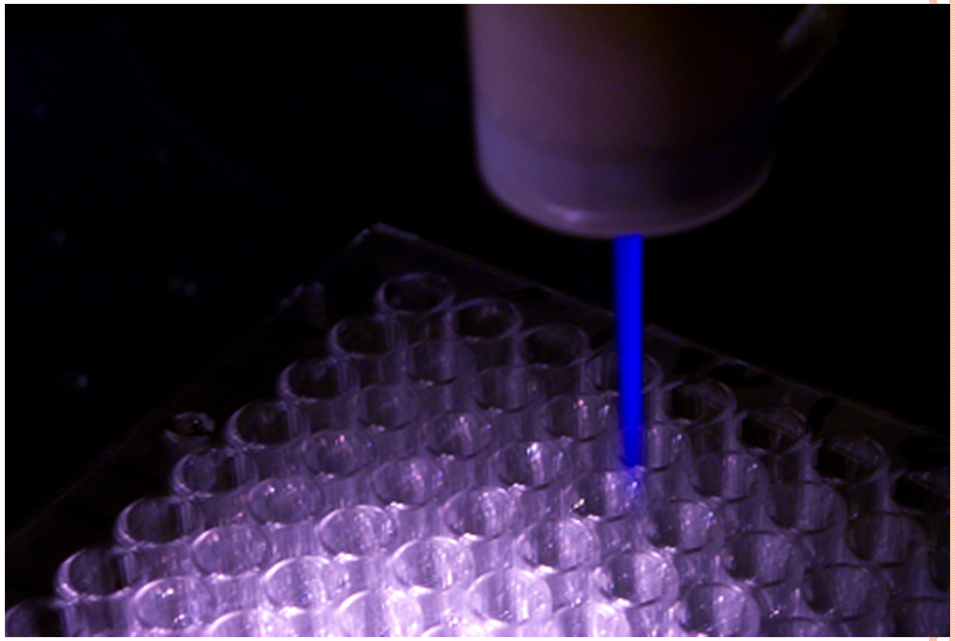
E. coli



P. aeuroginosa

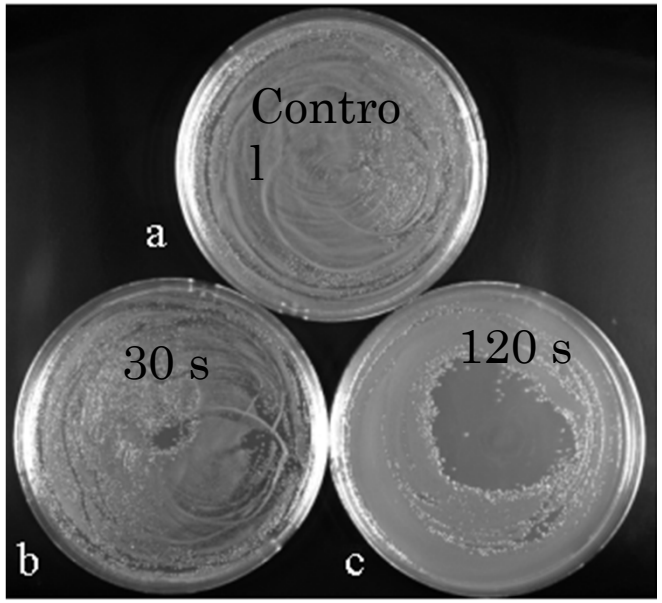
Experimental Setup for Biological Tests with the Plasma Pencil



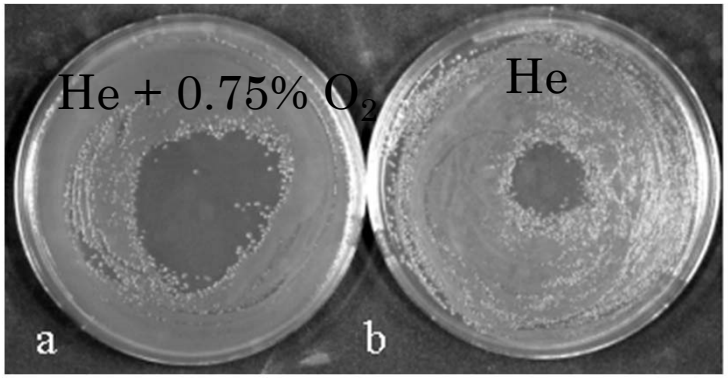


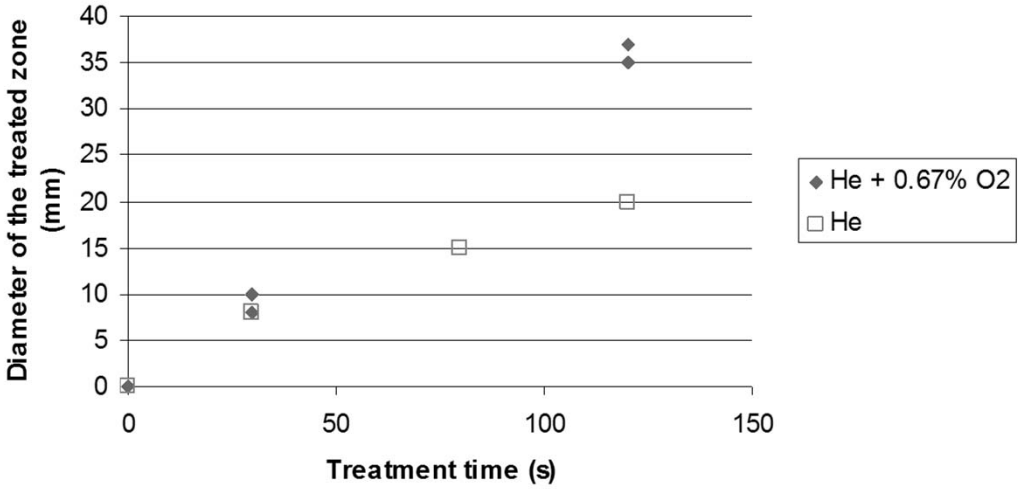
SIZE OF THE AFFECTED ZONE

E. coli

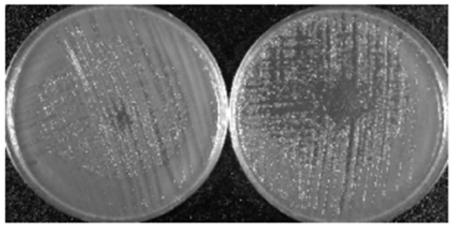


He + 0.75 O_2 ; TS agar



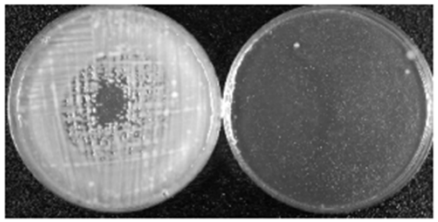


E. coli



undiluted 10^{-2} diluted

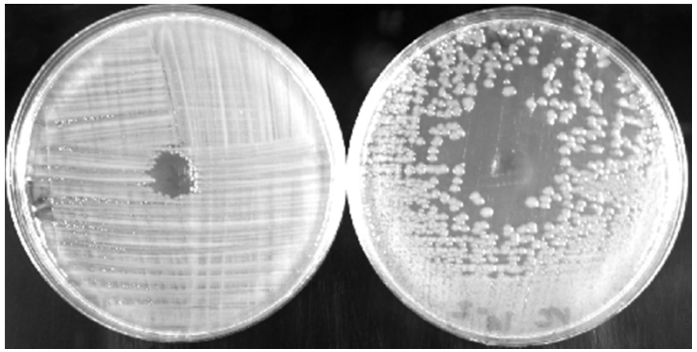
K. pneumoniae



undiluted 10^{-2} diluted

40 mm

Vibrio Cholerae

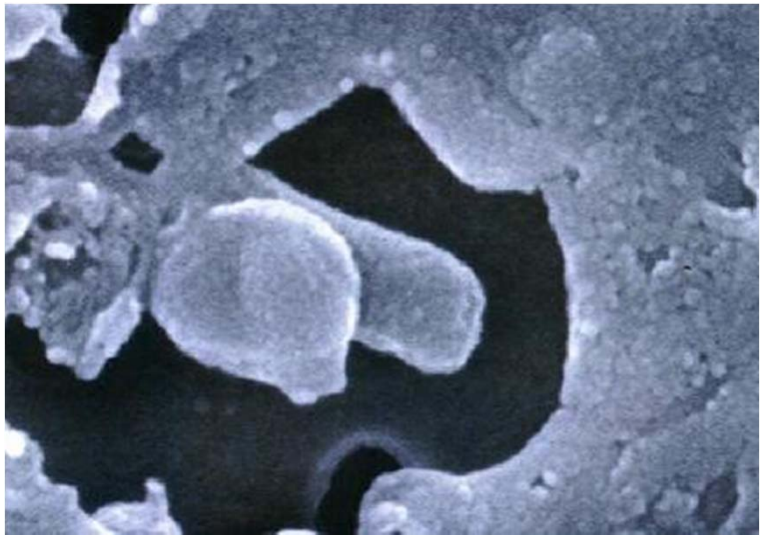


Undiluted 10^{-2} dilution

TS agar, 4 minutes treatment. Photos show the influence of both the type of bacteria and the initial concentration

MICROGRAPHS OF E.COLI (GRAM-NEGATIVE)

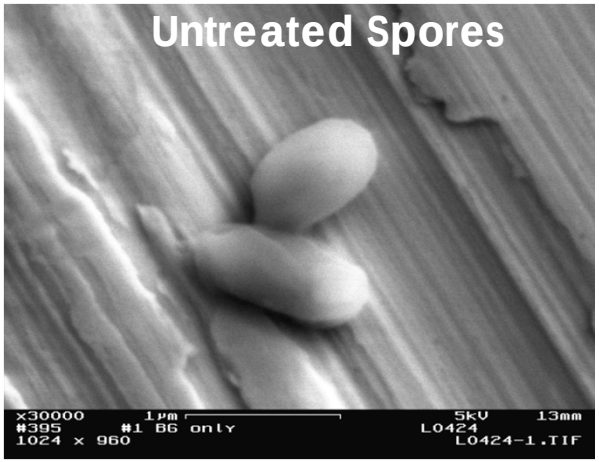
E.coli – Control

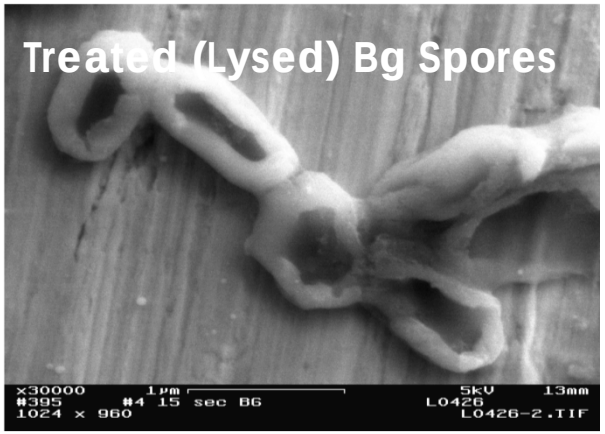


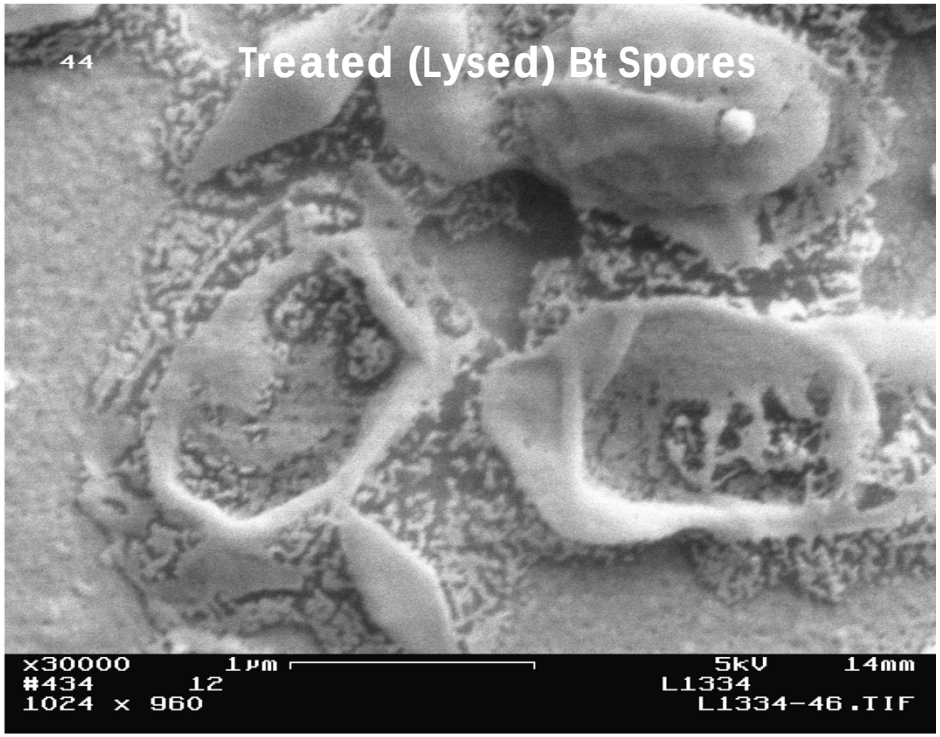
E.coli – After direct exposure to plasma

Micrographs of Bg/Bt Spores (gram-positive)

(Courtesy: J. Birmingham, μET)

