Engineering biosensors and nanocarriers using a novel photonic technology

NanoBiotechnology Group, Ultrafast Biospectroscopy Laser Lab - UBLL
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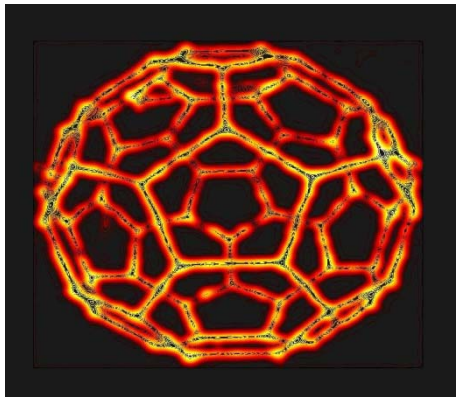
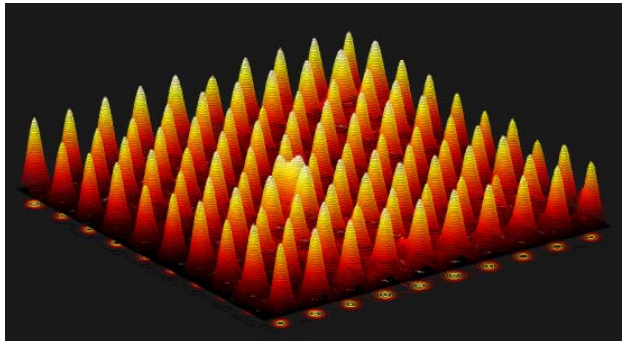
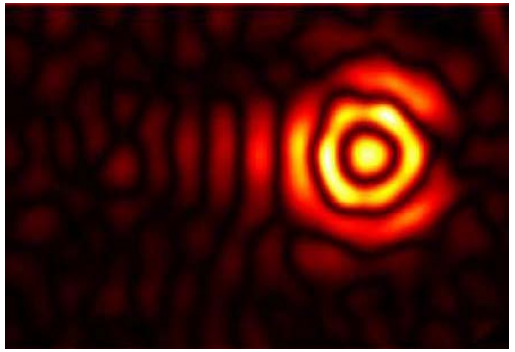
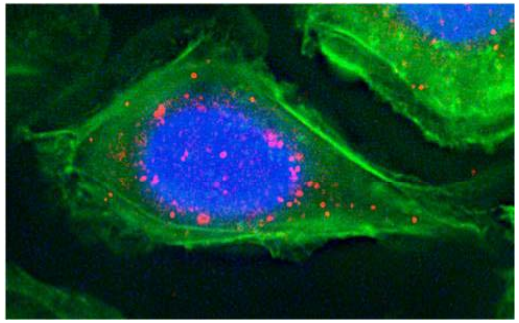








Biophotonics Nanomedicine
BioEngineering Bioimaging
Biophysics Bioinformatics

Key Messages

Bioinformatics leads to the discovery of an highly conserved structural motif in proteins that can be activated by light, leading to a new photonic technology for covalent immobilization of biomolecules,

Light **A**ssisted **M**olecular **I**mmobilization:

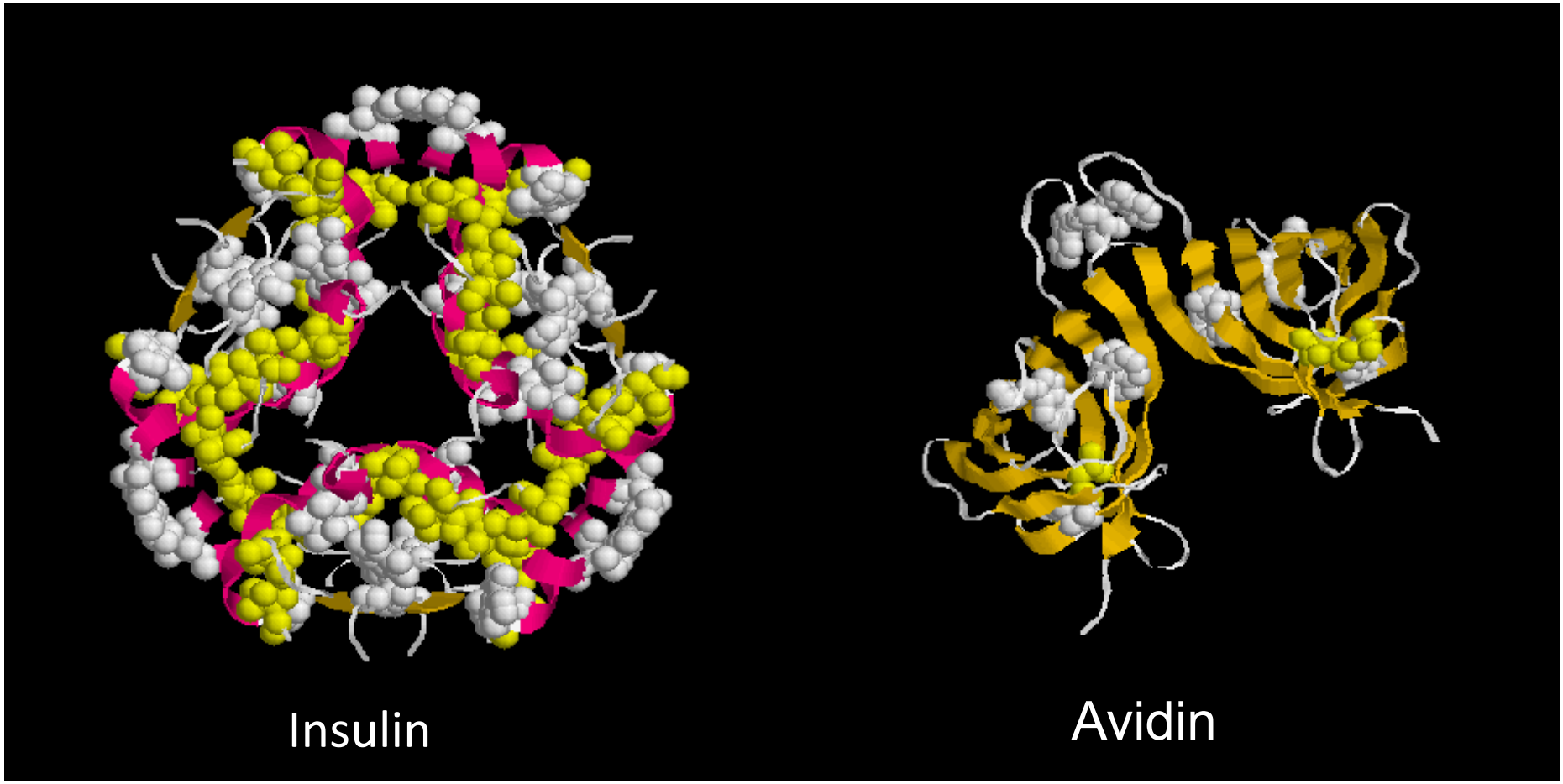
1- Key reaction mechanism induced by light and its time scales

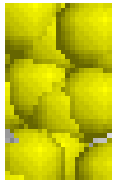
2- Applications:

- Biosensor microarraying
- derivatisation of surfaces with proteins with diffraction limited resolution (sub μm)
- nanoparticle based drug delivery systems

3- New light based cancer therapy – modulating cellular metabolism with light

Nature has evolved decorating proteins with light activated switches: SS-AROM in close spatial proximity

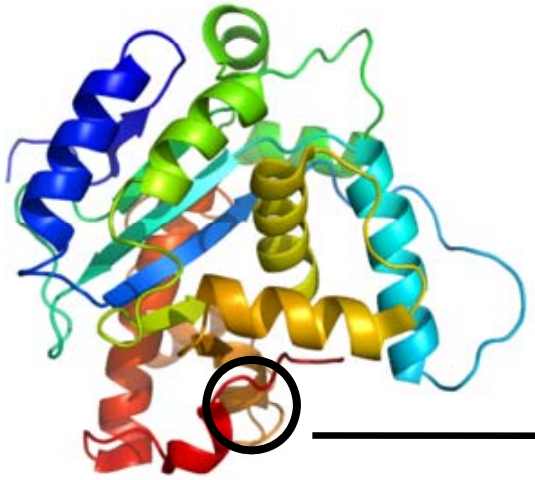




SS-Disulphide Bridges

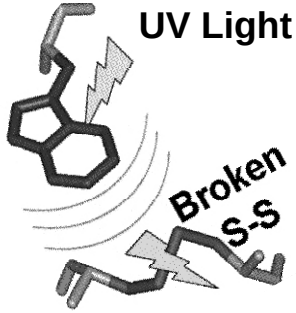
Aromatic residues (Trp, Try, Phe)

Light Induced Reaction

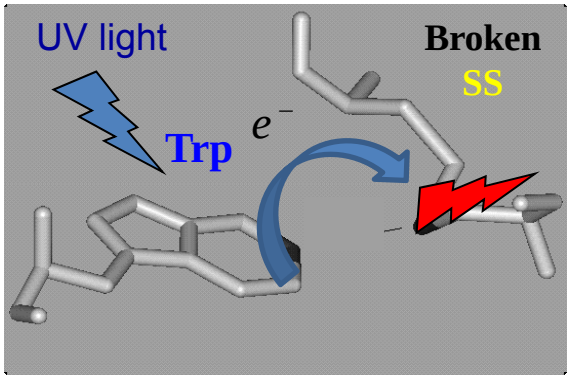


270-295nm

Free **SH** groups

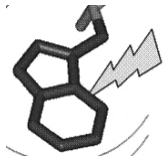


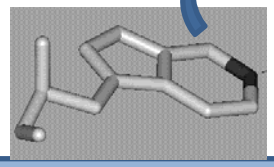
Reaction mechanism Time Scales



Formation of Solvated Electron ~12 ns after excitation

UV excitation





RSSR$^{-\cdot}$ lifetime 300-500μs

^{3}Trp lifetime 12μs

10^{-15} 10^{-12} 10^{-9} 10^{-6} 10^{-3}

Femto **Pico** **Nano** **Micro** **Milli**

Photon absorption

Vibrational relaxation

e^-_{aq} lifetime 300ns-2μs

Trp$^{\cdot}$ lifetime 200-400μs

Trp$^{\cdot+}$ lifetime 80μs

Internal convertion & Intersystem crossing

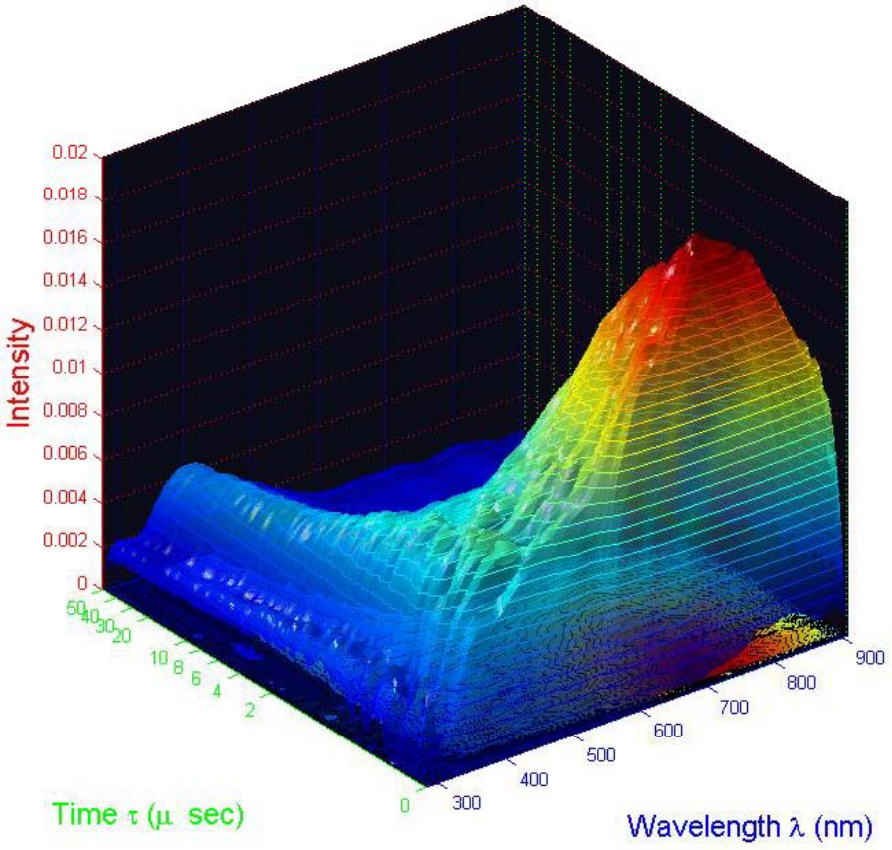
Neves-Petersen et al., Biophysical Journal 2009, Vol. 97, Iss. 1; pg. 211