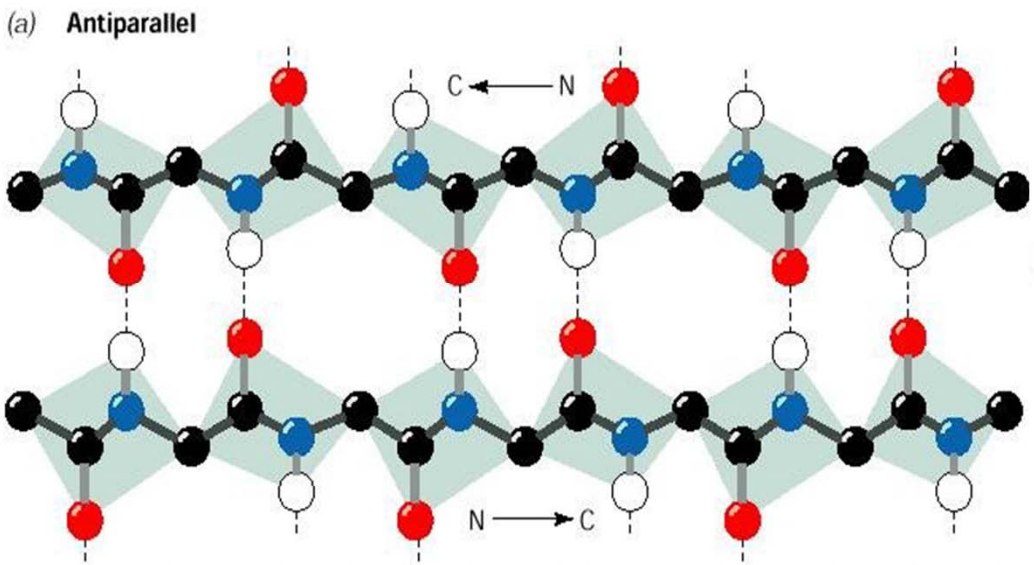






Destruction of Proteins

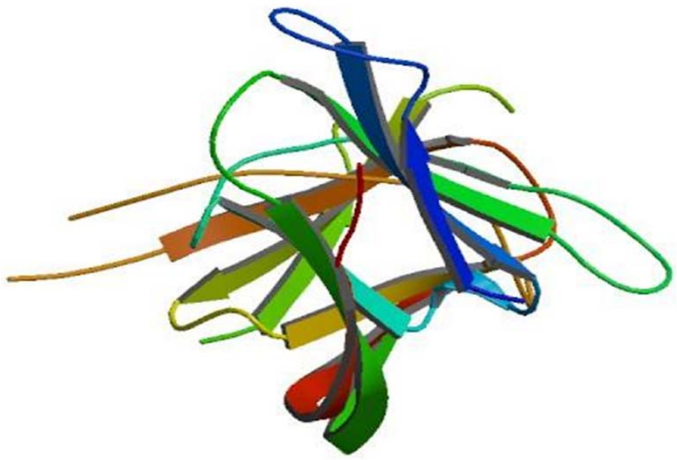
Model of a Beta-Sheet Structure



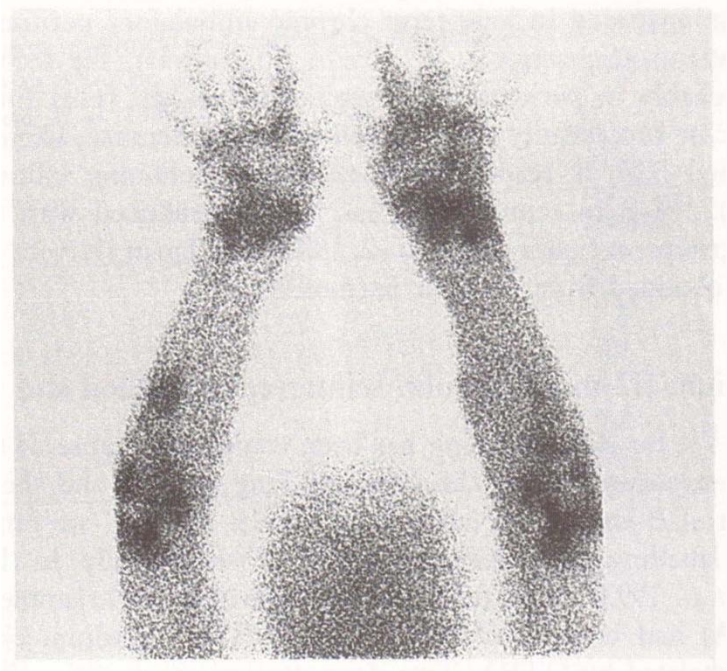
- Amyloid fibrils are composed of very stable beta-sheets which pack in a stacking manner into an elongated polymer.
- The individual proteins or peptides first form beta-sheet structures.

β2-Microglobulin: major component of hemodialysis-related amyloidosis

Fibrils are deposited in tissues including joint spaces



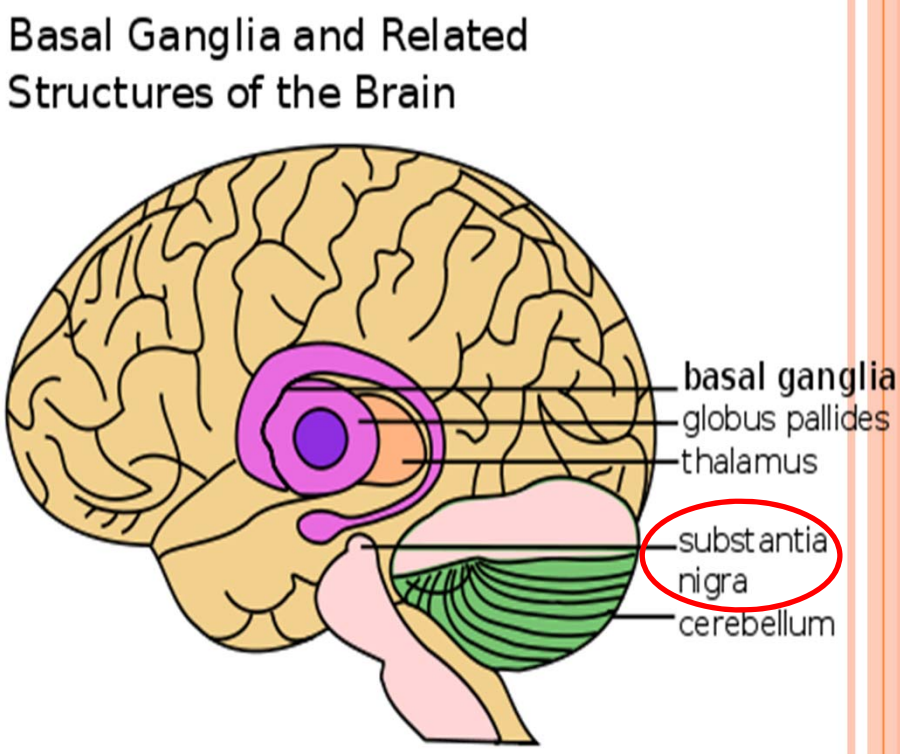
Native structure of β2-Microglobulin



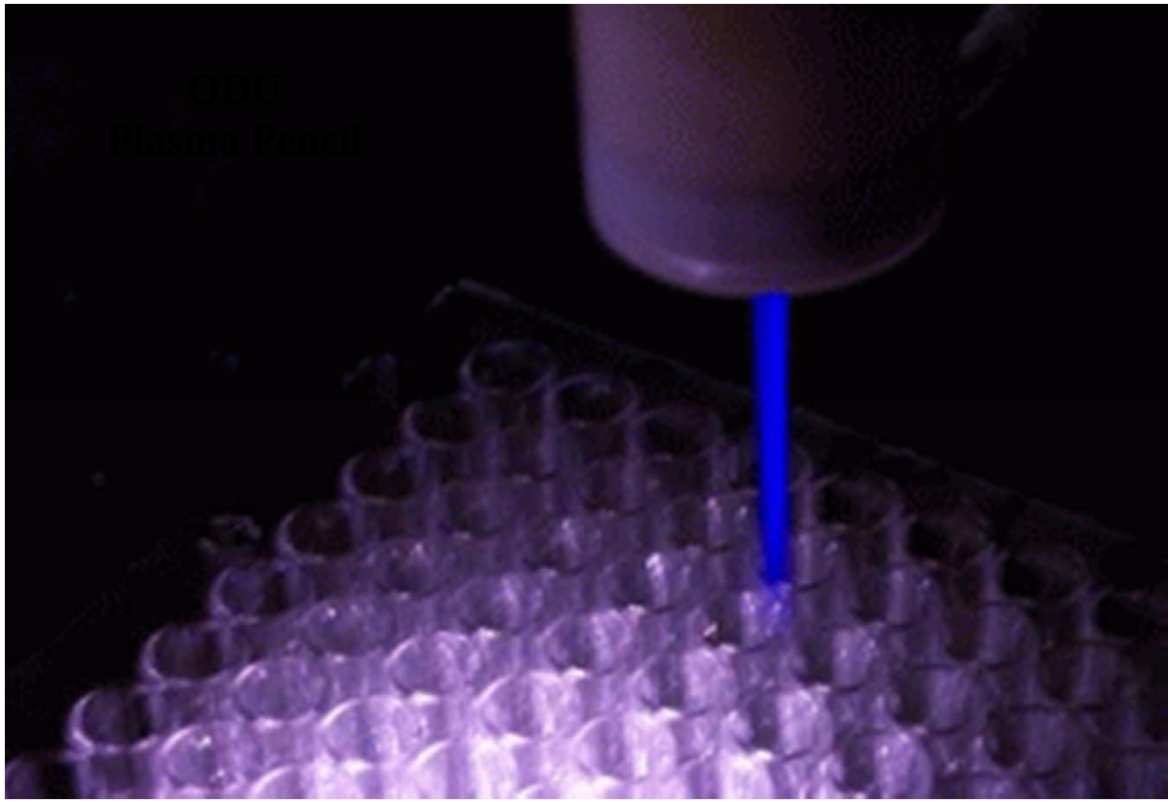
Dialysis amyloid. Edited by Ypersele and Drucke, Oxford medical publications (1996)

Parkinson's Disease

1. A neurodegenerative movement disorder.
2. It results from degeneration of dopaminergic neurons in the substantia nigra, a region of the brain that controls movement.
3. Death of dopaminergic neurons by the formation of amyloid fibrils with the protein alpha-synuclein results in decreased production of dopamine.
4. Lack of dopamine results in the major symptoms of Parkinson's disease: rigidity due to muscles constantly contracted; resting tremor; postural instability and slowness in initiating movement.



PLASMA PENCIL



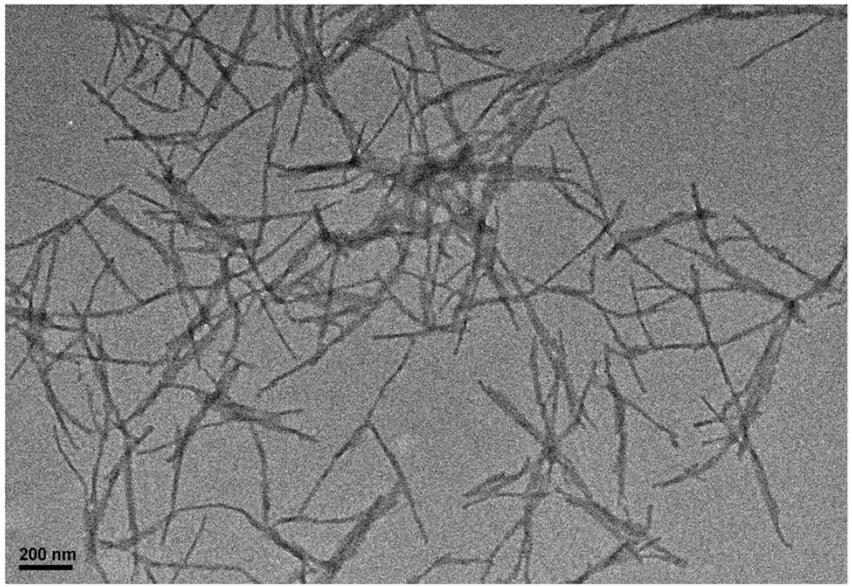
The amyloid fibrils in solution are placed into small tubes (0.2mls) or wells and exposed to the plasma pencil for varying lengths of time and intensity.

After exposure the fibrils are immediately fixed onto transmission electron microscopy grids for analysis of breakage.

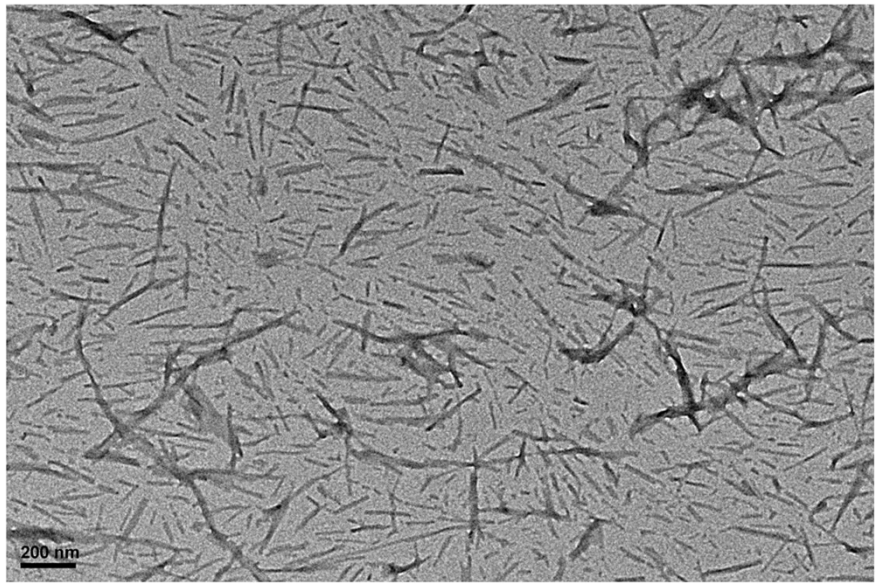
E. Karakas, A. Munyanyi, L. Greene, and M. Laroussi, "Destruction of a-Synuclein Based Amyloid Fibrils by a Low Temperature Plasma Jet", *Appl. Phys. Lett.* **97**, 143702 (2010).