Neves-Petersen et al., Biophysical Journal Volume 97 July 2009 211–226

$$Trp + h\nu \rightarrow Trp^{\bullet+} + e^-_{aq}$$ 710nm

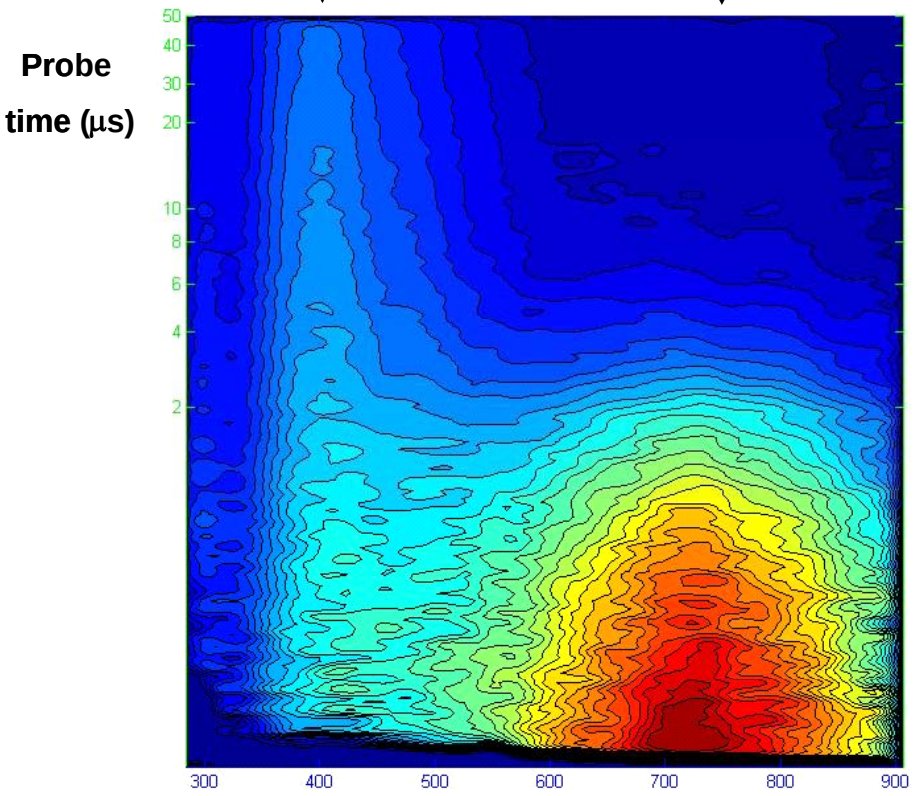
$$e^-_{aq} + RSSR \rightarrow RSSR^{\bullet-}$$ **410nm**

Flash Photolysis Studies

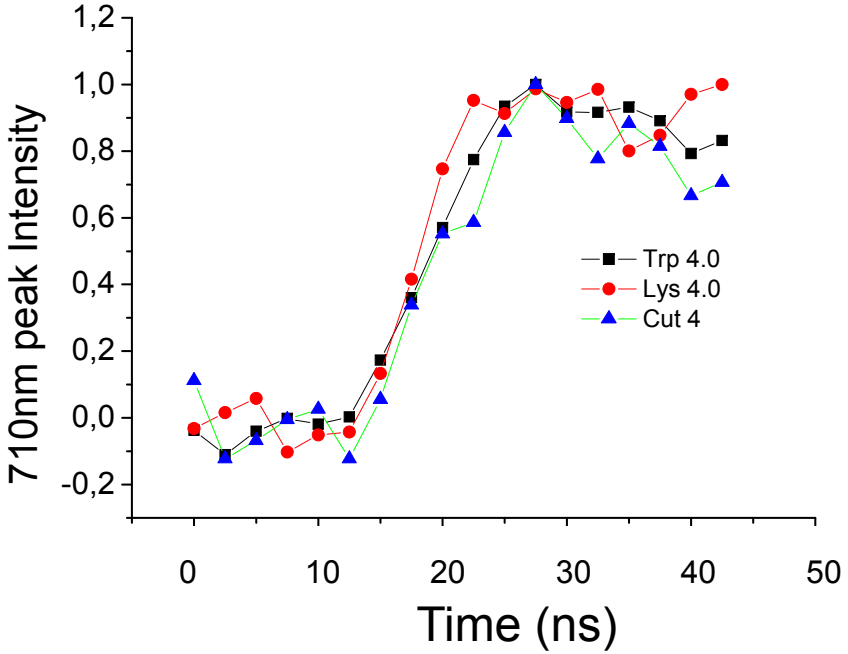


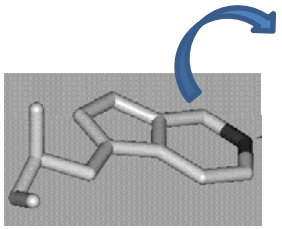
Disulphide electron adduct

Solvated electron absorption peak

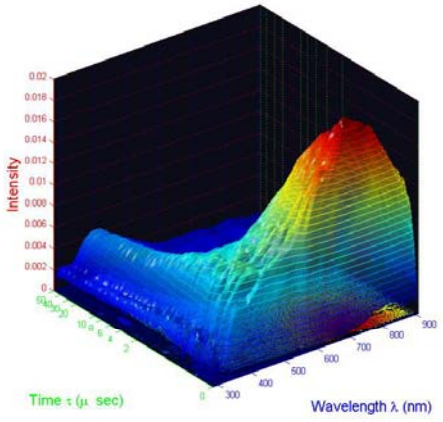


Nanosecond time resolved kinetics of solvated electron formation

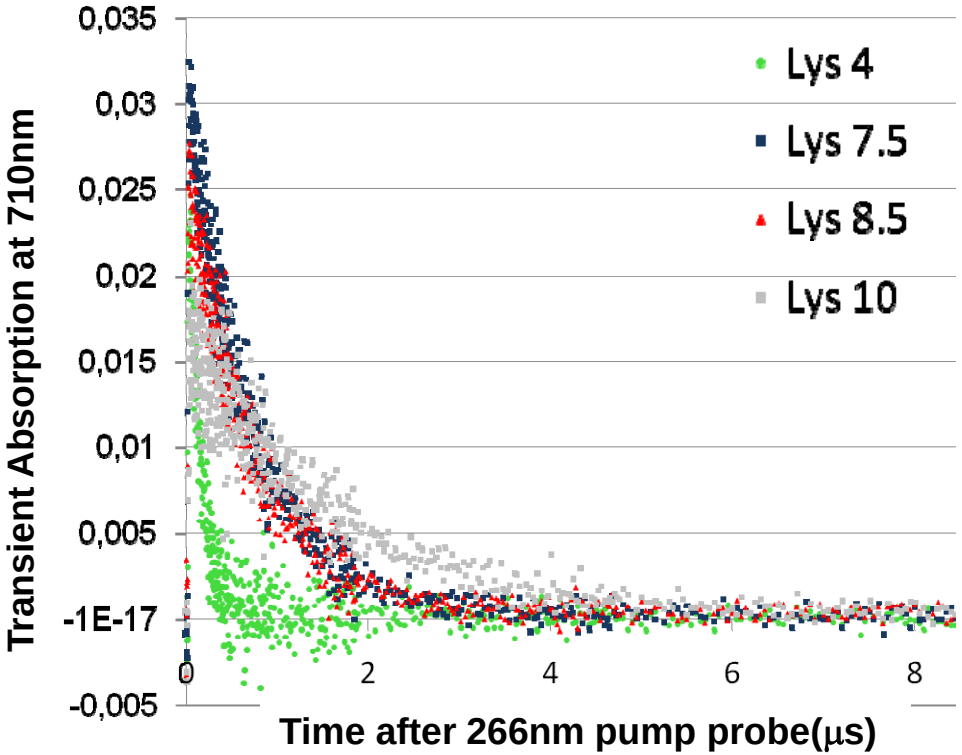




e^-_{aq}



Time resolved kinetics of solvated electron decay



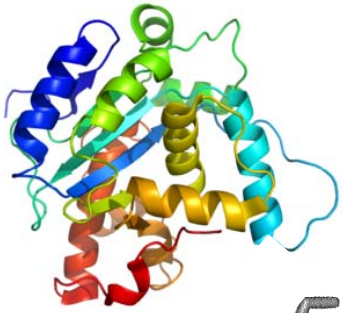
Biophysical Journal, Vol. 97, Iss. 1; pg. 211,, 2009

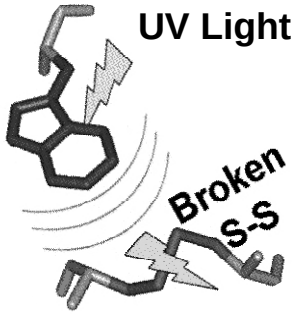
$$Trp + h\nu \rightarrow Trp^{\bullet+} + e^{-}_{aq}$$

Table 3- Lifetimes of the solvated electron in tryptophan, lysozyme and cutinase samples at different pH values.

The lifetimes, their normalised weights, and the associated errors obtained from global fits of the decay data for each molecule. The mean lifetime as a function of pH as well as goodness-of-fit parameters for each dataset are presented. The dominant lifetime has been high-lighted in **bold**.

Molecule	*pH*	*Mean lifetime*	*Lifetimes and pre-exponential factors for triple exponential global fitting*			*χ^2/DoF*	*R^2*
			τ_1 [µs]	τ_2 [µs]	τ_3 [µs]		
Tryptophan			0.17 ±0.01	1.11 ±0.045	2.93 ±0.07	1.4126E-6	0.99469
		<τ> [µs]	α_1	α_2	α_3		
	4.0	1.0	**0.98 ±0.01**	0.00 ±0.02	0.02 ±0.01		
	7.5	1.1	0.05 ±0.02	**0.95 ±0.02**	0.00 ±0.03		
	8.5	2.3	0.00 ±0.01	**0.61 ±0.02**	0.39 ±0.02		
	10.0	2.8	0.00 ±0.01	0.23 ±0.03	**0.77 ±0.02**		
			τ_1 [µs]	τ_2 [µs]	τ_3 [µs]		
Lysozyme			0.13 ±0.01	0.716 ±0.04	1.61 ±0.14	1.565E-6	0.97039
		<τ> [µs]	α_1	α_2	α_3		
	4.0	0.3	**0.99 ±0.02**	0.00 ±0.04	0.01 ±0.02		
	7.5	0.7	0.03 ±0.03	**0.97 ±0.04**	0.00 ±0.05		
	8.5	0.8	0.00 ±0.03	**0.97 ±0.04**	0.03 ±0.05		
	10.0	1.6	0.03 ±0.05	0.00 ±0.15	**0.97 ±0.12**		
			τ_1 [µs]	τ_2 [µs]	τ_3 [µs]		
Cutinase			0.15 ±0.01	0.97 ±0.07	2.11 ±0.13	1.0213E-6	0.97276
		<τ> [µs]	α_1	α_2	α_3		
	4.0	0.3	**1.00 ±0.03**	0.00 ±0.05	0.01 ±0.03		
	8.5	1.0	0.00 ±0.03	**1.00 ±0.06**	0.00 ±0.07		
	10.0	2.1	0.00 ±0.03	0.00 ±0.11	**1.00 ±0.09**		

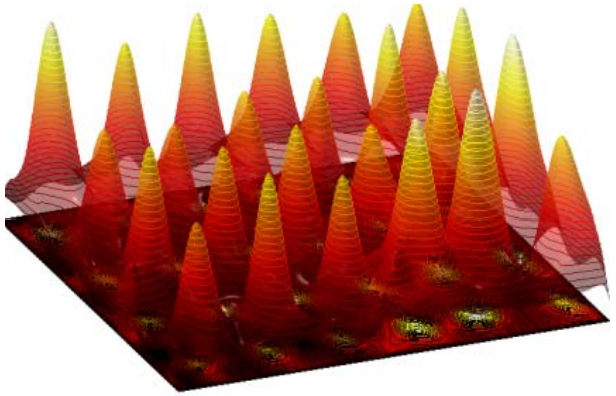




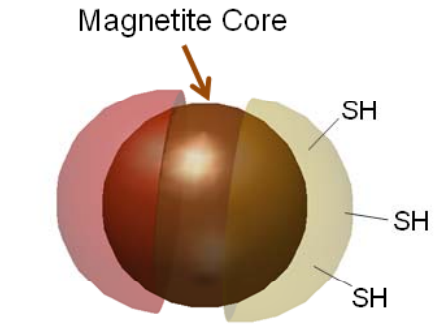
LAMI

Free **SH** groups

Protein microarray

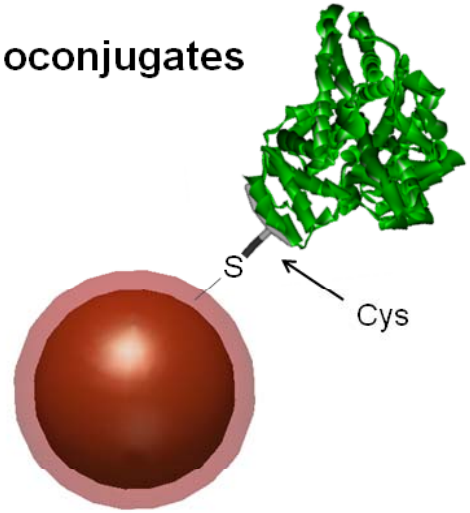


Coreshell Nanoparticles



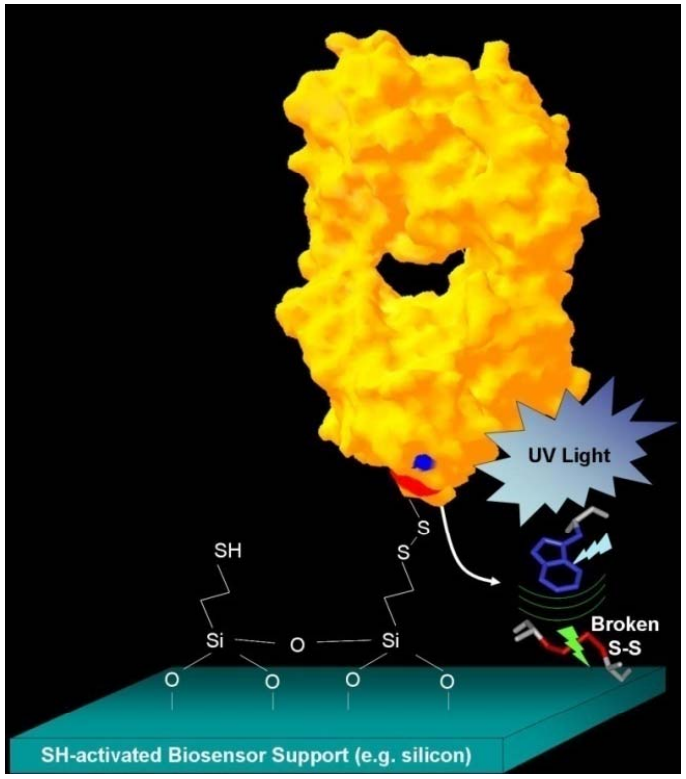
LAMI

Bioconjugates



Protein Mounted on Nanoparticles

Light Assisted Molecular Immobilization technology (LAMI)



Microarray Development of biomedical interest:

e.g. PSA and Fab arrays

Protein Science 2006 vol. 15, 343-351
Proteomics 2007, vol. 7 (19) 3432-3636
Protein Science 2010 19 (9) 1751—1759.

- Using a beam of **UV-light** molecules can be **covalently attached** to surfaces (oriented and specific immobilization)

- The **spatial resolution** of the technology is determined by the **size of the beam of light and can reach the diffraction limit of the optical setup used (nm)**

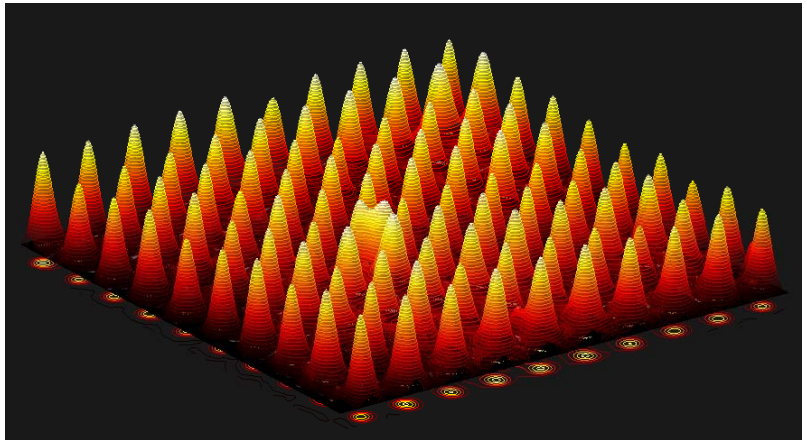
Microarray Development with new Photonic Technology

- ***Using a beam of*** **UV-light**, ***molecules can be*** **covalently attached** ***to surfaces.***
  The latter ranges from glass, quartz to gold, silicium as well as plastics.

- Currently we work with 5 micron spots arrays, 10 micron pitch leading to arrays with **10^4 spots inside a $1mm^2$ sensor !!**

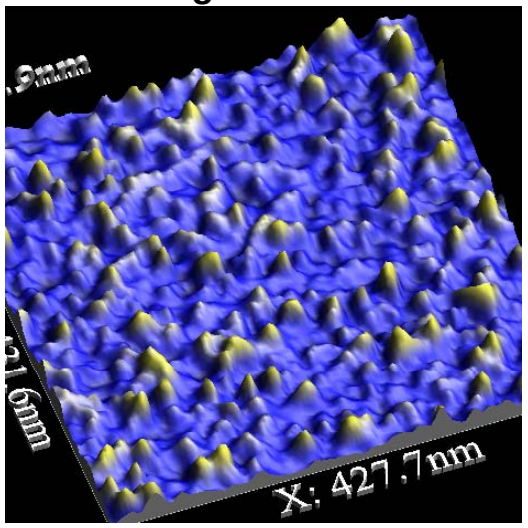
- **With Fourier Optics we have achieved 10^6 spots inside $1mm^2$ sensor**

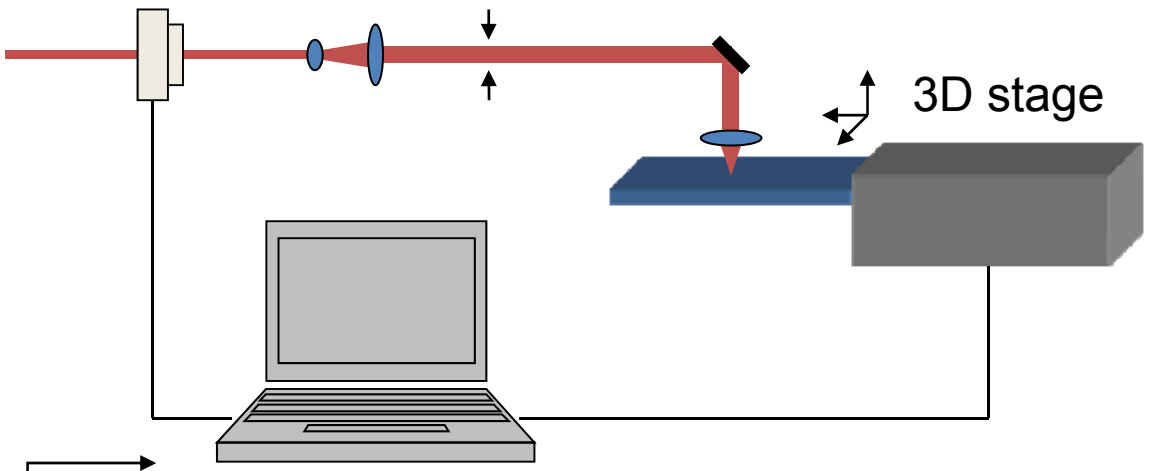
5 μm spots, 10 μm apart

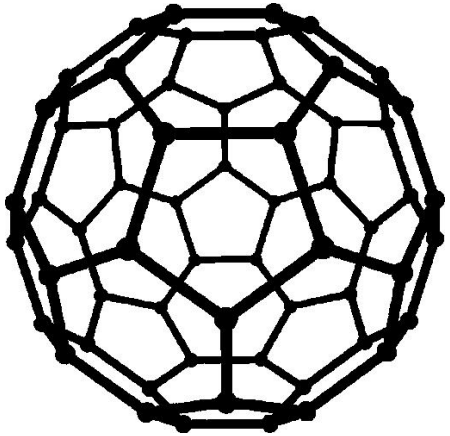


Proteomics 2007, Vol. 7, 3491-99

AFM observation of immobilised single molecules







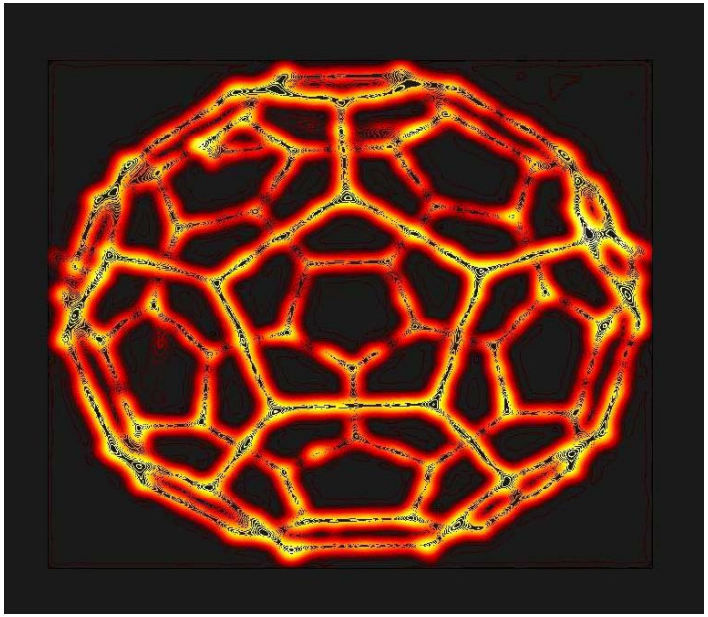
Fullerene Bitmap

- **Surface** is covered by a **molecular film**
- A **bit map** is loaded
- The surface is illuminated according to the bitmap, i.e., light will hit the surface reproducing the image in the bit map
- **Molecules will only be immobilised on the surface if they have been illuminated**
- The **fluorescence** of the immobilised molecules can be observed

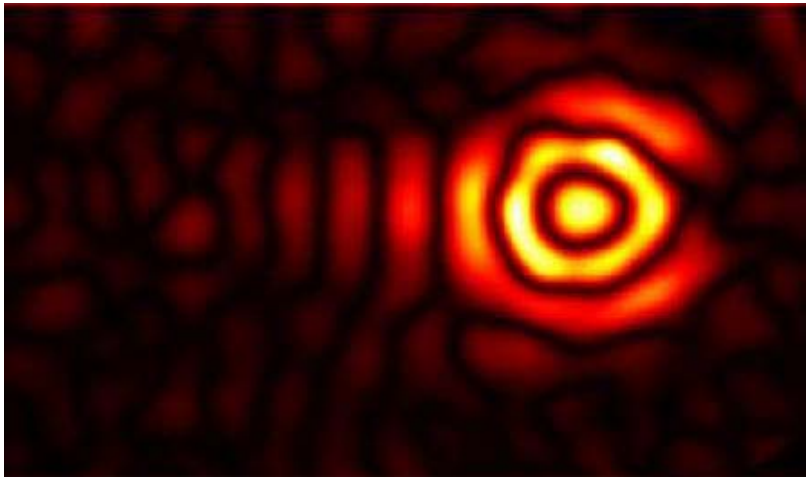
Fullerene nanostructure

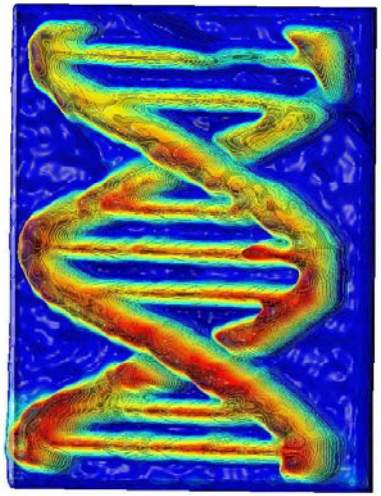
1260 μm x 1220 μm

10μm resolution



700nm resolved patterns





DNA

10μm resolution

Parracino et al., **Protein Science** 2010, 19 (9) 1751—1759.

Skovsen et al., **Proteomics**, 2009, 9, 1–4.

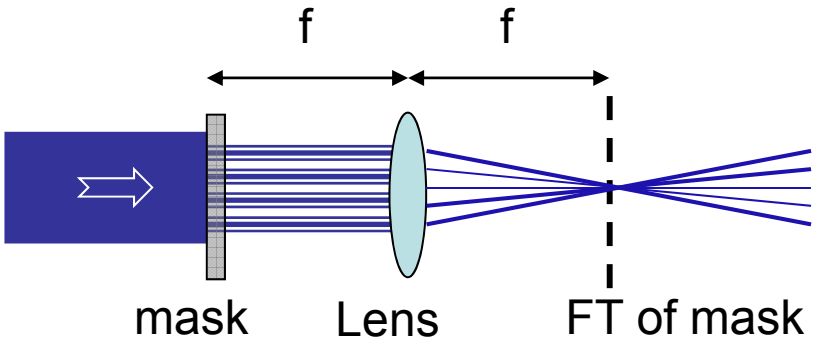
Skovsen and Neves-Petersen et al. **, Journal Nanoscience and Nanotechnology**, 9, 4333–4337 (2009)

Neves-Petersen et al., **Journal Nanoscience and Nanotechnology**, 9, 6, 3372–3381 (2009).