Destruction of Amyloid Fibrils with Low Temperature Plasma

M. Laroussi - ODU



Mature intact alpha-synuclein fibrils

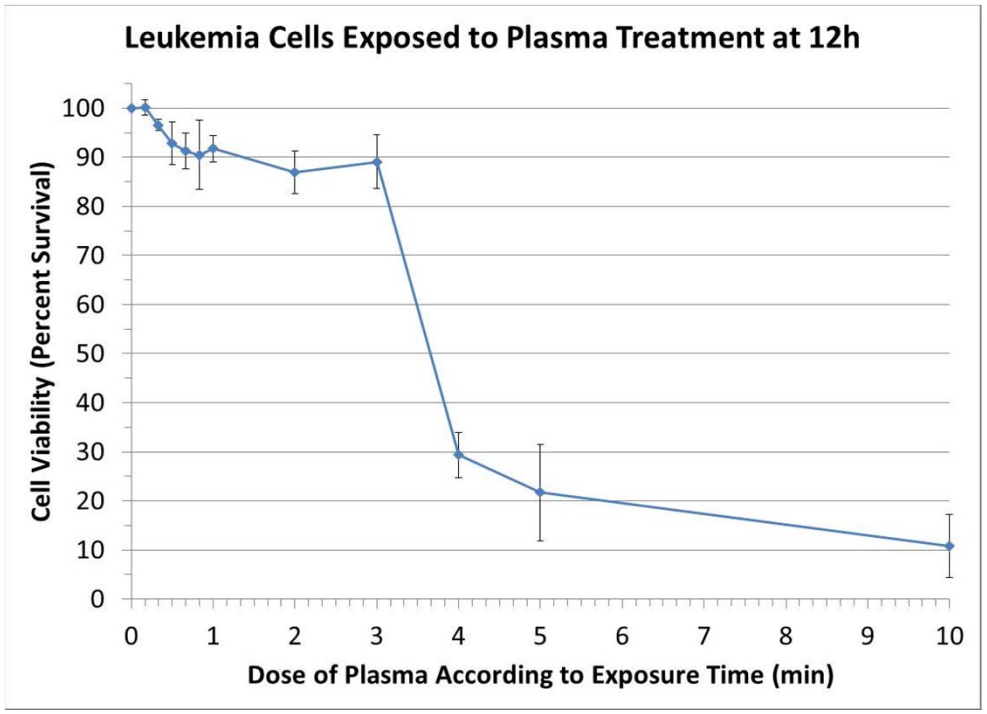


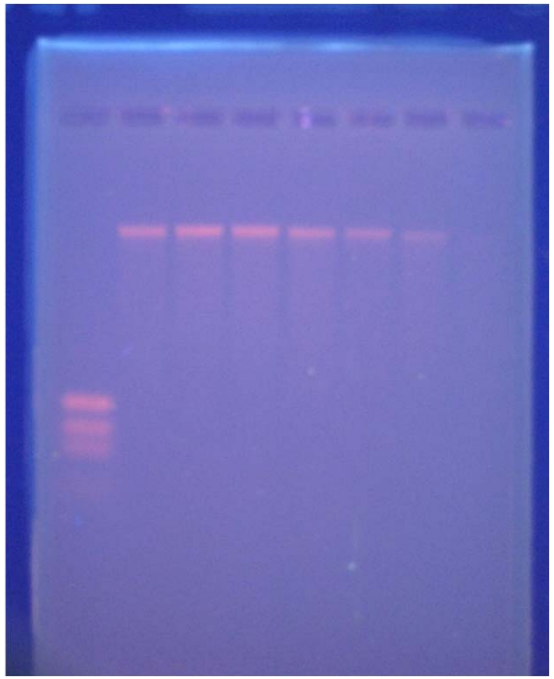
Exposure of fibrils to Helium with plasma for **6 minutes**

E. Karakas, A. Munyanyi, L. Greene, and M. Laroussi, "Destruction of a-Synuclein Based Amyloid Fibrils by a Low Temperature Plasma Jet", *Appl. Phys. Lett.* **97**, 143702 (2010).

KILLING OF CANCER CELLS

CCRF-CEM human T-cell leukemia cells

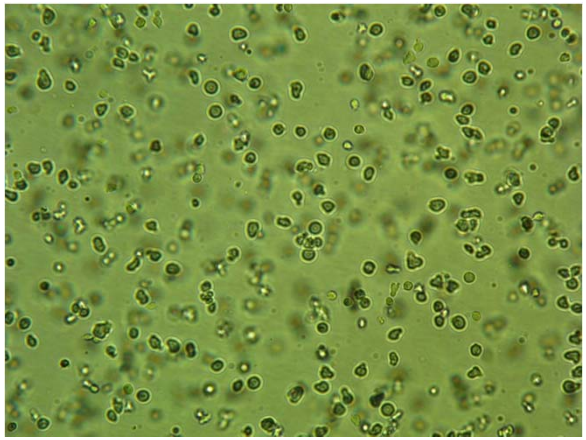




Plasma Pencil parameters: He = feed gas (flow rate at 5 L/min); 7.5 kV; 5 kHz; pulse width = 500 ns.

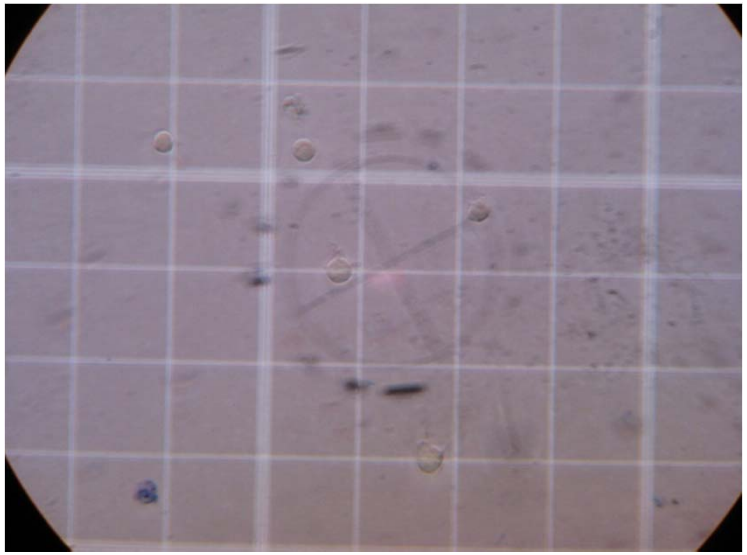
N. Barekzi and M. Laroussi,. J. Phys. D: Appl. Phys. 45 (2012) 422002.

Trypan Blue exclusion assay

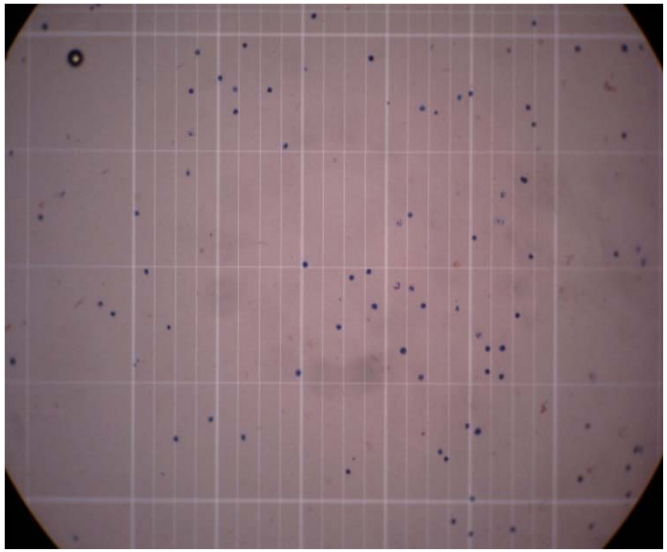


Untreated
RPMI-1640 media

2 minutes



10 minutes

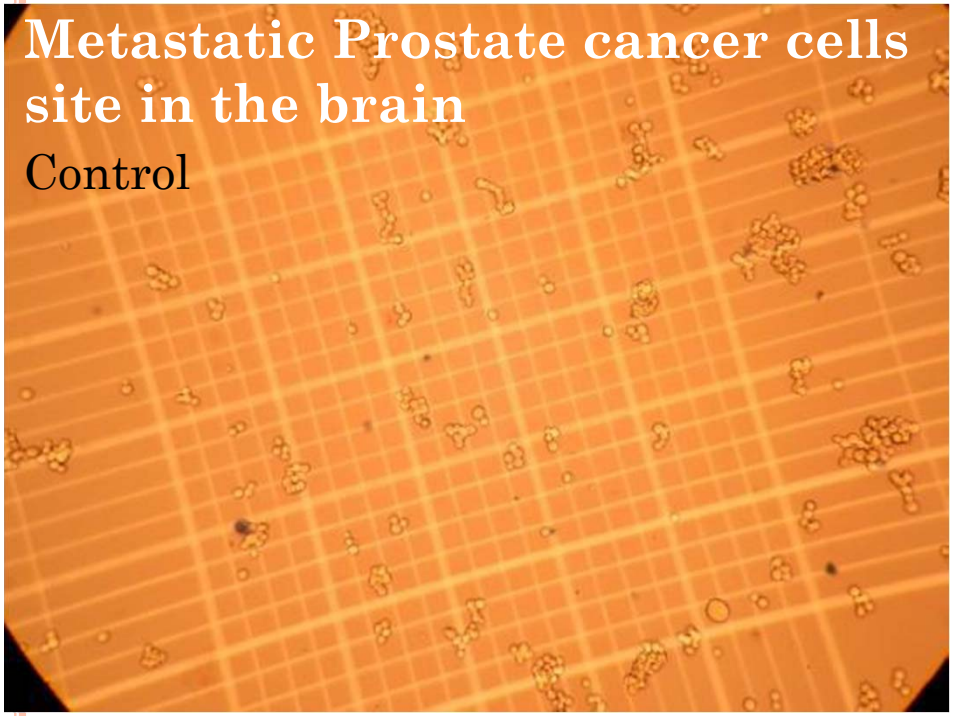


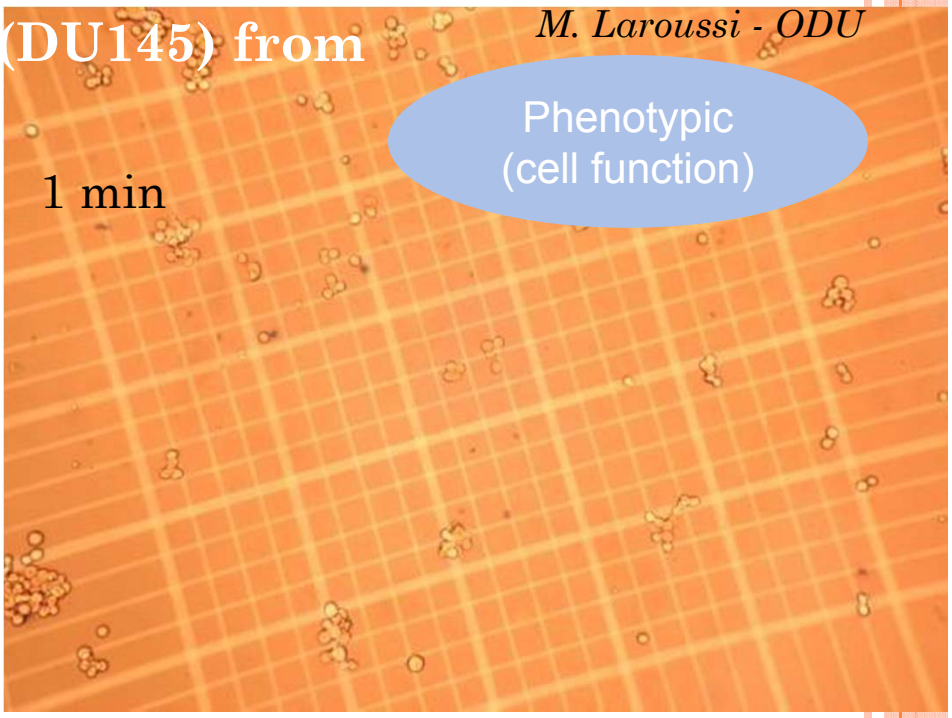
N. Barekzi and M. Laroussi,. J. Phys. D: Appl. Phys. 45 (2012) 422002.

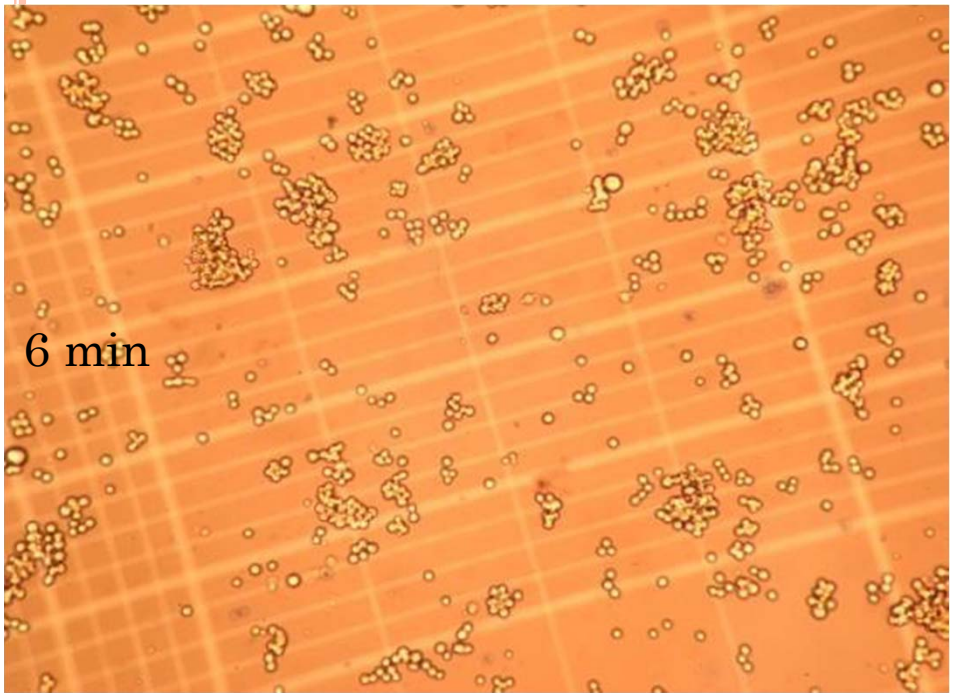
Metastatic Prostate cancer cells (DU145) from site in the brain