Skovsen et al., 2007, **Journal of Optomechatronics**, Volume 1, Issue 4 October 2007 , pages 383–391

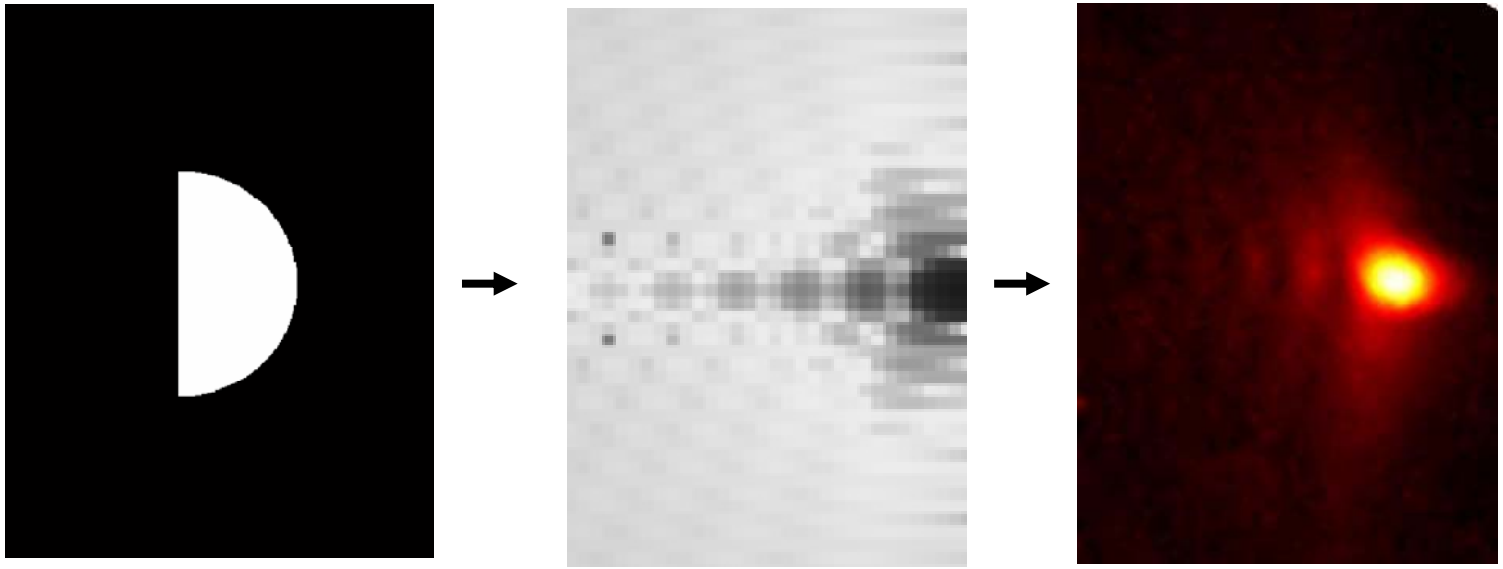
Achieving nm resolved protein patterns

Fourier transform properties of lenses



According to the Fourier transform properties of lenses, a transmission mask placed in the back focal plane of a focusing lens will result in a light pattern in the front focal plane, which is identical to the Fourier transform of the pattern transmitted by the mask.

Immobilizing according to diffraction patterns



Aperture

Diffraction pattern

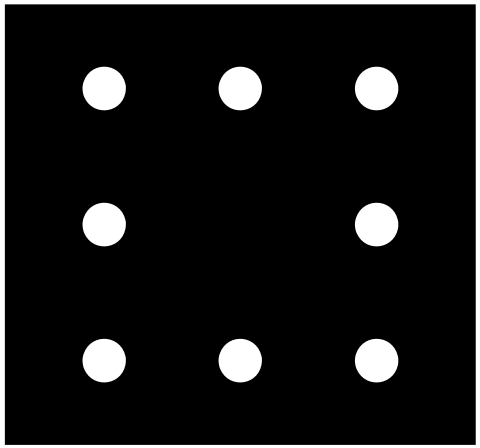
Protein immobilization

Skovsen and Neves-Petersen et al. , Journal Nanoscience and Nanotechnology, 9, 4333–4337 (2009)

Arraying with **700nm** resolution using a **cm** sized mask

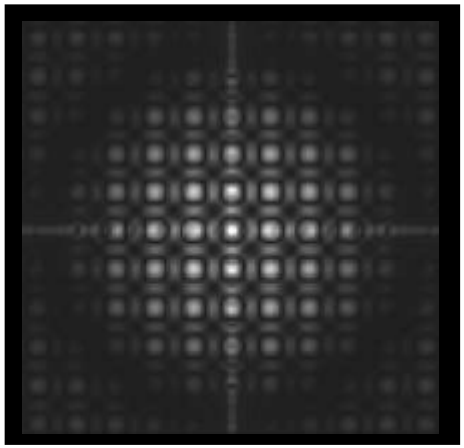
Photonic Immobilization of High Density Protein Arrays Using Fourier Optics.
Proteomics 2009, *9*, 1–4 Front Page issue

Mask pattern



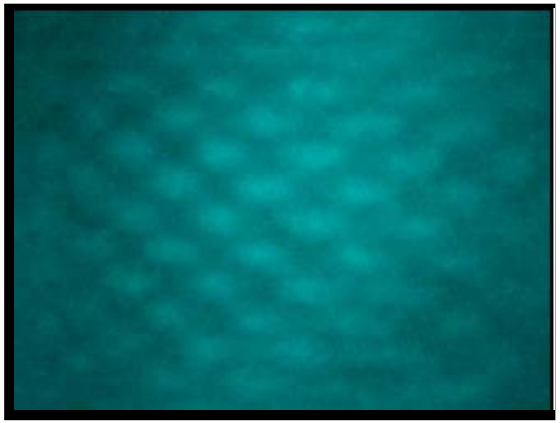
10mm x 10mm

FFT of Mask

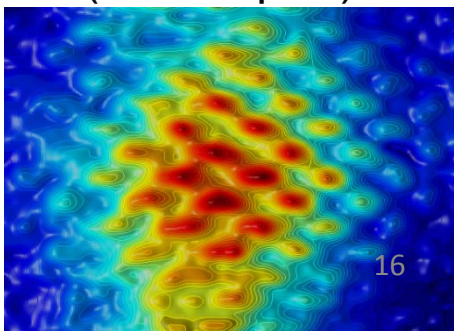


(central part)

Immobilized pattern

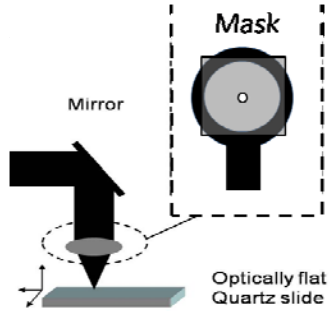


8μm x 8μm
(central part)

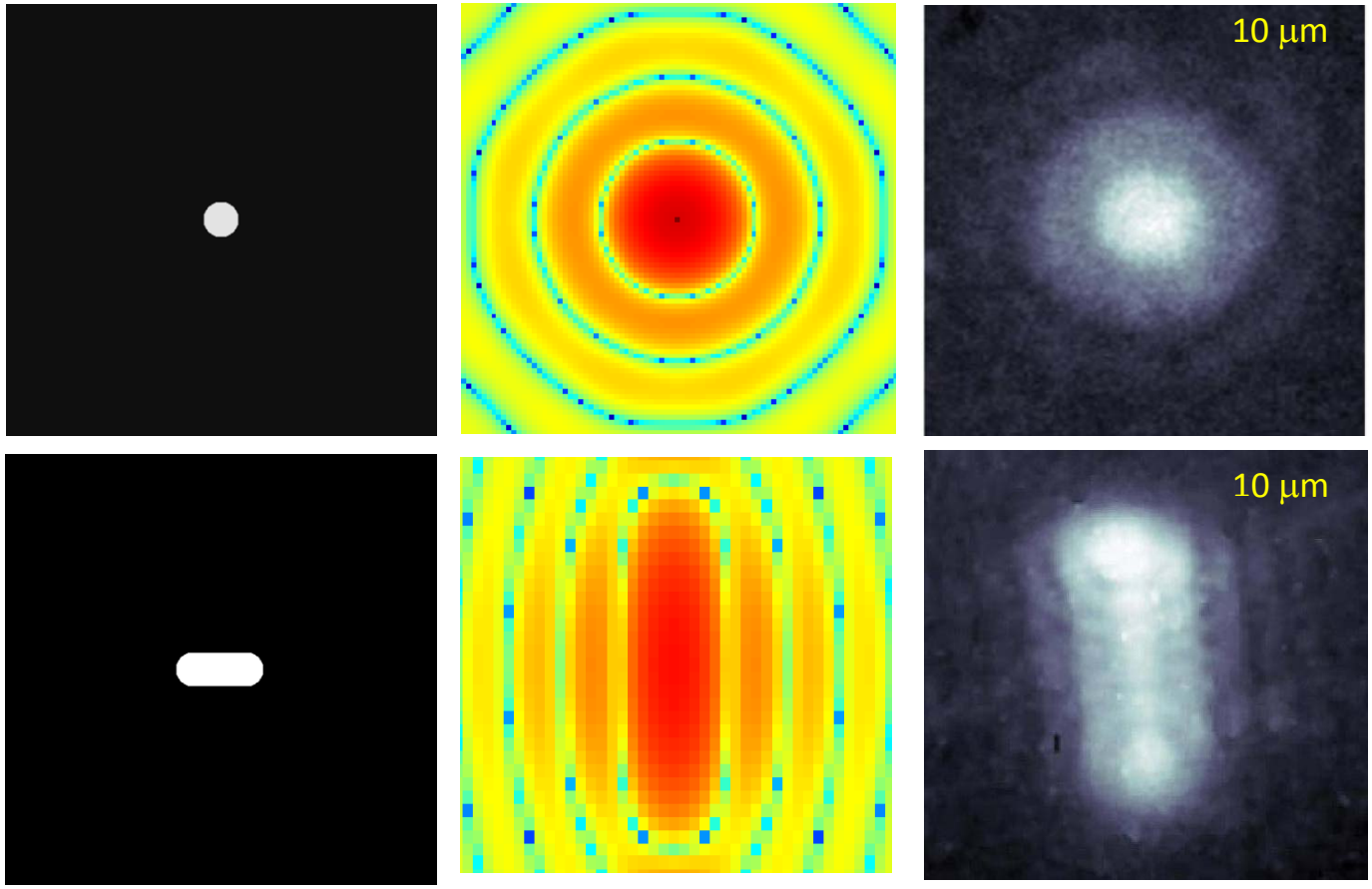


700nm resolved pattern
(optical resolution of the setup was 512nm)

Single aperture masks



Mask | Fourier Transform | Protein immobilization

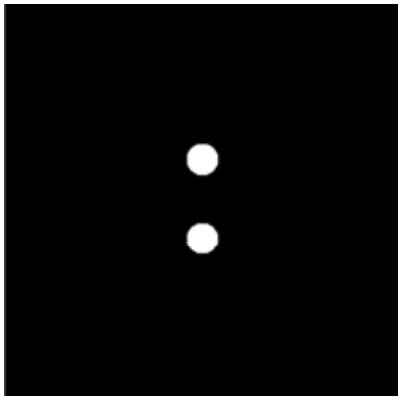


Immobilization of biomolecules onto surfaces according to UV-light diffraction patterns
Petersen et al., Applied Optics 2010, in print

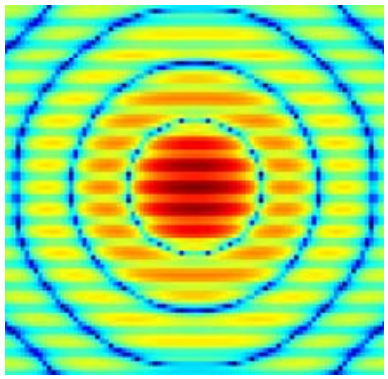
Immobilization of biomolecules onto surfaces according to UV-light diffraction patterns
Petersen et al., **Applied Optics 2010, in print**

Dual Aperture Mask

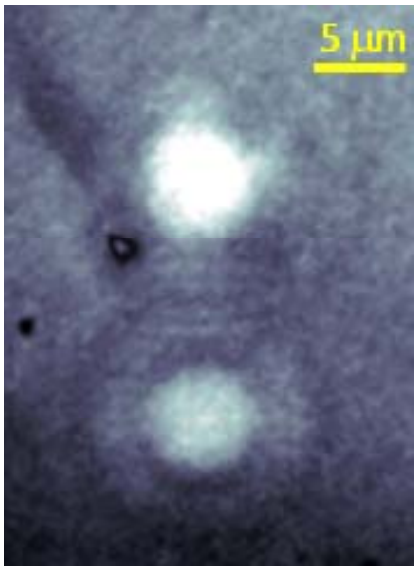
Mask



Fourier Transform



Protein immobilisation pattern



If a dual aperture spatial mask is used, the results are different from the expected fourier transform pattern of the mask. It appears as a **superposition of two diffraction patterns** produced by the two apertures **with a fine structured interference pattern superimposed**.

The Airy pattern observed around both circular images agrees with the predicted pattern for a single pinhole.

One possible reason leading to the two circular apertures is **partial loss of coherence between the parts of the laser beam that passes through the two apertures.**

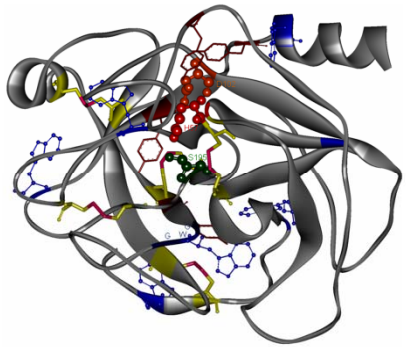
Longitudinal coherence depends on:

1) $\lambda^2/\Delta\lambda$, where $\Delta\lambda$ is the bandwidth of the laser line,
2) the duration of the short pulses used, and
3) **the presence of detectors** (biomolecules)

Careful analysis of the laser bandwidth, geometry of the optical setup, maximum path length difference from the apertures to the slide makes us conclude that loss of coherence due to 1) and 2) is not likely to happen.

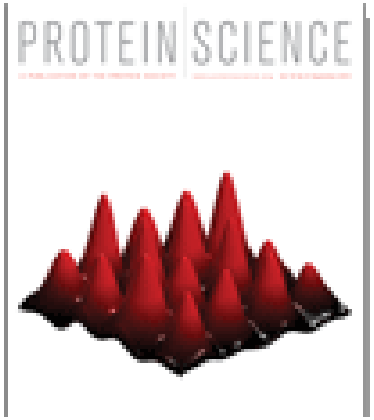
3- **The presence of a photon detector, the biomolecules, can prevent interference from happening**. When a photon is absorbed by a biomolecule, that photon is no longer available for interference. Even if the photon is reemitted, this is likely to take place ps/ns delayed relative to the initial excitation.

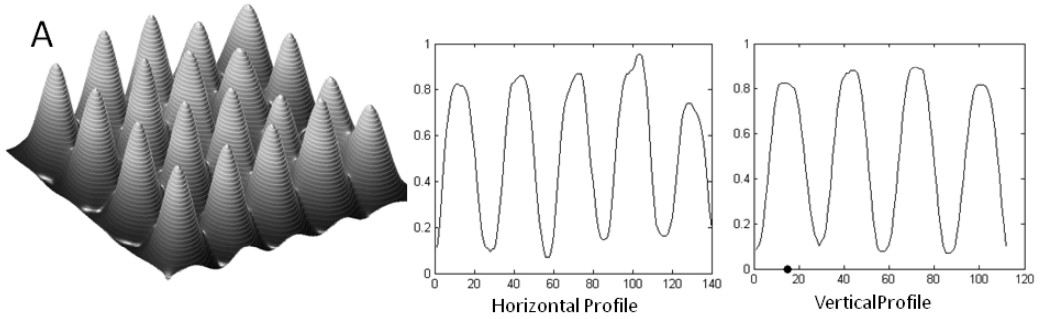
We therefore speculate that molecular absorption of photons prior to the image plane effectively abolishes the interference processes between photons passing the two apertures. However, the residual coherence is still sufficient to result in a detailed interference pattern.



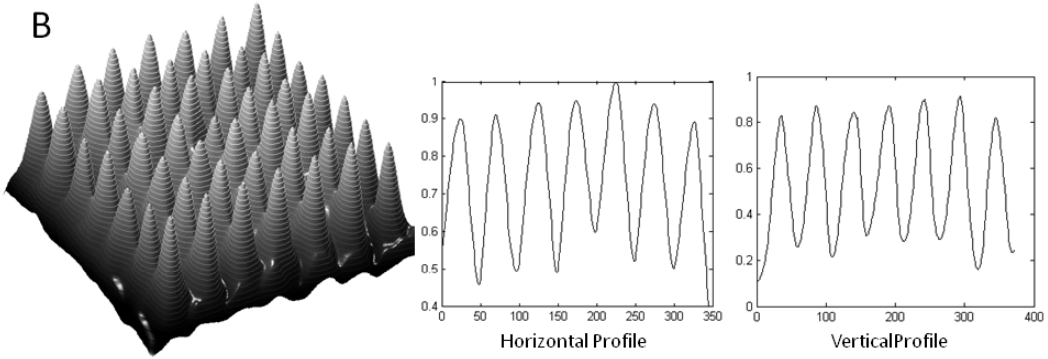
Arraying prostate specific antigen PSA and Fab anti-PSA

Protein Science 2010, 19 (9) 1751—1759



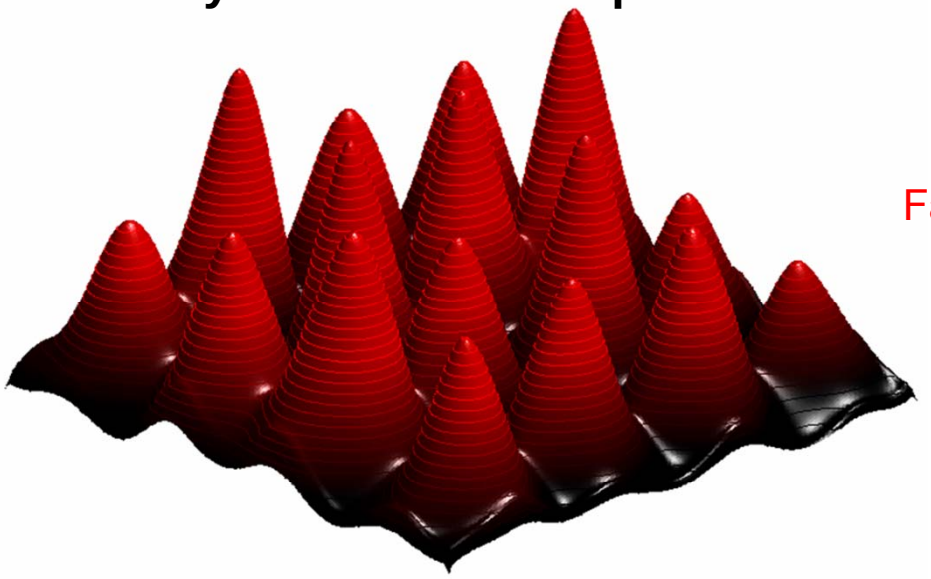


A - Arrayed **PSA-FITC** with vertical and horizontal fluorescence intensity profiles;



B - Arrayed **Fab-AF647 5A10 anti-PSA** with a vertical and horizontal fluorescence intensity profiles.

Currently we can detect 7 pM PSA. Detection limit of commercial kits is 160 pM



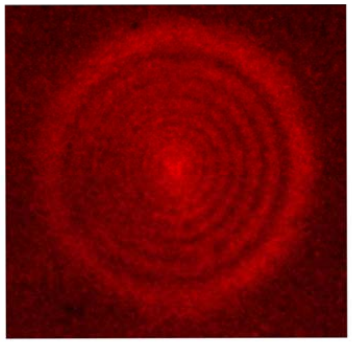
Fluorescence intensity of Fab-AF647 anti-PSA-5A10 after cross-reaction with immobilized PSA.

Protein Science 2010, 19 (9) 1751—1759

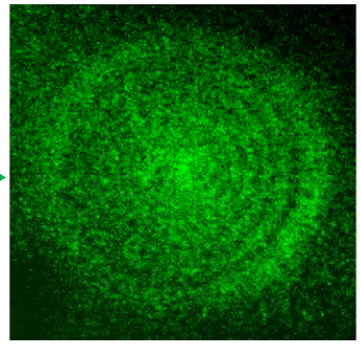
25μm

A

Fluorescence from immobilized PSA-AF647



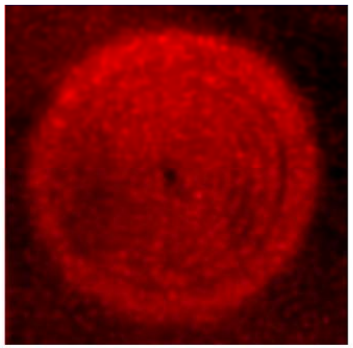
B



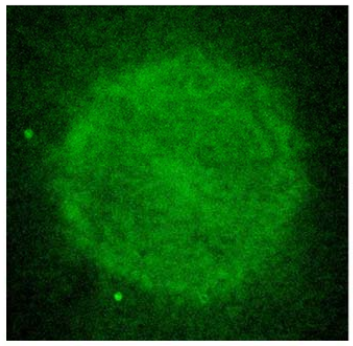
Fluorescence from **Fab-AF488 anti-PSA** after incubation with immobilized PSA-AF647