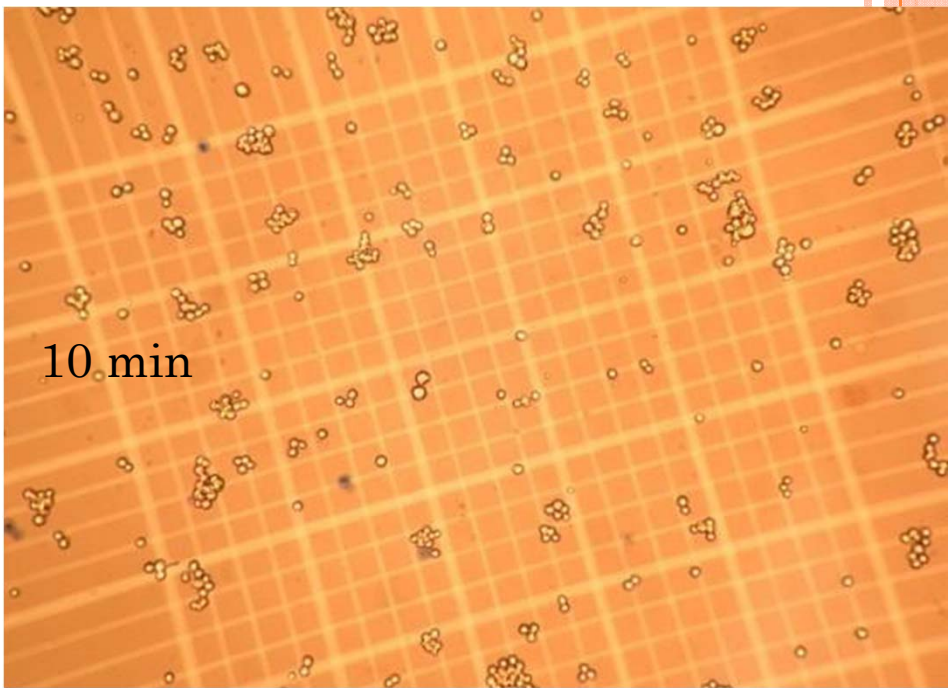
M. Laroussi - ODU

Phenotypic
(cell function)



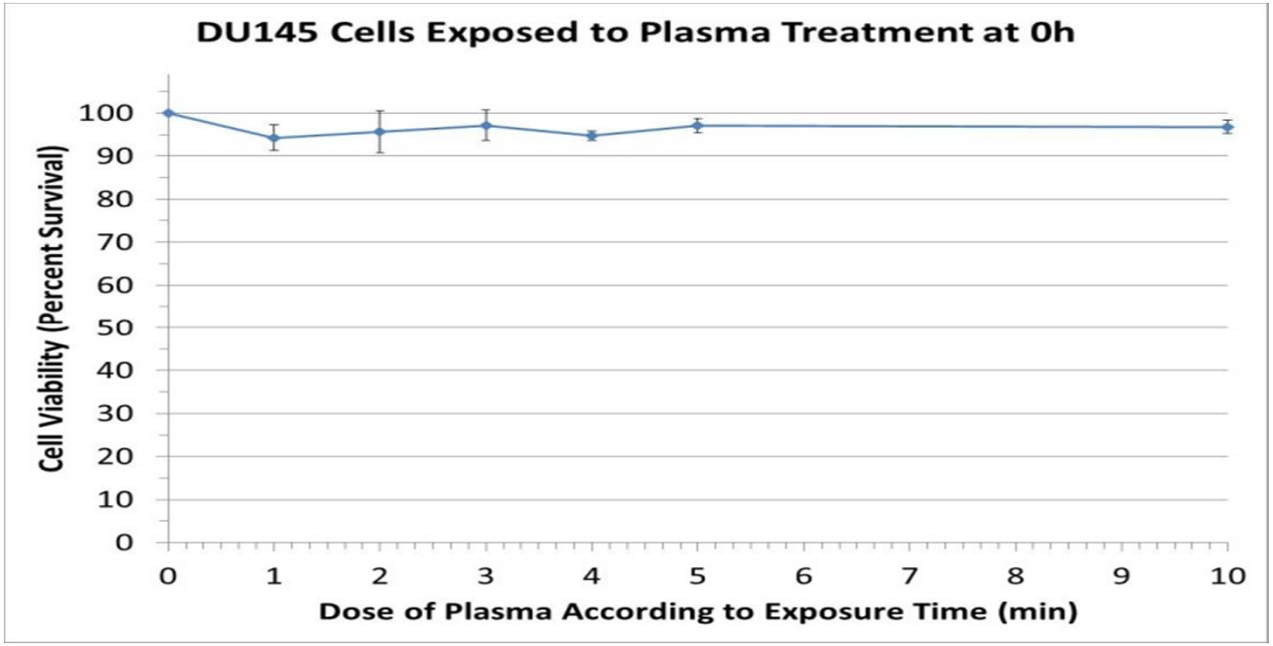




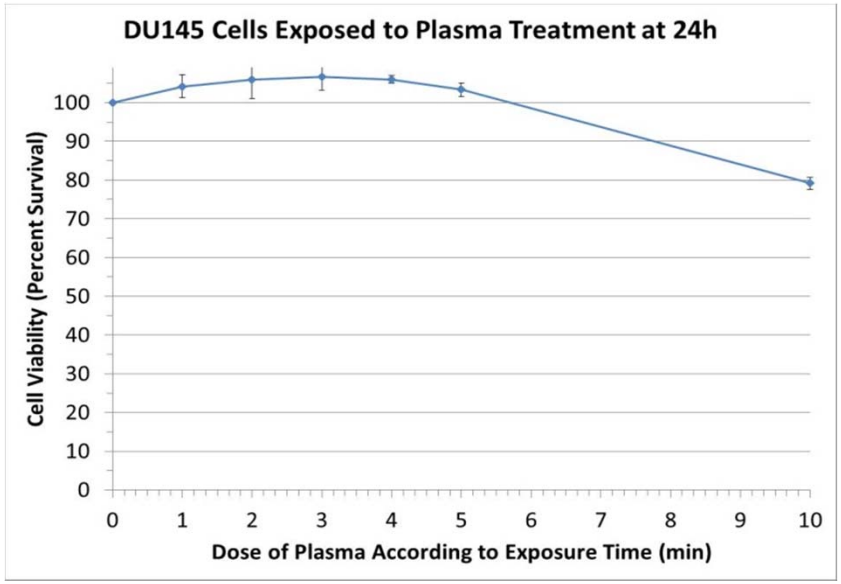


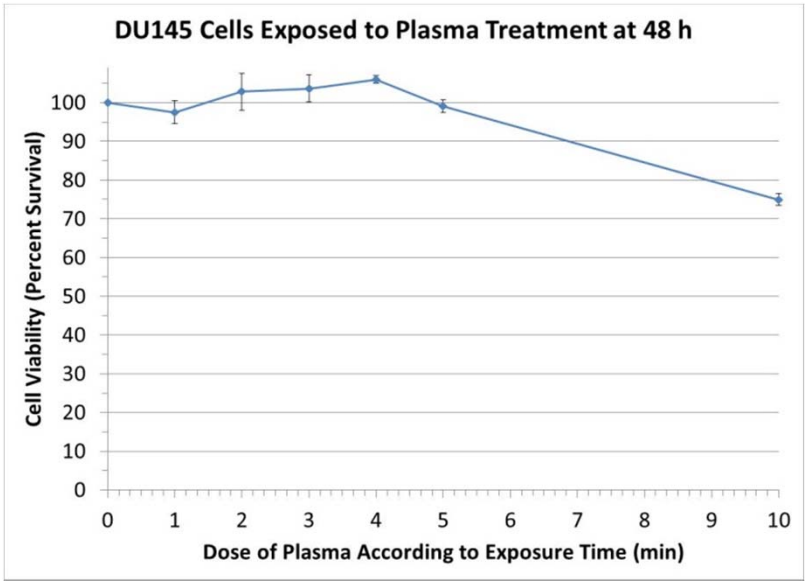
Treatment time less than 10 minutes. 0 hour post treatment

He = feed gas (flow rate at 5 L/min); 7.5 kV; f = 5 kHz; pulse width of 500 ns.

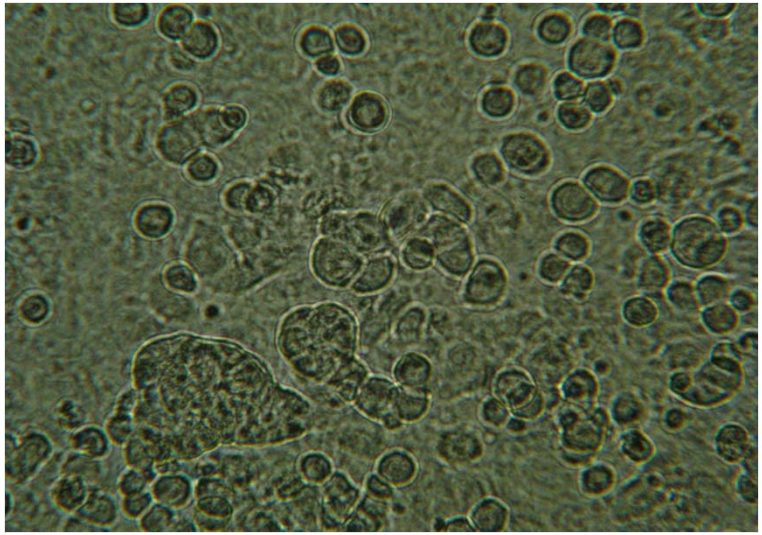


24 hours and 48 hours post treatment; Treatment time = 10 minute

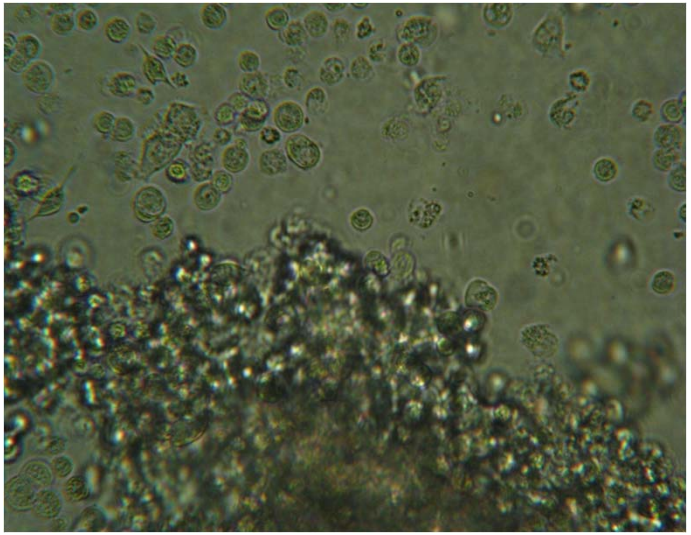




Cell viability determined by Trypan Blue assay.



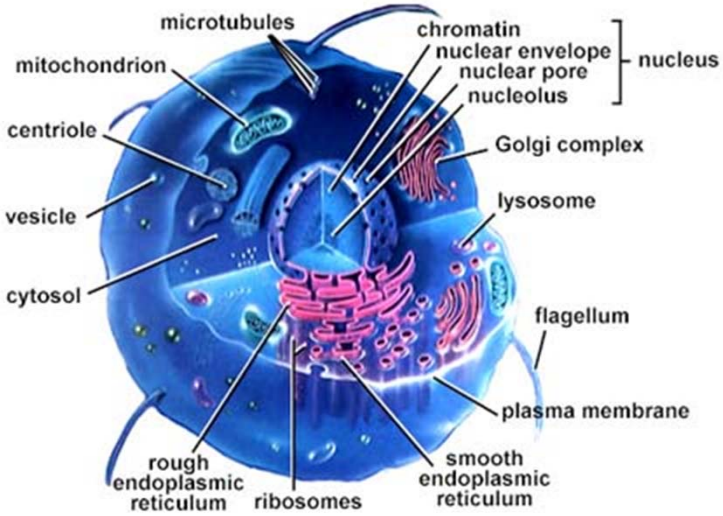
Untreated DU 145 cells



DU 145 cells after 10 minutes plasma exposure. 24 h post treatment.

EFFECTS ON MAMMALIAN CELLS

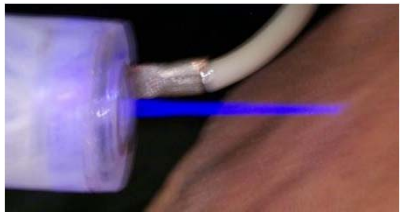
(NON-CANCEROUS)



Experimental design using mammalian cells

- **Grow mammalian cells *in vitro***
  - **Using media in vented flasks**
  - **37°C containing 5% CO_2**

- **Treat cells with Low Temperature Plasma**
  - **Plasma pencil**



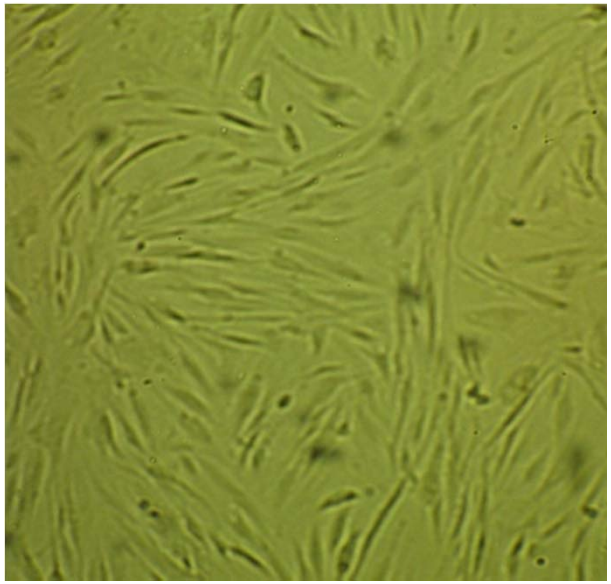
- **Determine morphological changes**

- **Determine cell viability**
  - **Trypan blue exclusion assay**

- **Plot results**

Eukaryotic cell morphology

XPV fibroblasts



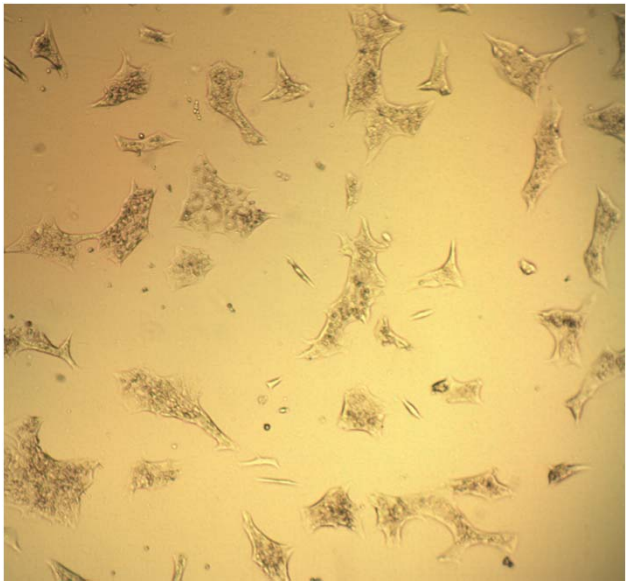
Xeroderma pigmentosum variant fibroblasts