C

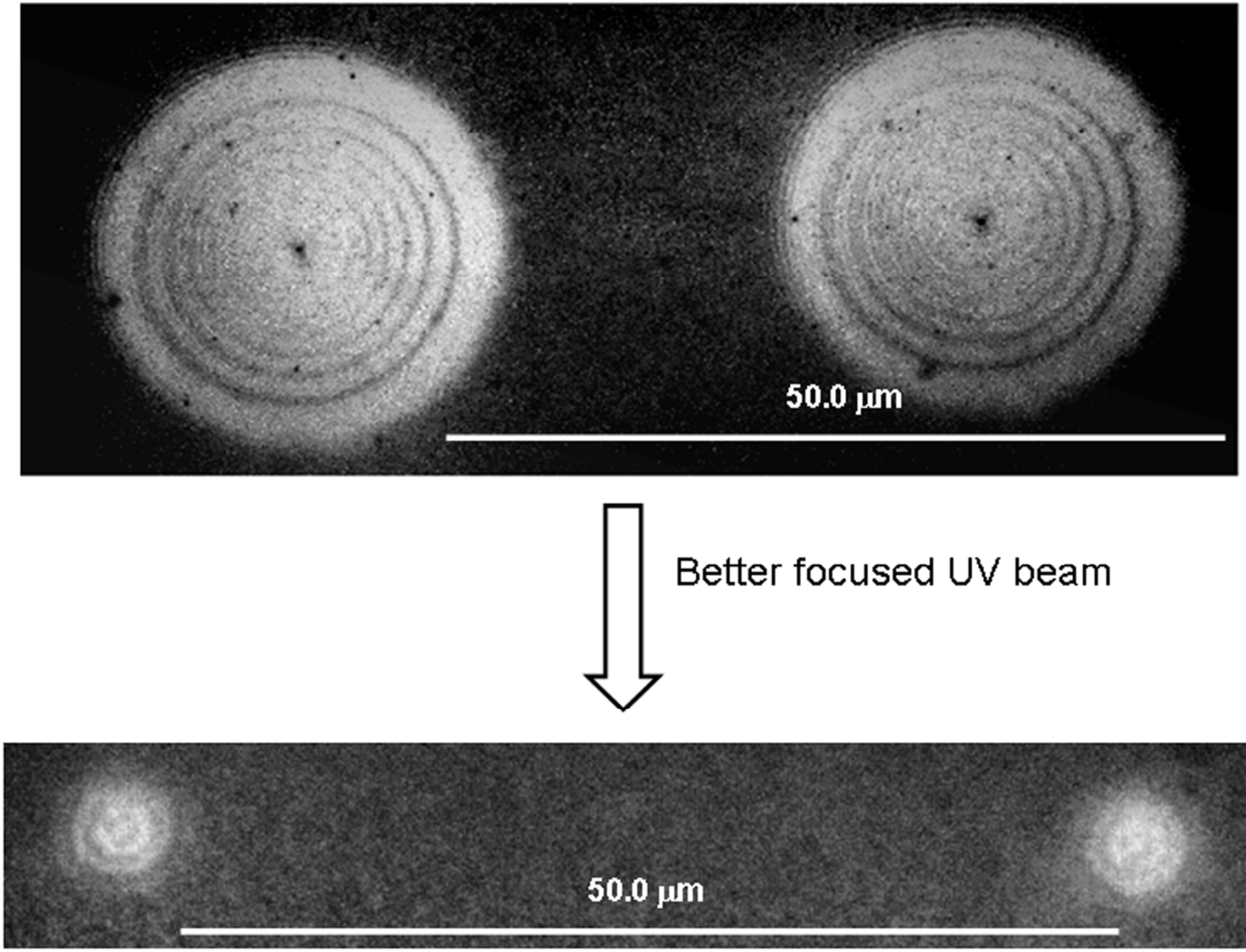
Immobilized Fab-AF647 5A10 anti-PSA



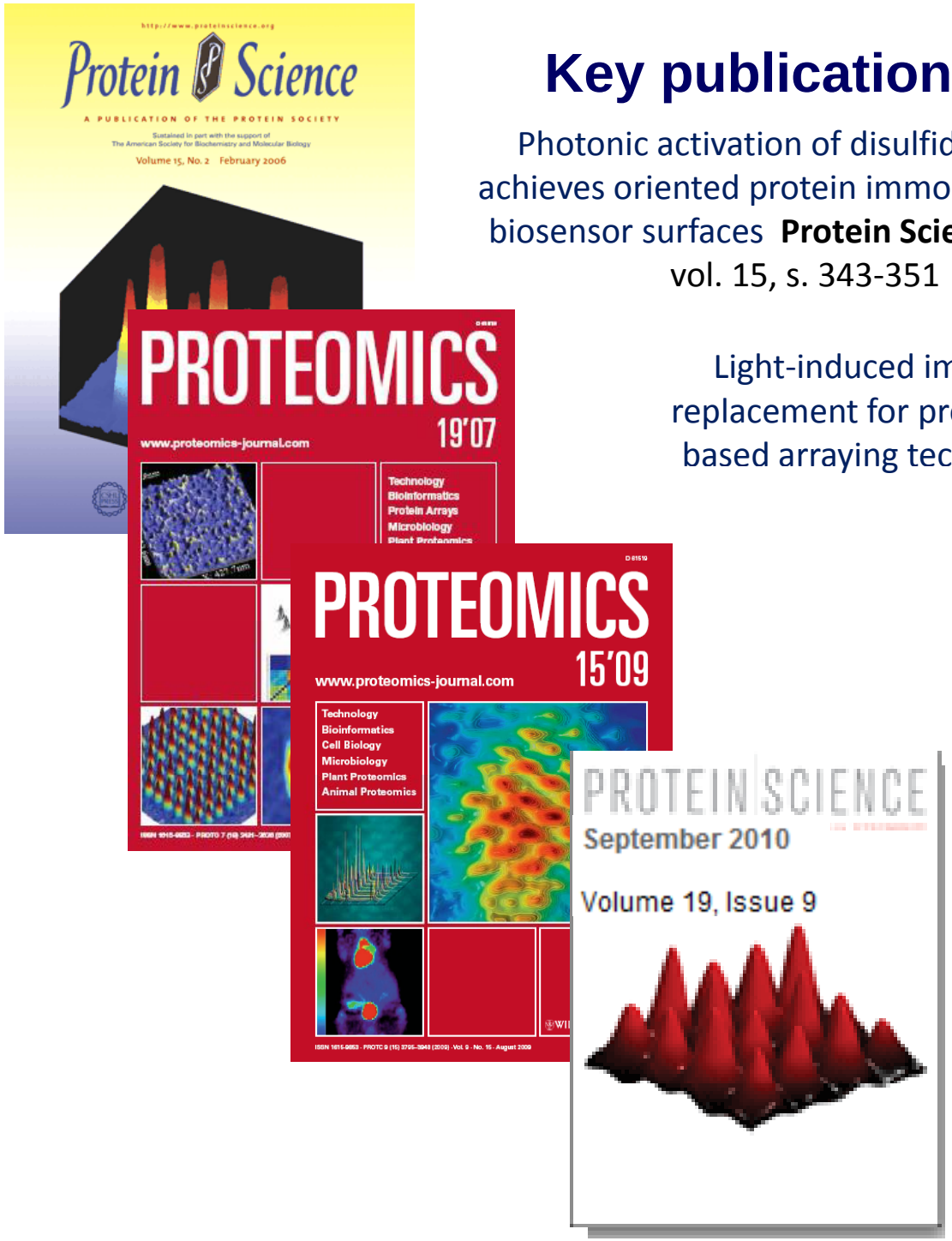
D



Fluorescence from **PSA-AF555** after incubation with immobilized Fab-AF647 5A10 anti-PSA



Dimensions of a typical array spot imaged with fluorescence microscope: after tighter focus of the UV light used to immobilize the protein (PSA-AF555), the spot dimensions decrease from **25 to 5 micrometer.**



Key publications

Photonic activation of disulfide bridges achieves oriented protein immobilization on biosensor surfaces **Protein Science 2006** ; vol. 15, s. 343-351

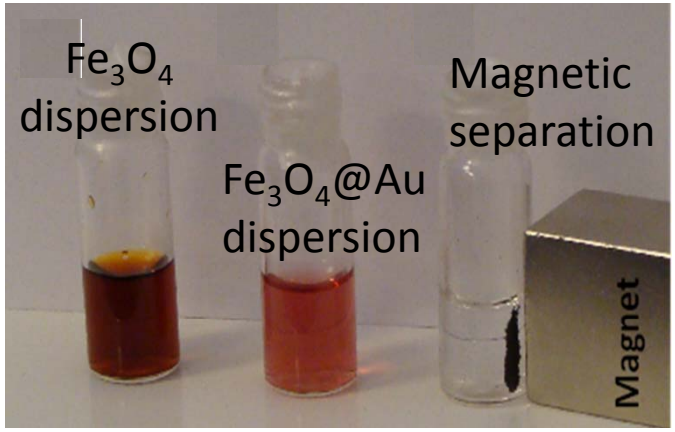
Light-induced immobilisation of biomolecules as a replacement for present nano/micro droplet dispensing based arraying technologies. **Proteomics**. **2007**, Vol. 7, 3491-99.

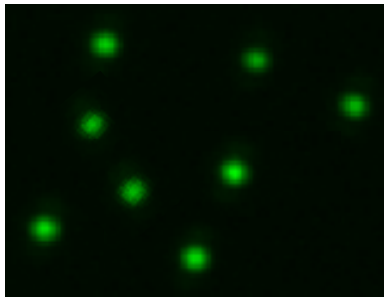
Photonic Immobilization of High Density Protein Arrays Using Fourier Optics. **Proteomics 2009**, 9, 1–4

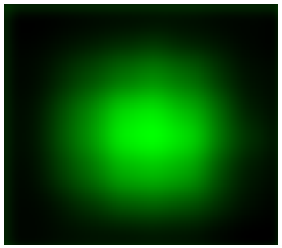
Immobilization of biomolecules onto surfaces according to UV-light diffraction patterns **Petersen et al., Applied Optics 2010, in print**

High density microarrays for biomarkers detection: arraying **prostate specific antigen** and Fab anti-PSA using UV light. **Parracino et al., Protein Science 2010,** 19 (9) 1751—1759**.**

Engineering nanoparticle based molecular carriers using LAMI





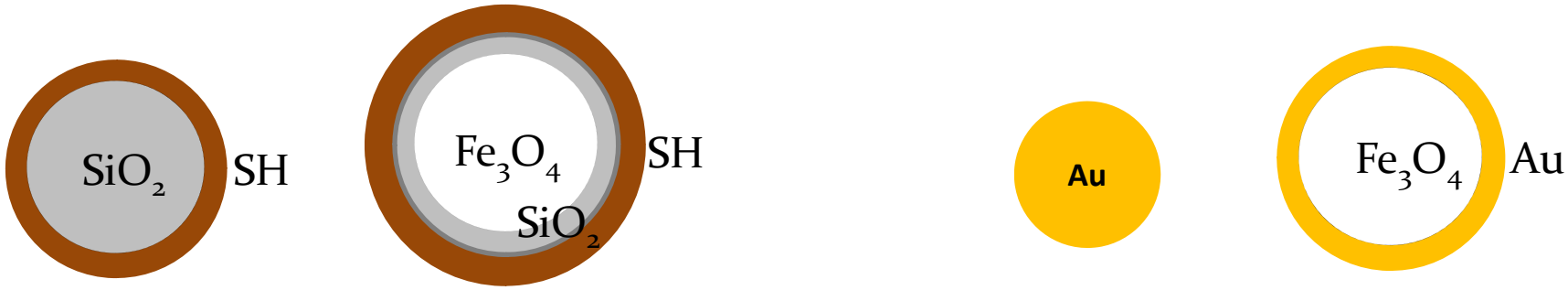


Proteins mounted on thiol reactive nanopaticles

NANOMEDICINE

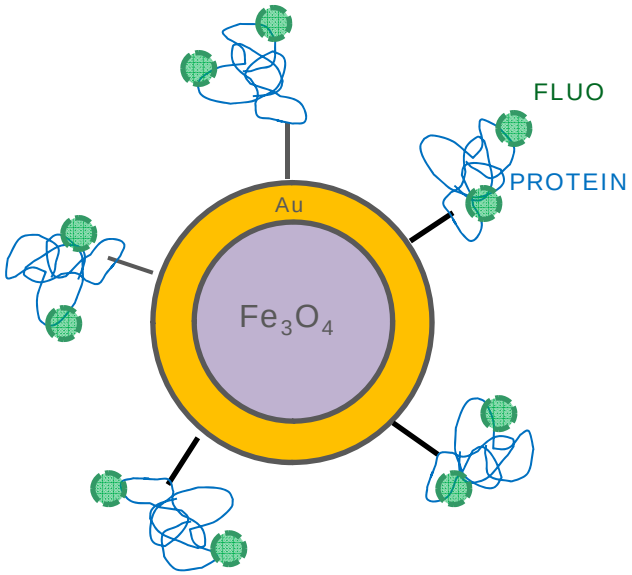
Nanoparticles as molecular carriers

- **Nanoparticles** are rendered **biologically active using LAMI**
- **Prostate Specific Antigen (cancer marker), Bovine Serum Albumin, Insulin** have been coupled to thiol derivatised silica nanoparticles
- Targetting (drug delivery into cells: **gene therapy, cancer therapy**
- Particles can be tracked inside cells with **confocal microscopy**



Applications - Bioconjugation

Insulin and Bovine Serum Albumin immobilized with LAMI



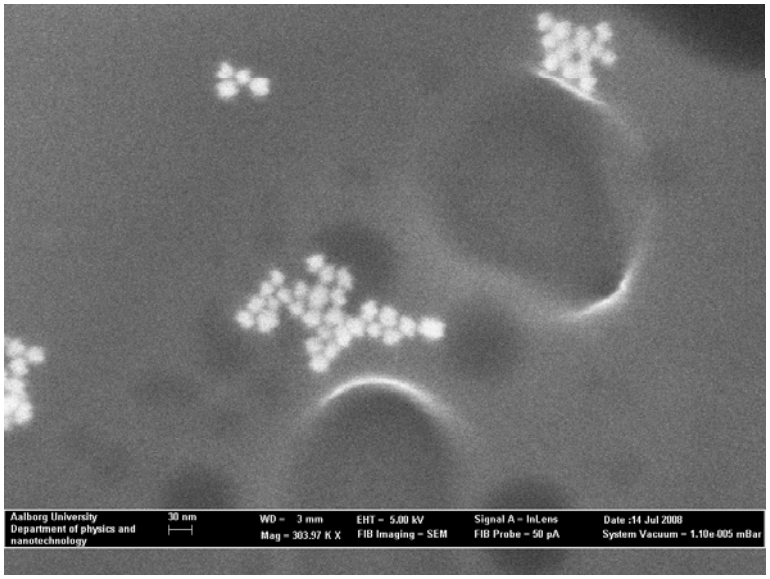
•Covalent and oriented immobilization

Caracterization

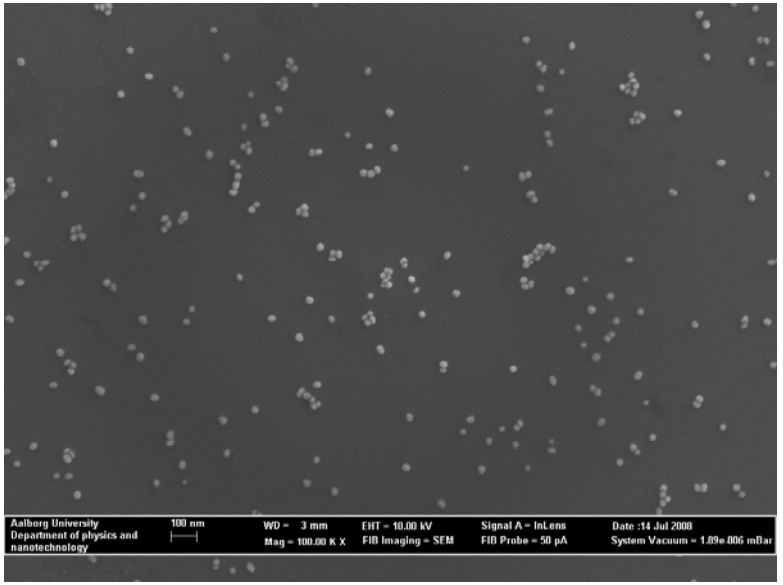
Fluorescence spectroscopy
Absorbance Spectroscopy
Dynamic Light Scattering
Fluorescence confocal microscopy
Scaning Electron Microscopy

SEM image for Insulin immobilized Au-nanoparticles

Au-Nanoparticles alone



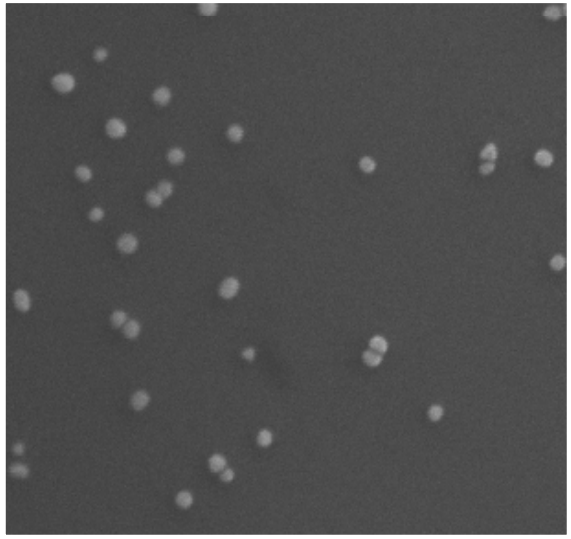
Insulin – Au Nanoparticles



The immobilized particles were washed twice with water and imaged.

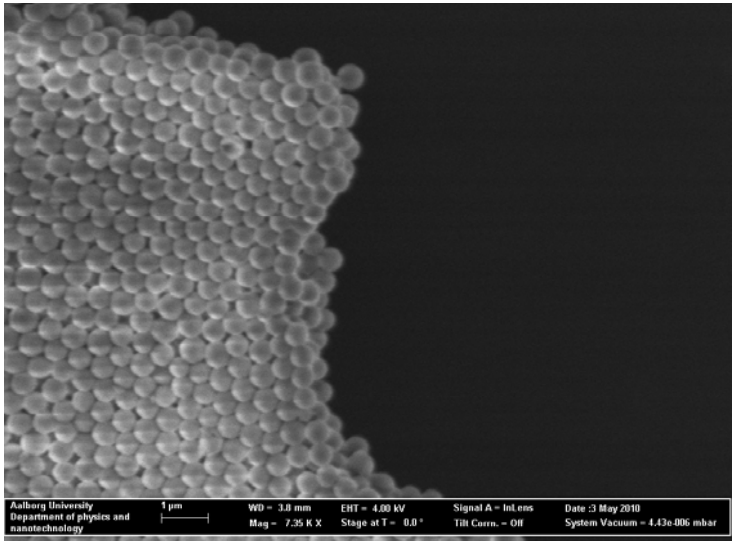
The protein did not precipitate after washes. The particles are in isolated state.

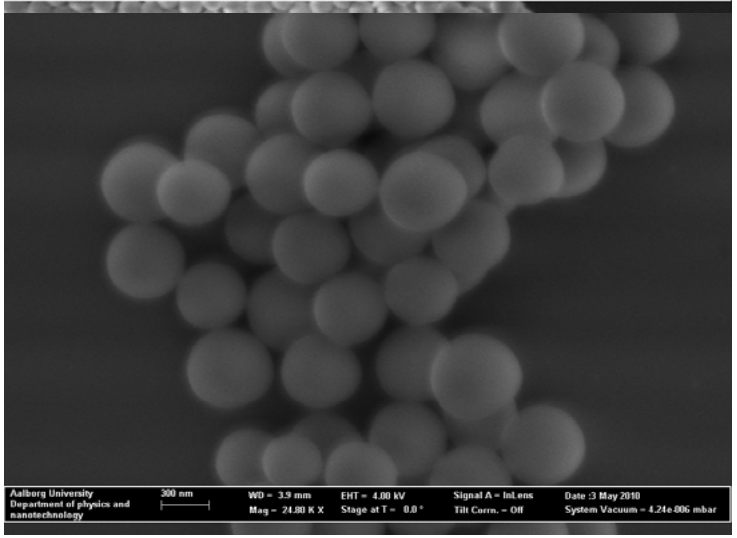
Particles are well stabilized with protein and more individual after immobilization



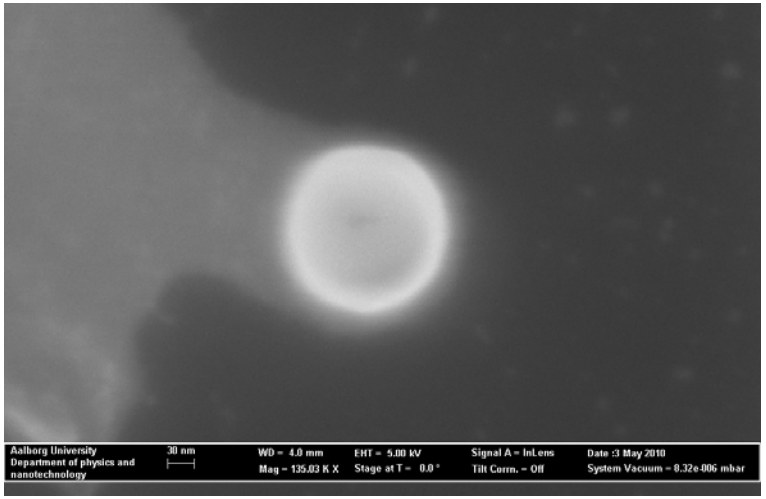
Silica particles with different sizes

310nm

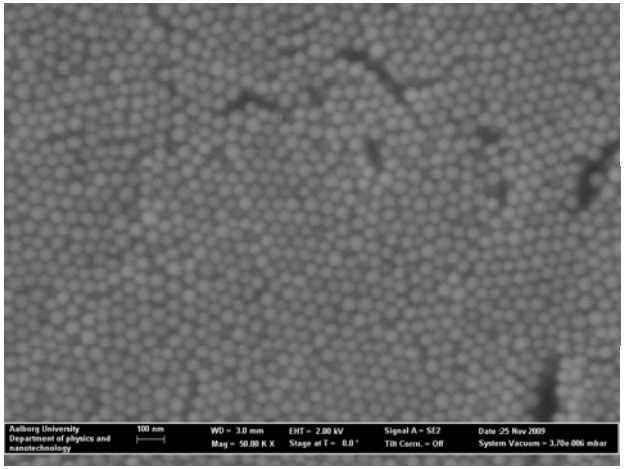




200nm



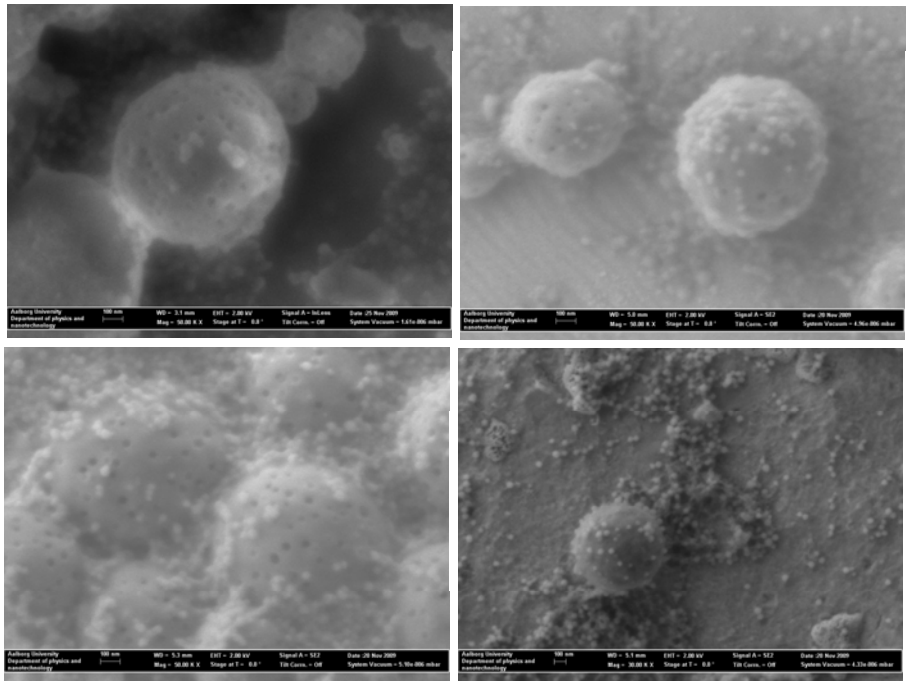
50nm



Thiol Functionalization

80 nm silica particles + 0.5M 300 µl **MPA**.
Particles washed and re-dispersed in water

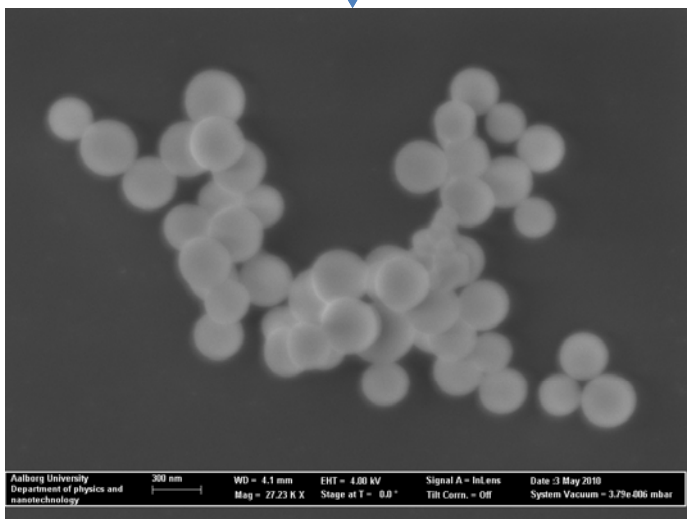
Stirring at 700 rpm for 3 days at 20 C Temperature



Particles aggregated and grown up to approx. 600 nm

200 µl TEOS + 0.5 ml 25% Ammonia + 9.8 ml 96% ethanol ---→ washed and dispersed in 12 ml ethanol (**250 nm** silica particles)

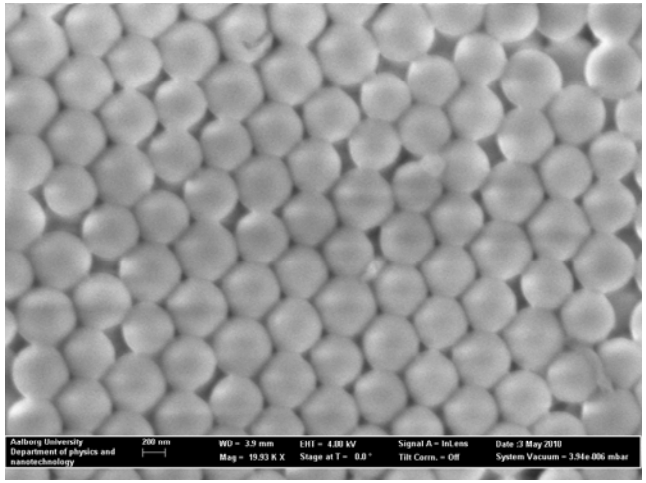
10 ml of above particles dispersion + 150 ml 96% ethanol + 7 ml water + **pH adjusted to 8.0-8.5 with ammonia** + 40 µl MPA + 1 hour at 200 rpm stirring. Then **solution heated up to 80 C and refluxed for 3 hours** (until final solution volume become 50 ml)



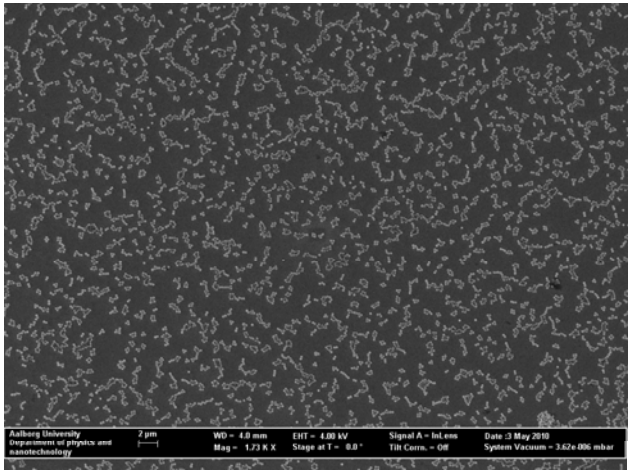
MPA: (3-Mercaptopropyl) trimethoxy silane

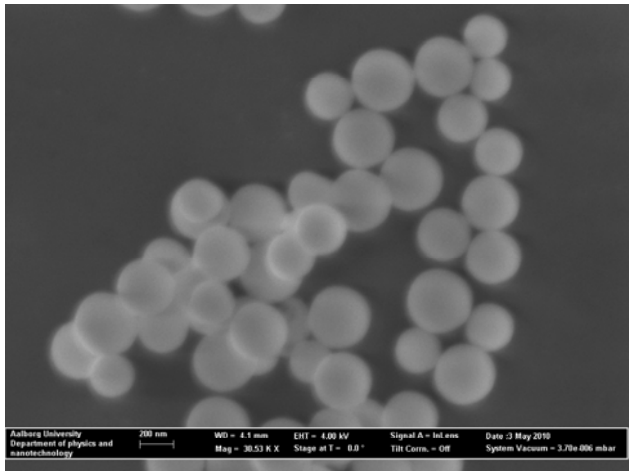
TEOS: Tetra ethyl orthosilicate

SiO_2 nanoparticles



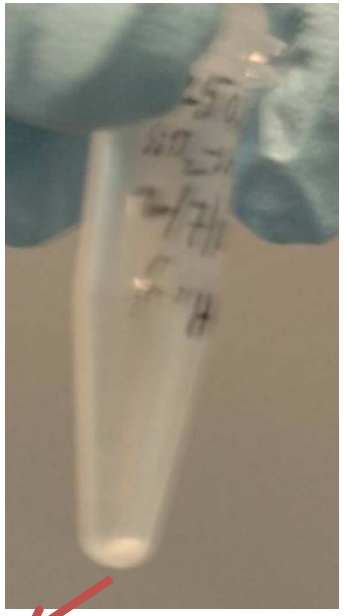
SiO_2-SH nanoparticles



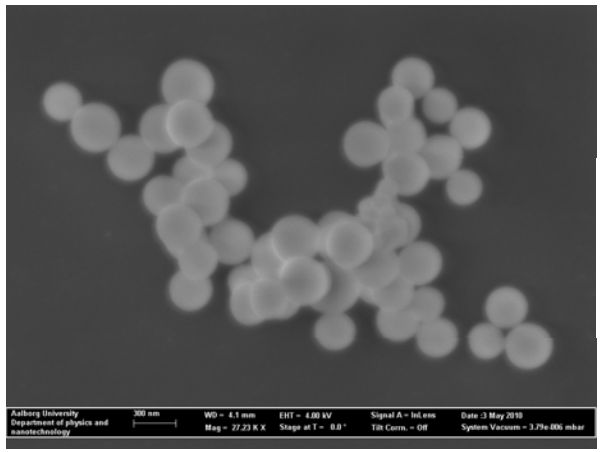


Silica Particles checked for thiolization

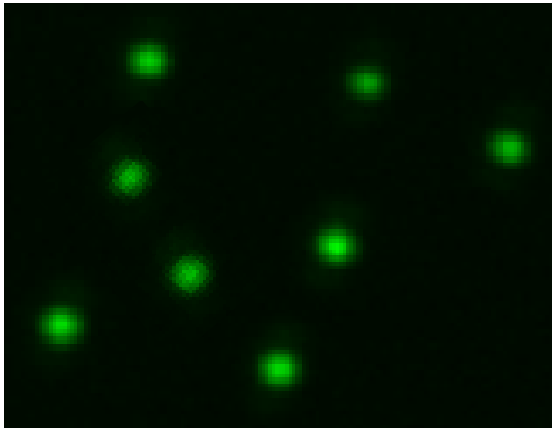
SEM, DLS and Fluorescence microscopy shows no particle aggregation