- **XPV fibroblasts (XP30RO)**
- **Adherent parallel clusters**
- **Speckled nucleus**
- **Branched cytoplasm**

Growth Media:

- **Eagle's MEM, 10% FBS, 1% non-essential amino acids, 1% Pen/Strep, 1% glutamine**

Epithelial cells



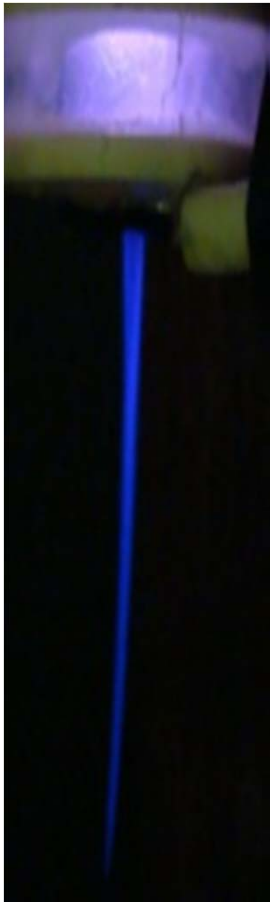
Adherent epithelial cells

- **CCL-22 (ATCC)**
- **Cuboidal**
- **Central nucleous**
- **Isolated from steer kidney**

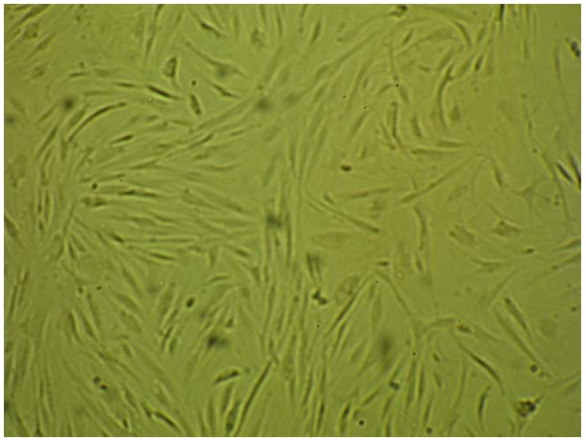
Growth Media:

- **Eagle's MEM, 10% Horse serum, 1% Pen/Strep, and 1% glutamine**

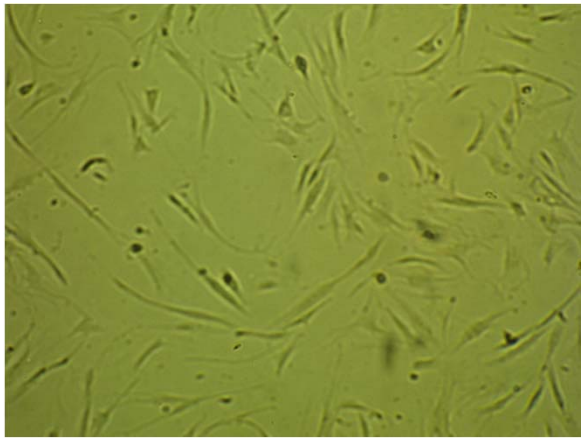
EFFECTS ON FIBROBLAST CELLS



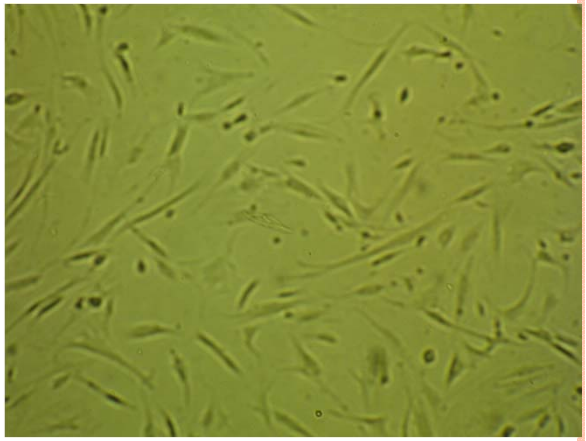
XPV Fibroblasts: Xeroderma pimentosum variant fibroblasts (XP30RO)



Untreated in EMEM media



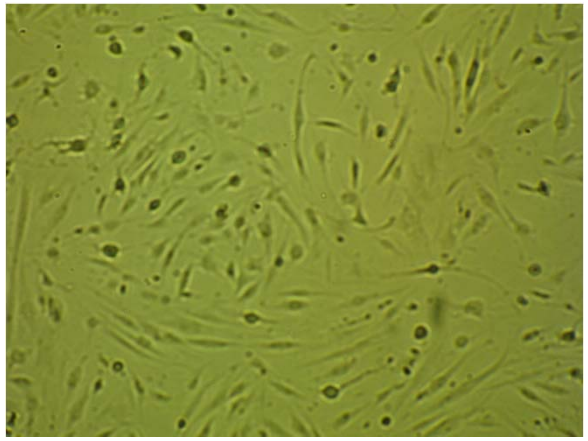
10 seconds, 12 h post exposure



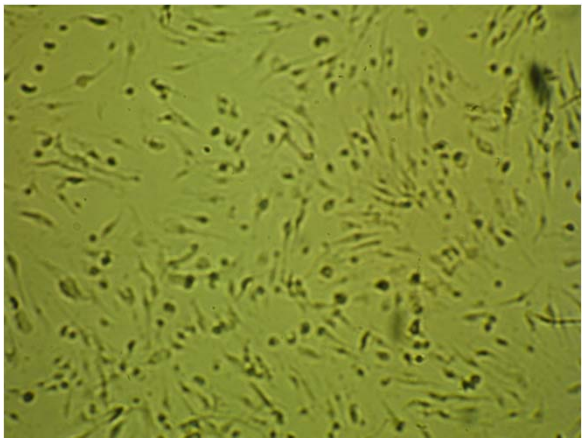
30 seconds, 12 h post exposure

Up to 30 seconds plasma exposure appear not to cause damage to the cells

XPV Fibroblasts



1 minute, 12 h post exposure



2 minutes, 12 h post exposure

Plasma exposure 1 minute or greater leads to significant kill

EFFECTS ON EPITHELIAL CELLS

CCL-22 adherent epithelial cells controls

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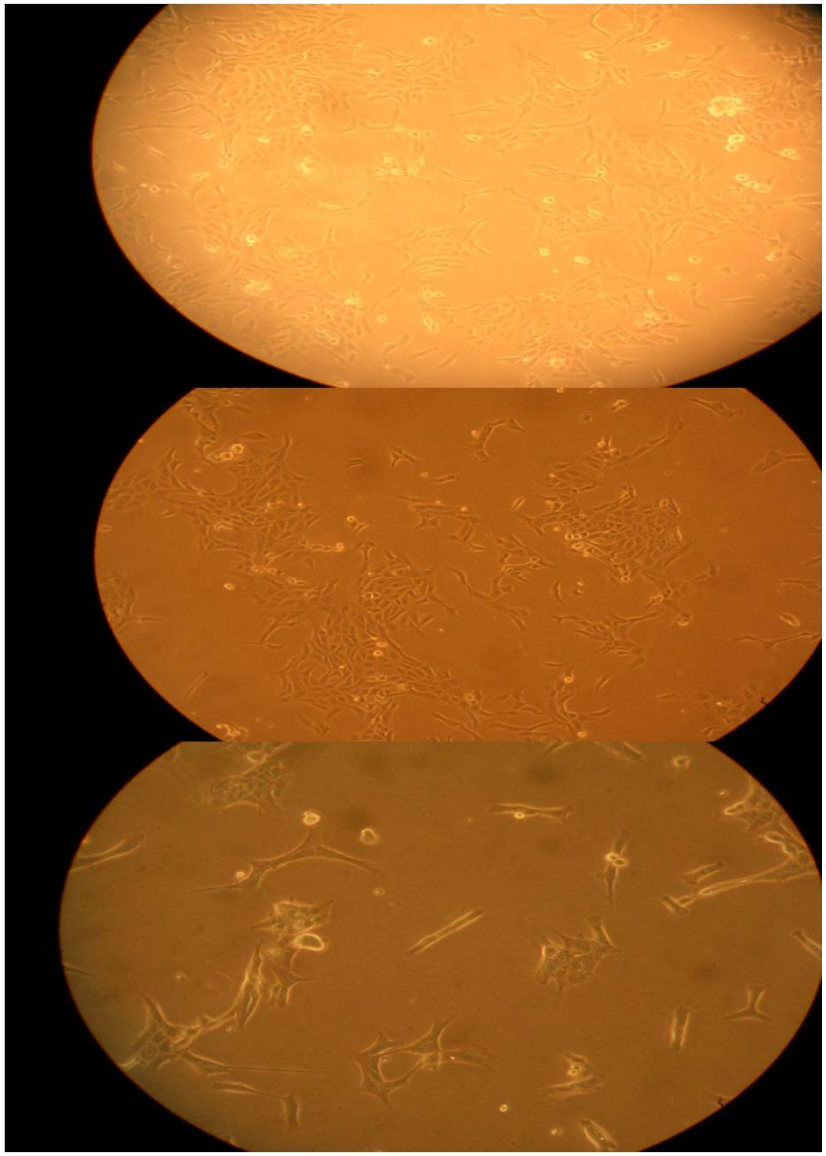


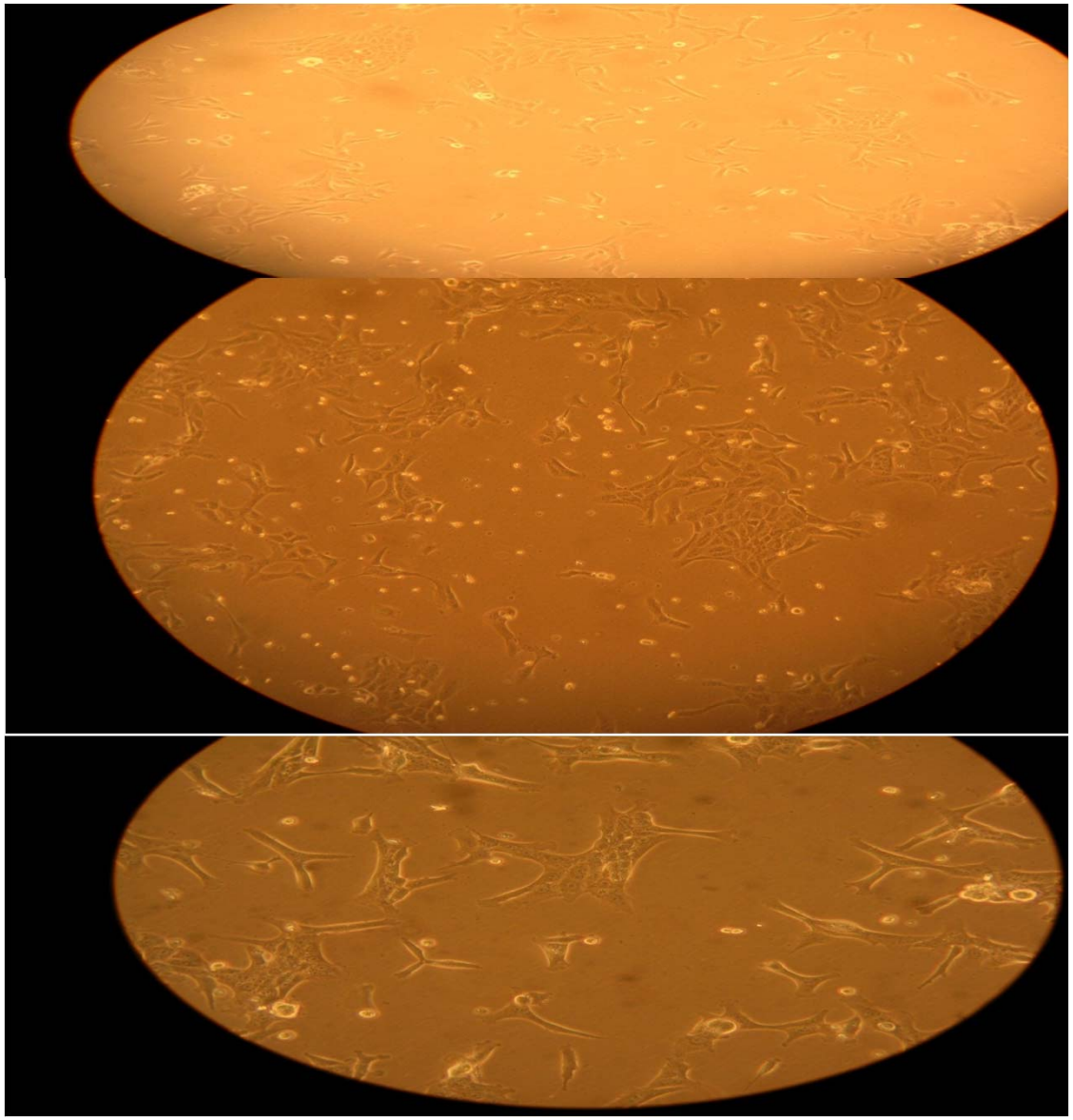
4 h

8 h

24 h

M. Laroussi - ODU

CCL-22 adherent epithelial cells

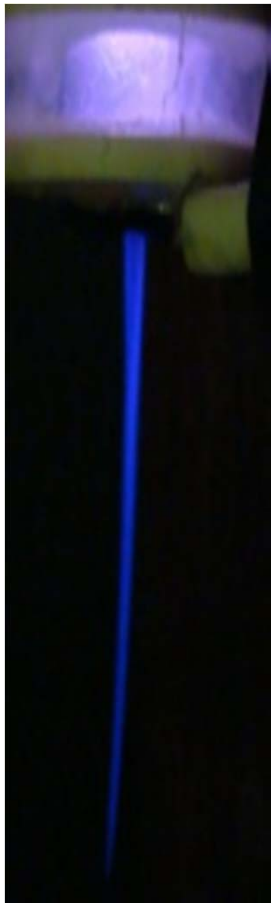


4 h

8 h

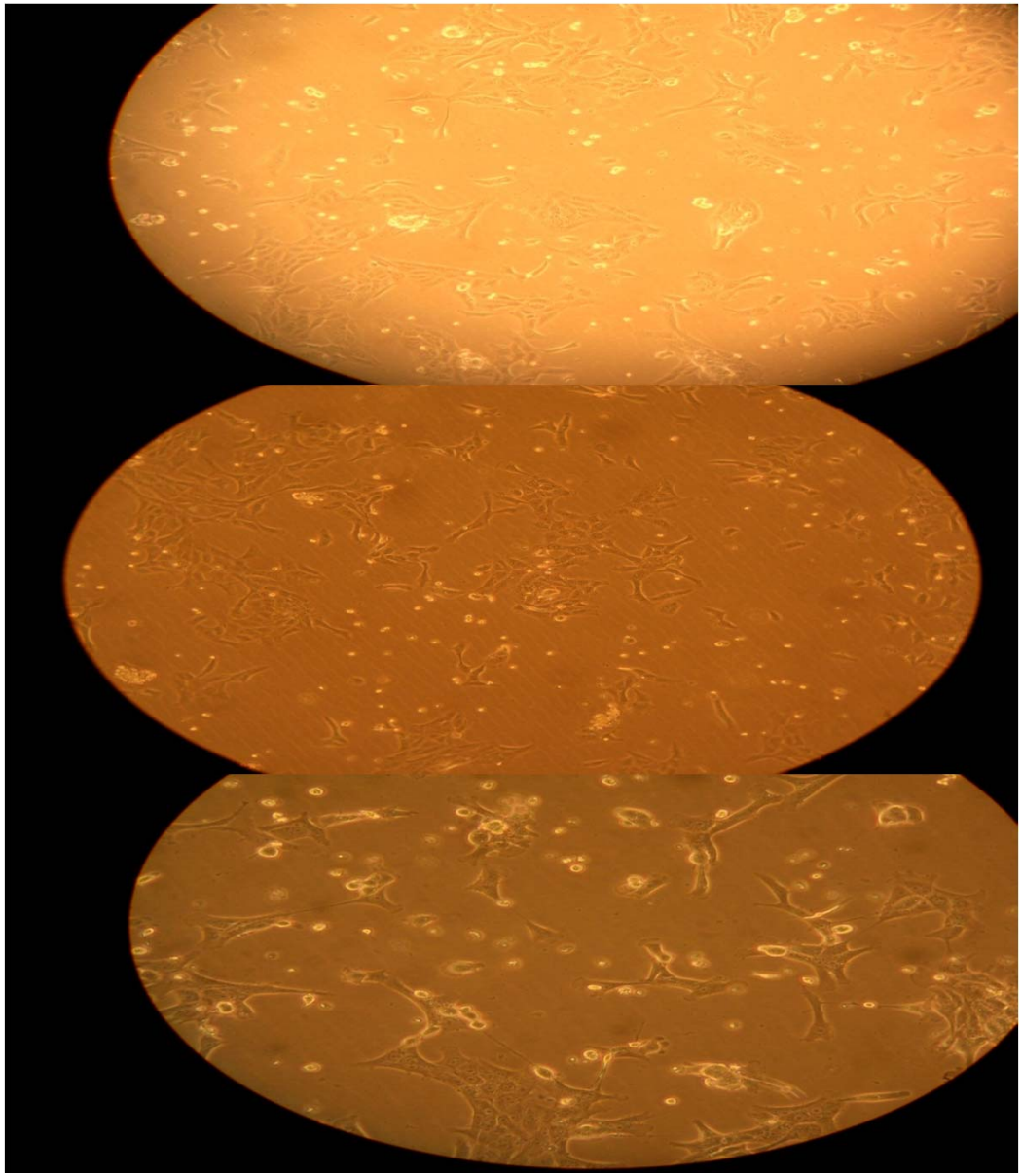
24 h

30 s plasma



M. Laroussi - ODU

CCL-22 adherent epithelial cells

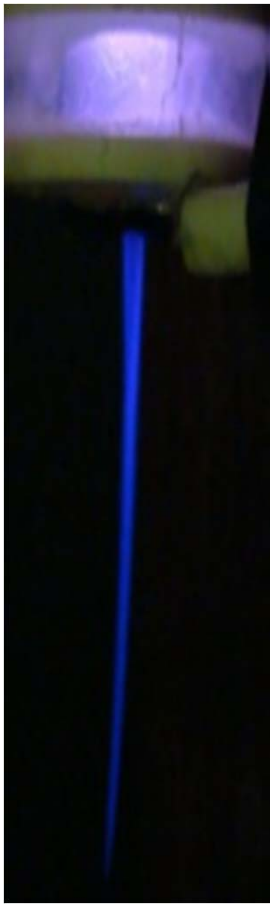


4 h

8 h

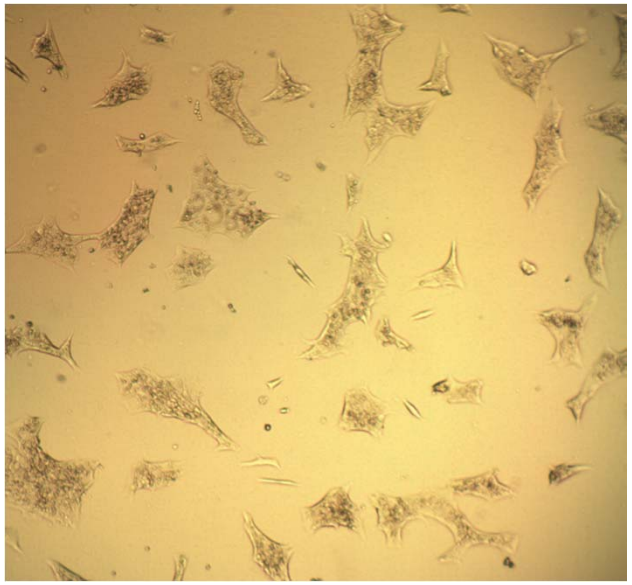
24 h

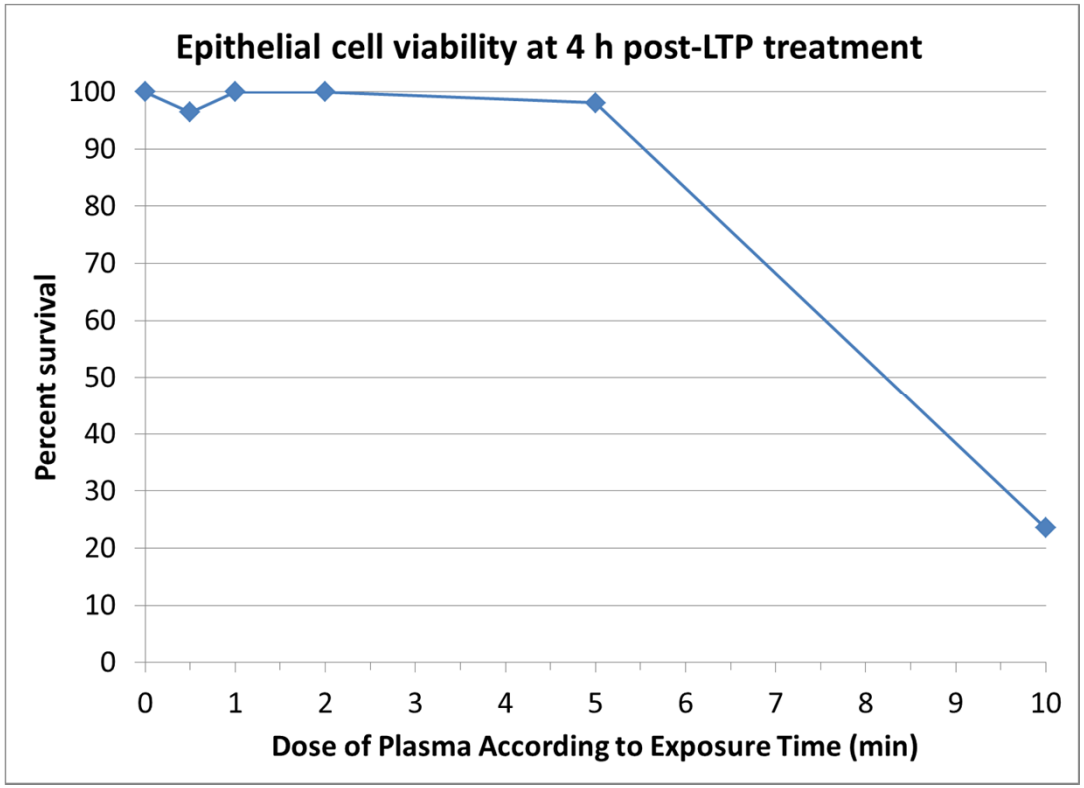
1 minute plasma



Epithelial cells treated and assayed at 4 h post-LTP

Epithelial cells

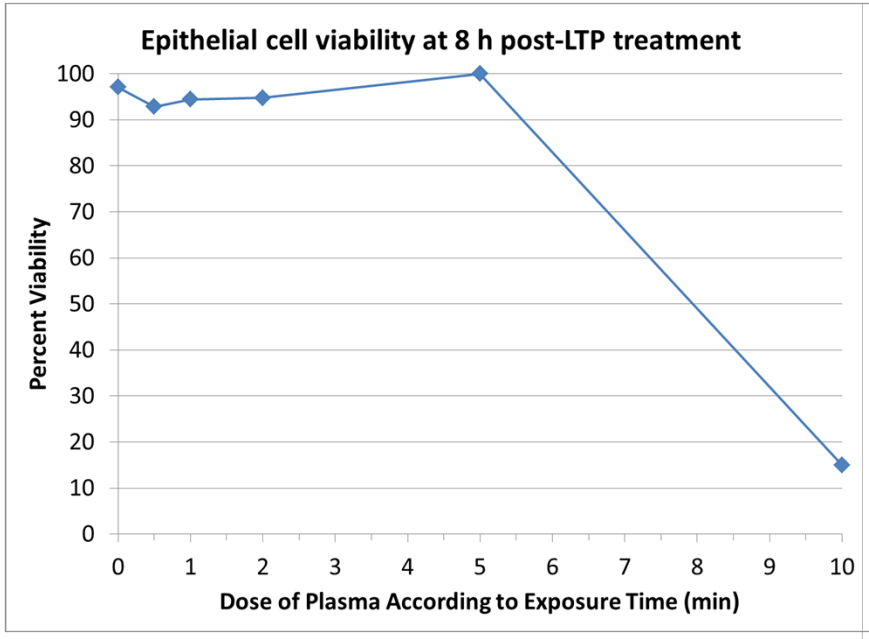


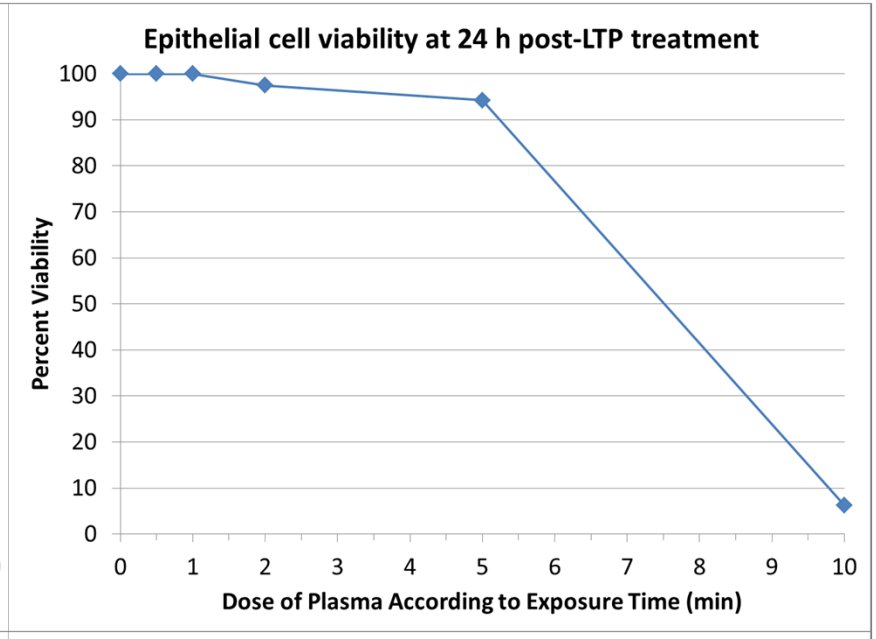


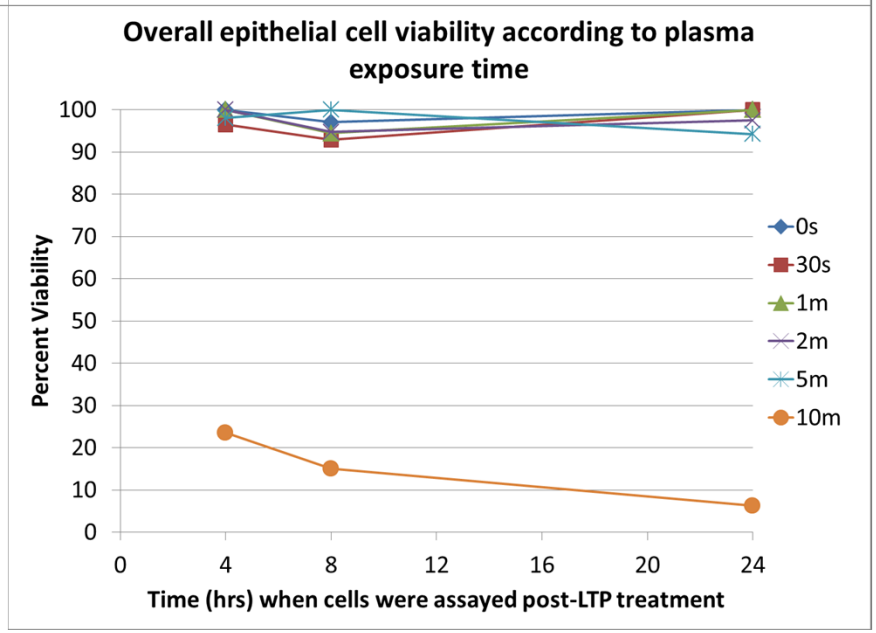
Gas flow: He
HV: 7.5kV
Freq: 5kHz
Pulse width: 500ns
Treatment: 0, 30 sec, 1, 2, 5 and 10 min.

Significant cell death evident with a single 10 min plasma exposure at 4 h.

Epithelial cells assayed at 8 and 24 h post-LTP



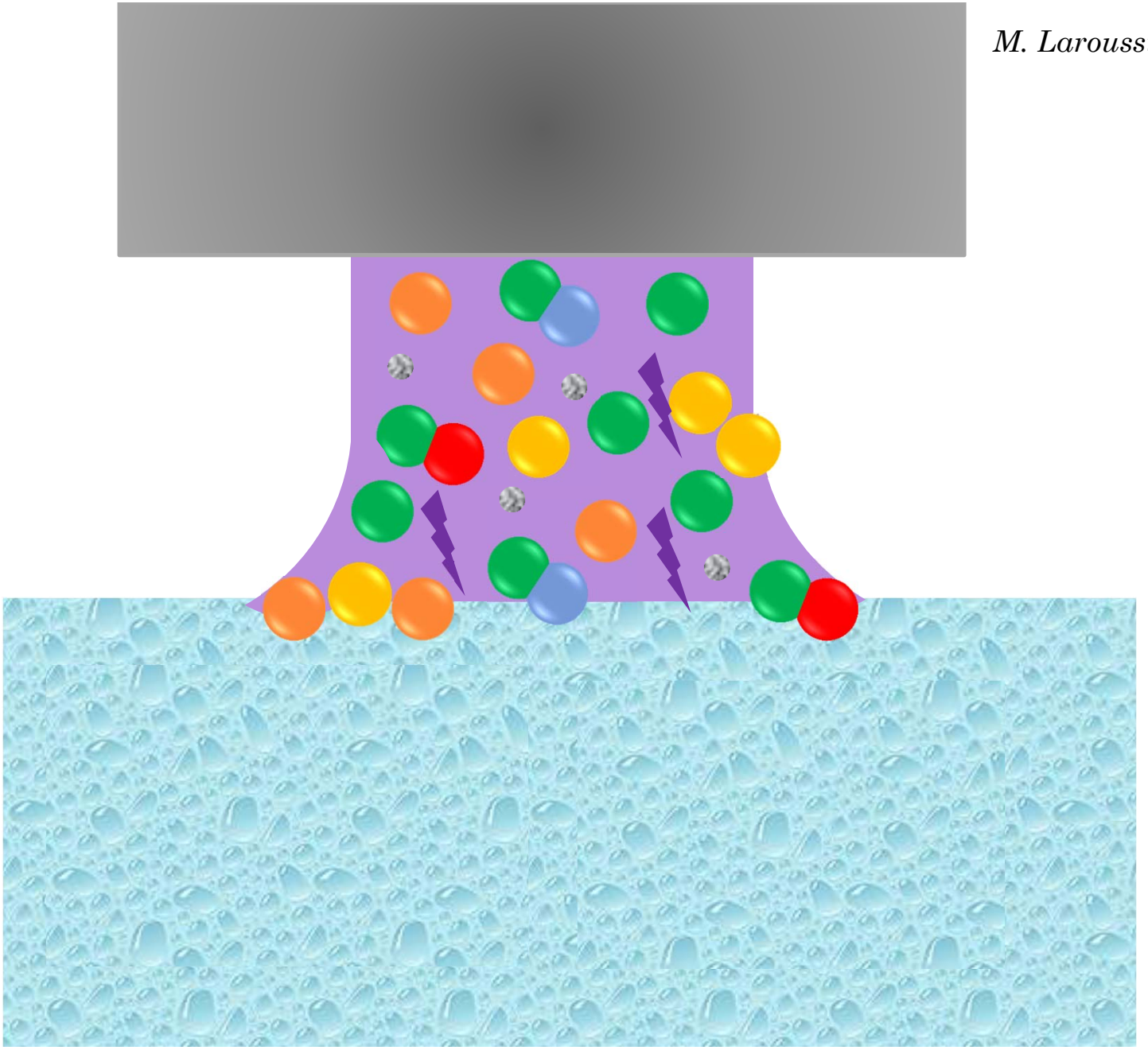


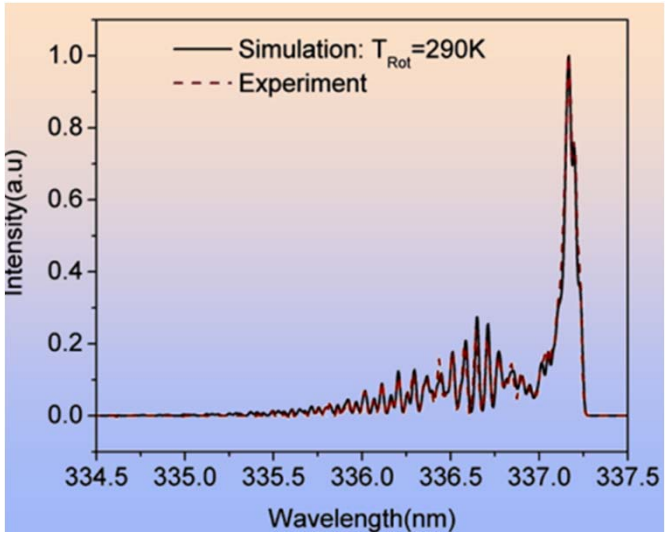


The overall cell viability according to the plasma exposure time revealed that the 10 min dose was the most harmful to epithelial cells.

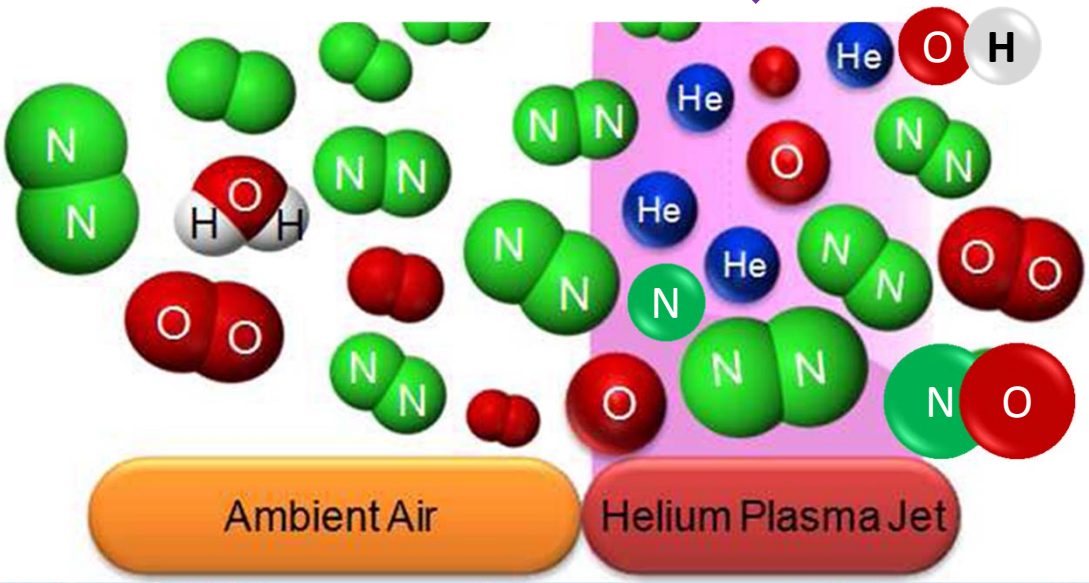
Plasma – Cell Interaction: The burning questions

Cells are surrounded by fluids (media):
Plasma-liquid interface?
Plasma-generated reactive species in liquids –
Identification and measurements of the fluxes?
Detailed mechanisms of interaction of ROS and RNS with cells?
Intracellular ROS generation via plasma exposure?
Cell signaling pathways initiated by plasma exposure?
Selectivity of plasma effects? prokaryotes vs. eukaryotes
Cytotoxicity?

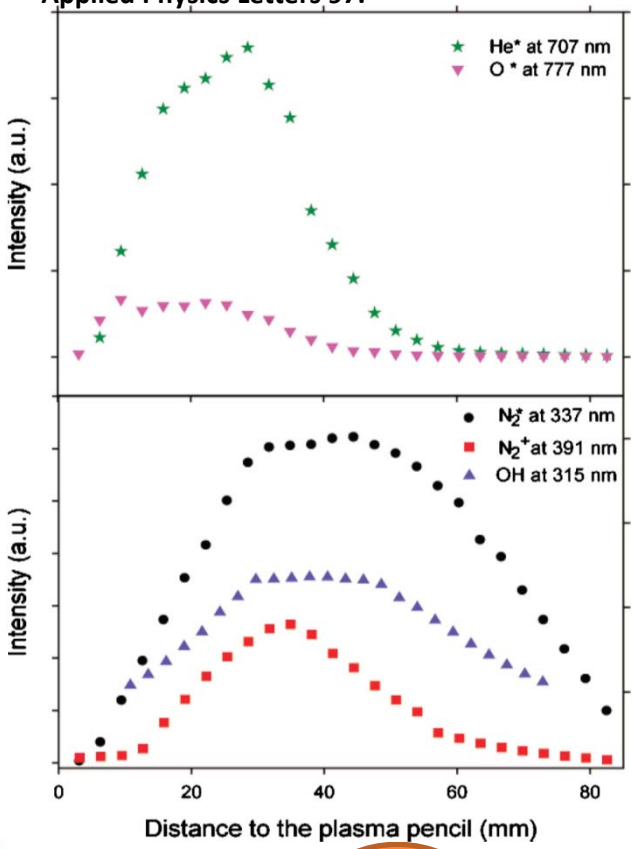




Laroussi and Lu. 2005 Applied Physics Letters 87.



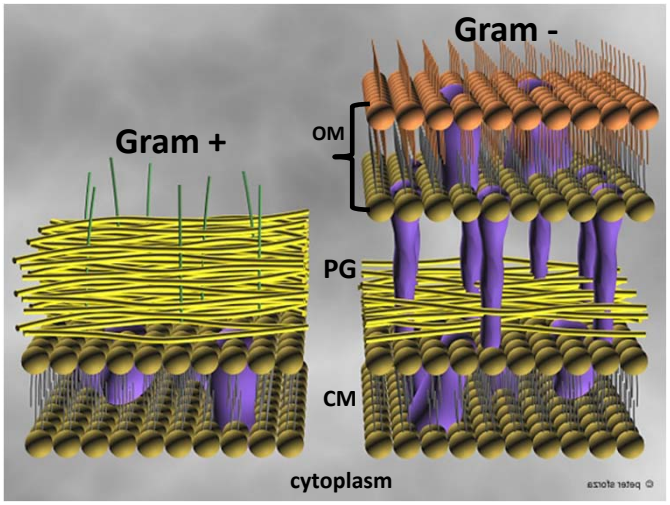
Karakas, Munyanyi, Greene, and Laroussi. 2010 Applied Physics Letters 97.

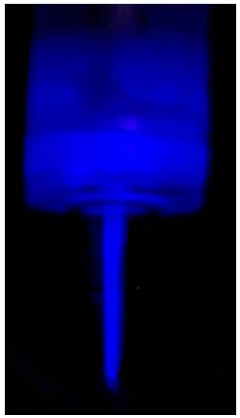


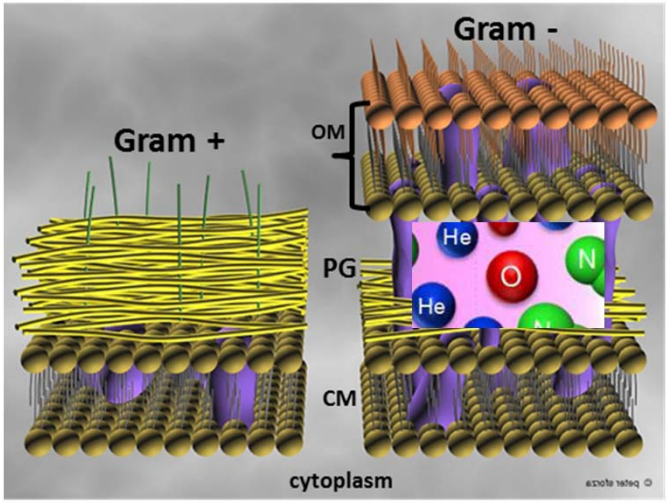
ROS
RNS

- **Cell morphology**
- **Cell signaling**
- **Cancer**

What is happening at the molecular level?



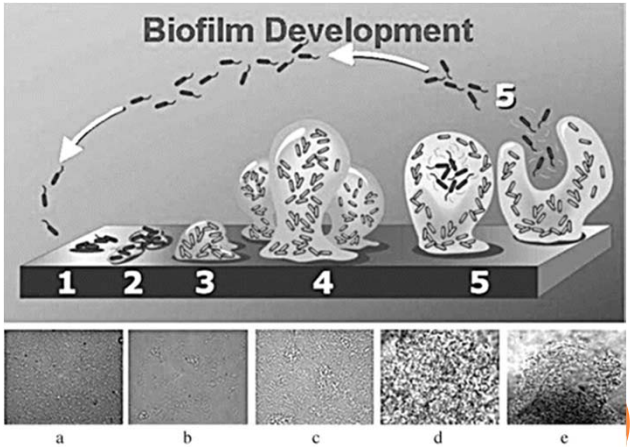




Studies in progress:

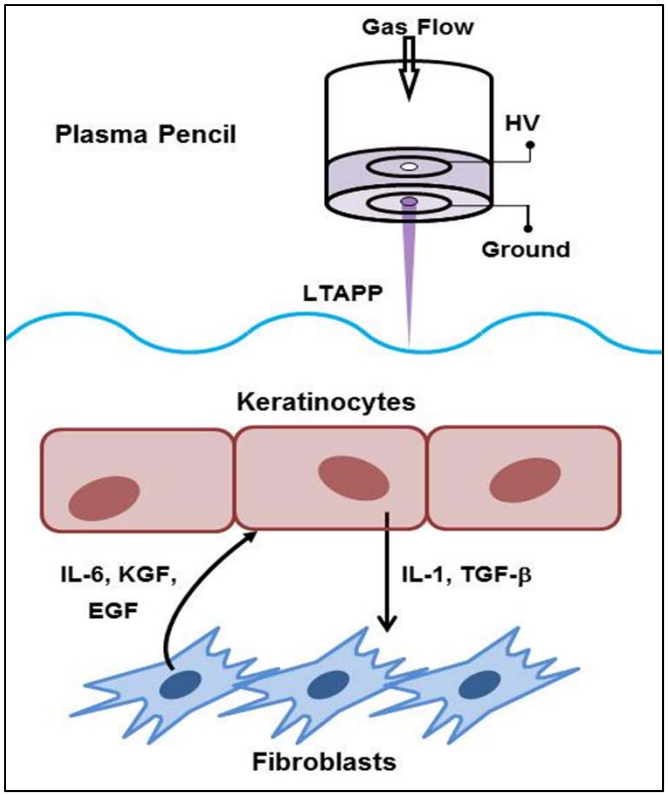
- membrane proteins
- metabolic signals
- molecular signals
- Sigma factors
- Quorum sensing
- Euk. Cell Invasion
- Biofilm Development

Stoodley, Sauer, Davies, and Costerton. 2002 Annual Rev. of Microbiology



Developing New Experimental Assays

Effect of LTP on eukaryotic signals



A generalized keratinocyte-fibroblast tissue culture model

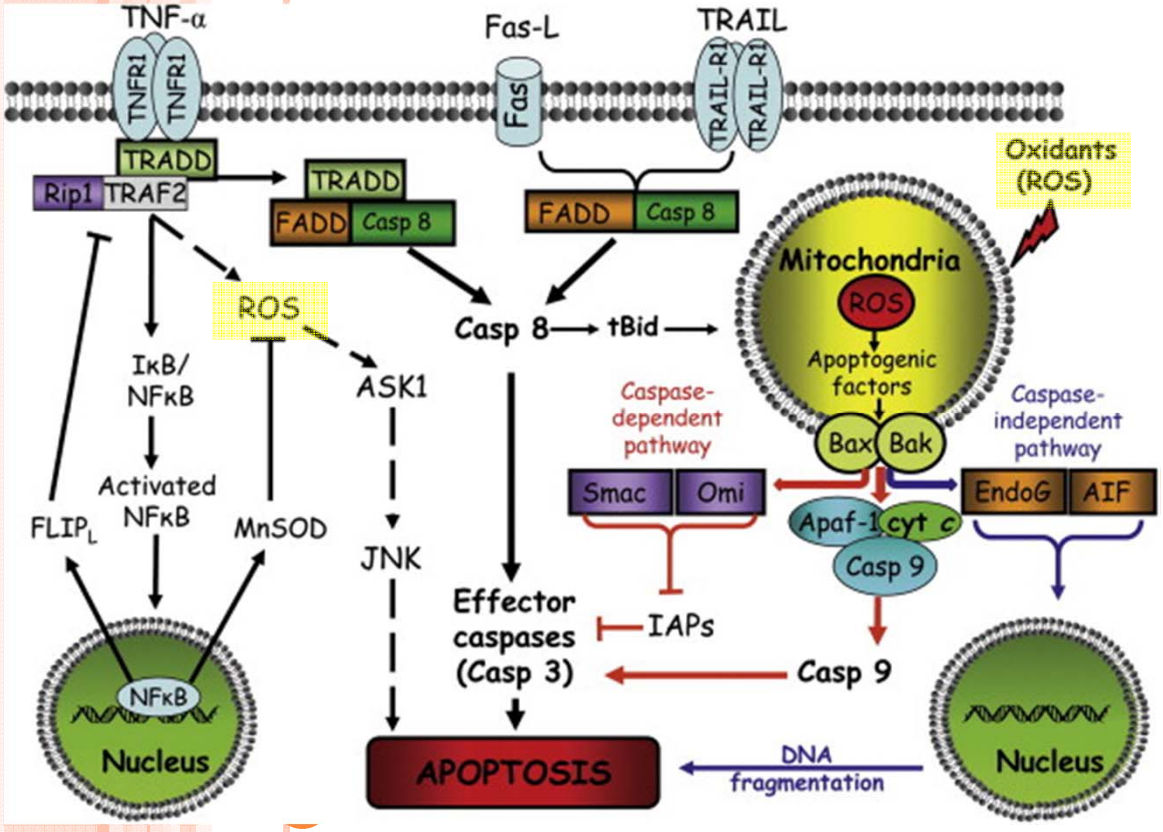
- Application of low temperature atmospheric pressure plasma (LTAPP)
- With and without trauma to cells (scratch assay)
- Detect cytokines and signals
  - IL-1 and TGF-β in keratinocytes
  - IL-6, KGF, and EGF in fibroblasts
  - Membrane proteins (integrins, cadherin)
- Assays
  - Proliferation, morphology, differentiation, protein expression, DNA integrity
  - Include bacterial contaminants (invasion study using *S. aureus* (MRSA)

Determine the role of ROS and RNS on eukaryotic signals

Do plasma reactive species trigger intracellular signaling?

How are the internal cell signaling pathways normally modulated by iROS affected by RONS generated by plasma?

Circu, M. L. and T. Y. Aw (2010). "Reactive oxygen species, cellular redox systems, and apoptosis." Free Radical Biology and Medicine 48(6): 749-762.



Extrinsic Pathway

Intrinsic Pathway

Plasma effects:

- DNA fragmentation (gamma-H2AX)
- Caspase 3

Does plasma reactive species alter these targets?

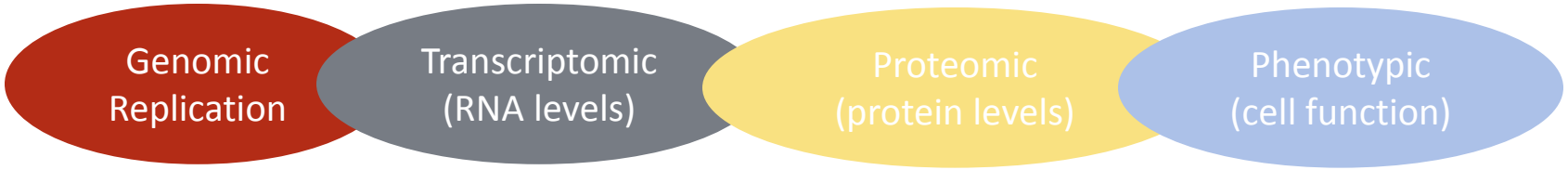
- TNF-α
- NFκB
- Other cytokines

Intracellular eukaryotic signaling after LTP

Molecular Biology: Study of biology that deals with the molecular basis of biological activity

- DNA
- RNA
- protein biosynthesis

Tools: PCR, Blotting, Microarray, Flow cytometry, etc.



Molecular biology approach would be helpful in determining the effects of LTP:

- Effect of ROS/RNS on cells
- Intracellular signaling
- Apoptosis or necrosis
- DNA degradation
- Protein denaturation

Plasma generates reactive species:

- He, O, N based species
- He^m, O_3, O_2^{*-}, NO, NO_2

Do these reactive species signal the same pathways in eukaryotes:

- growth, proliferation, apoptosis, inflammation?

Questions have to be investigated, characterized and resolved empirically!

CONCLUSIONS

- **Low temperature plasmas offer effective and controlled means to inactivate bacteria**
- **Possibility of using plasma jets to treat diseases caused by pathogenic proteins**
- **Killing of cancerous cells**
- **Low doses of plasma have no cytotoxic effects on fibroblasts and epithelial cells**
- **Mechanisms of interaction on the subcellular and molecular levels remain not well understood**

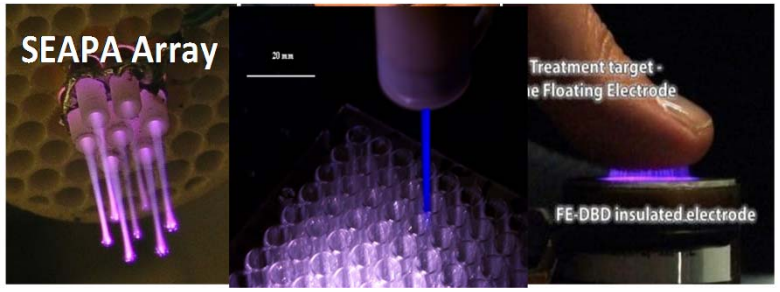
Prospects

M. Laroussi - ODU

Medieval Surgery

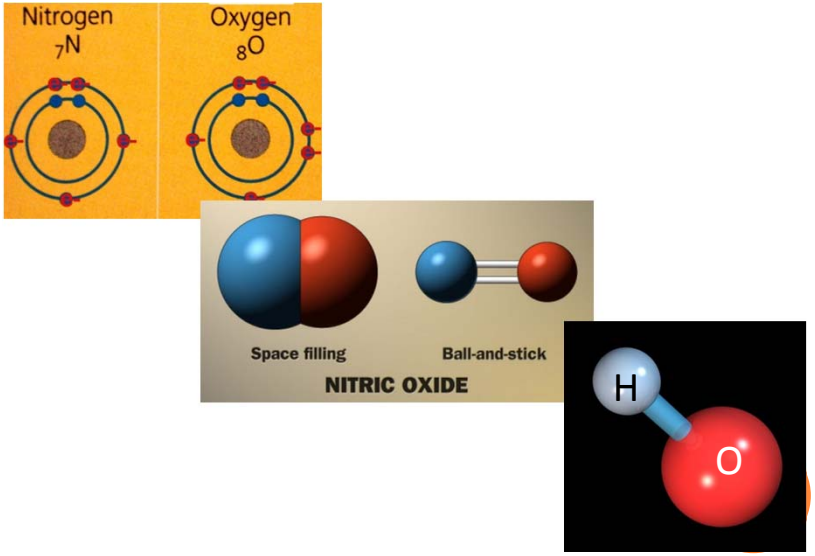
Nature, 2007, 450, 796.

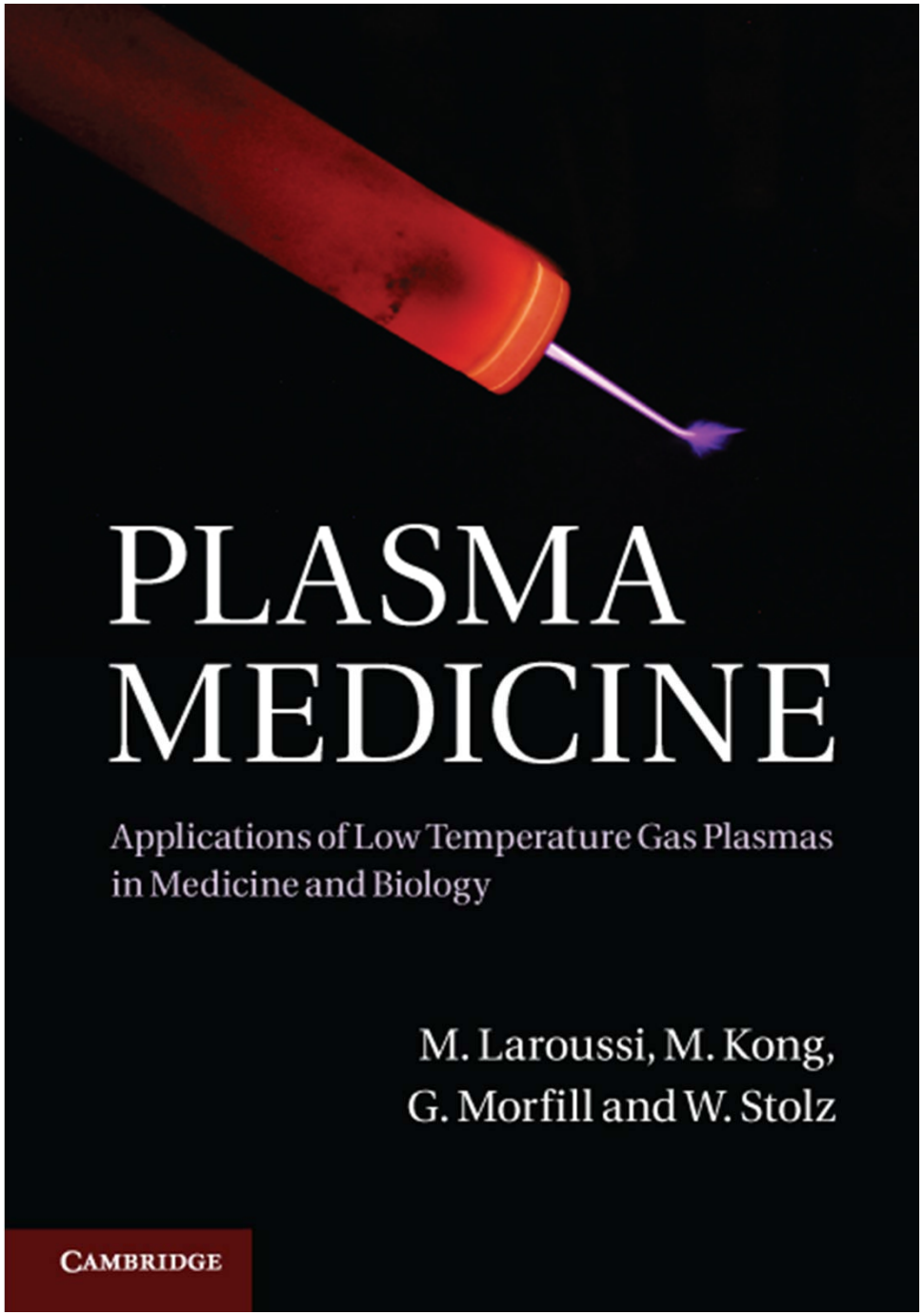
Plasma Technology – Today's toolbox



Plasma Kill → Plasma Heal

Plasma Medicine – future's toolbox





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Questions?