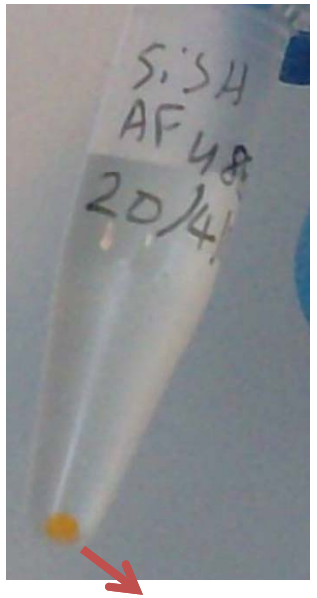


250nm Silica Particles upon centrifugation



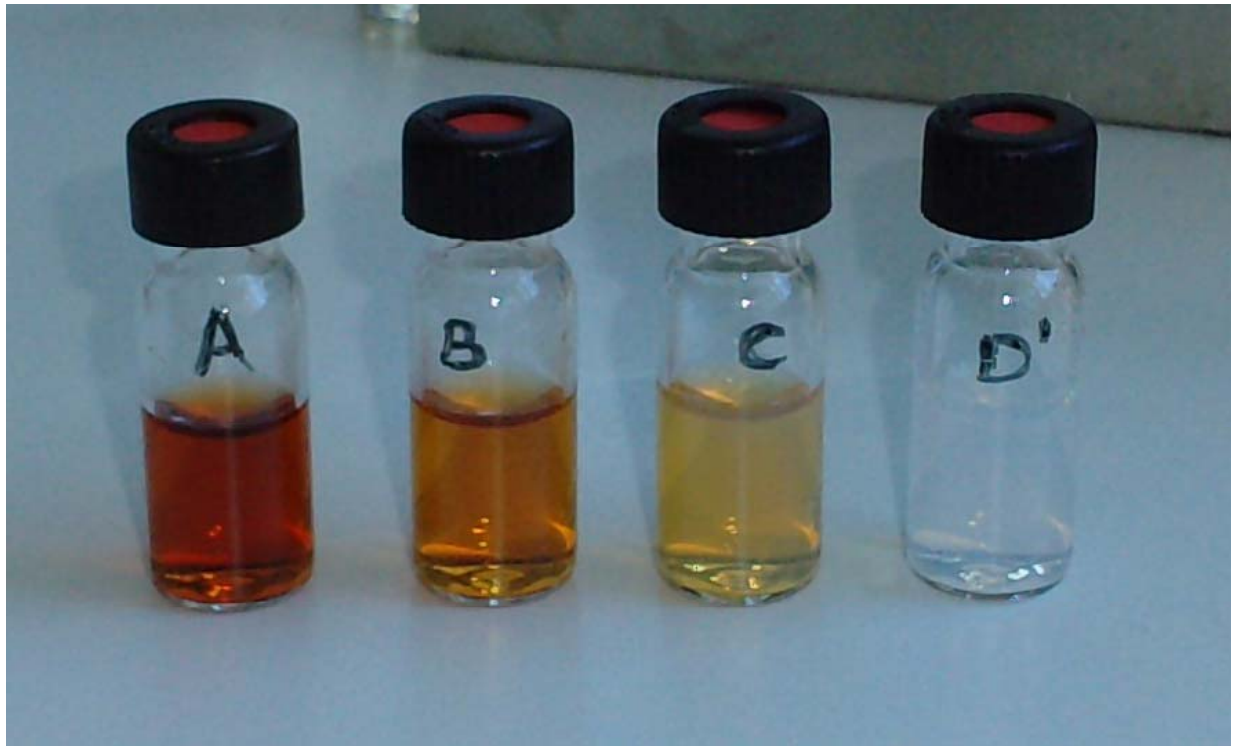
Particles labelled with AF 488





AF488 labeled 250nm Silica particles upon centrifugation

Fe_3O_4@SiO_2 core-shell nanoparticles



A : **10 nm** average size **Magnetite** nanoparticles (Total average diameter

B : **15 nm silica layer** over the **10 nm** diameter **magnetite** nanoparticles

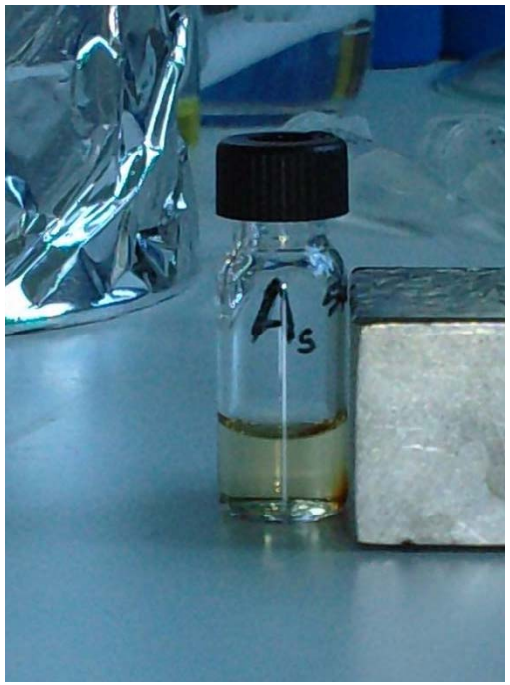
C : **30 nm silica layer** over the **10 nm** diameter **magnetite** nanoparticles

D: **60 nm silica layer** over the **10 nm** diameter **magnetite** nanoparticles

Magnetic nature

40 nm thiolated magnetic silica nanoparticles separation from dispersion in the presence of field with time

No Field



In Field: After a 1 day

After 2 days

After 5 days

40nm Fe_3O_4@SiO_2-SH-AF488

0 min

MAGNET

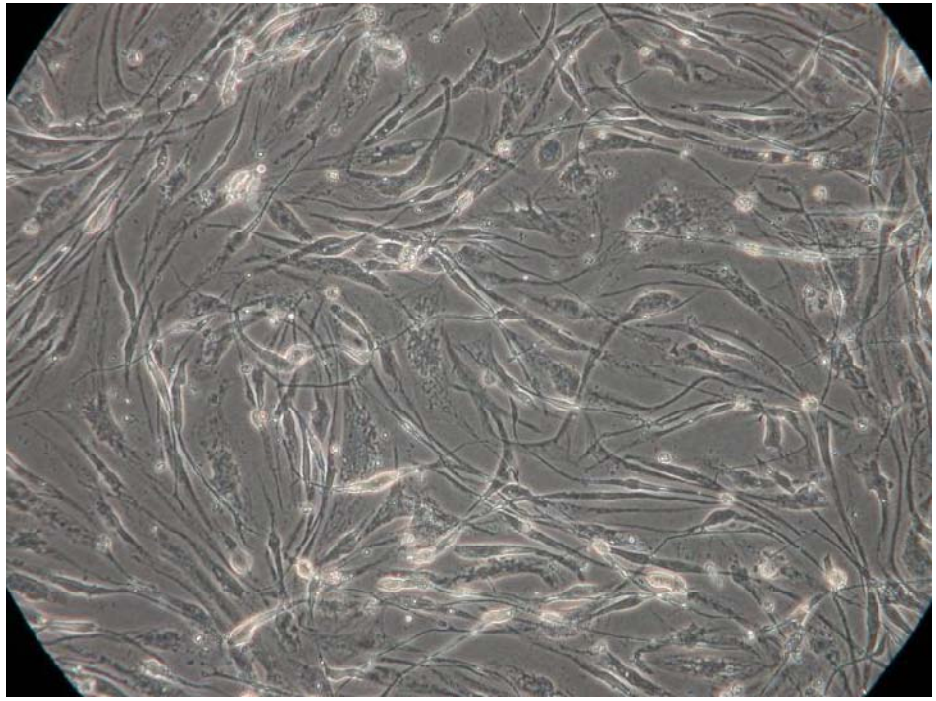
397 min

4527min

MAGNET

Exc 488nm

Cell targetting – **We aim at following drug delivery mediated by derivatised nanoparticles (e.g. insulin derivatised nanoparticles) and at tracking metabolic changes induced by ligand-receptor interaction**



Muscle Cells – Dr Per Bendix, Aarhus University Hospital, Denmark

Key Messages

Bioinformatics leads to the discovery of an highly conserved structural motif in proteins that can be activated by light, leading to a new photonic technology for covalent immobilization of biomolecules,

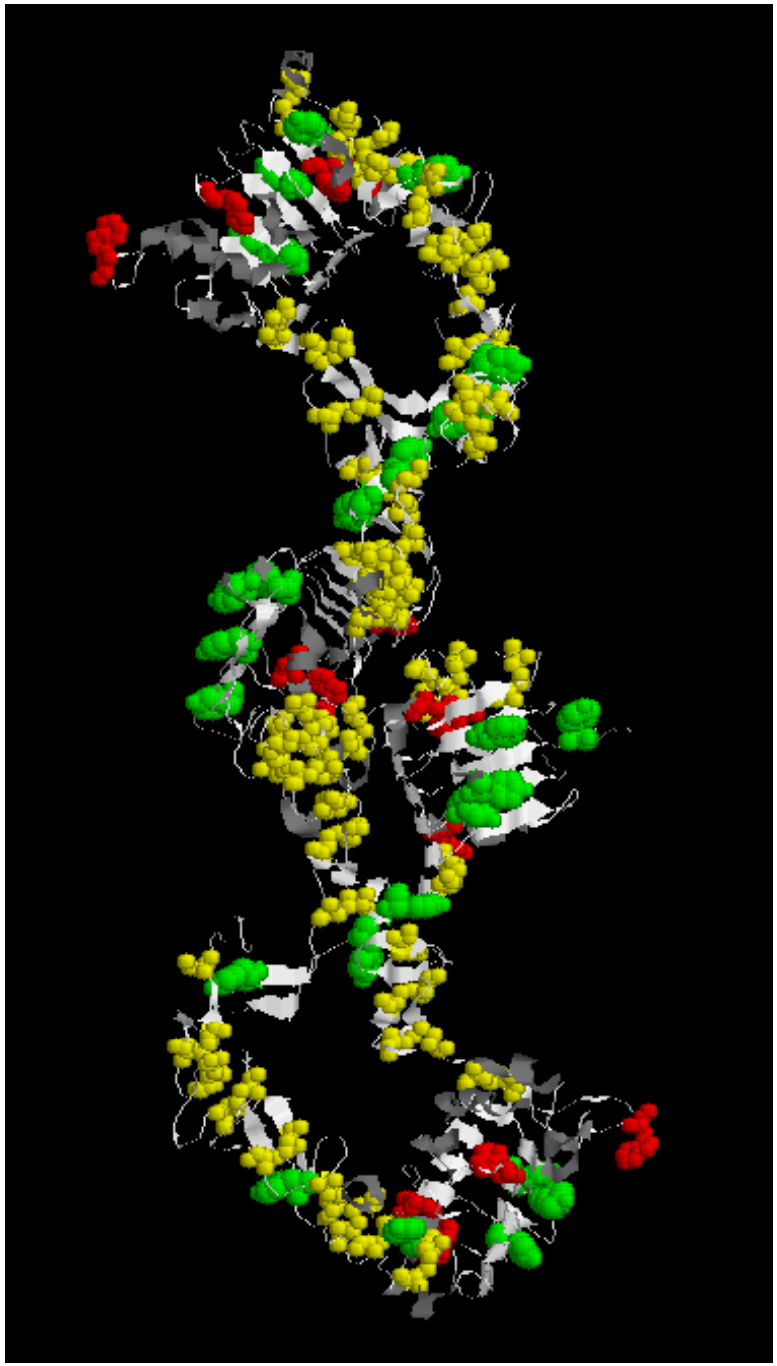
Light **A**ssisted **M**olecular **I**mmobilization :

1- Key reaction mechanism induced by light and its time scales

2- Applications:

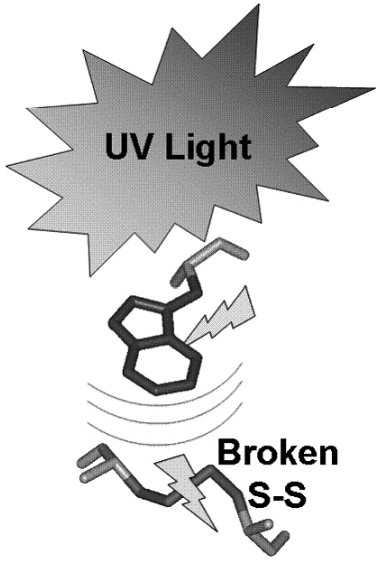
- Biosensor microarraying
- derivatisation of surfaces with proteins with diffraction limited resolution (sub μm)
- nanoparticle based drug delivery systems

3- New light based cancer therapy – modulating cellular metabolism using light

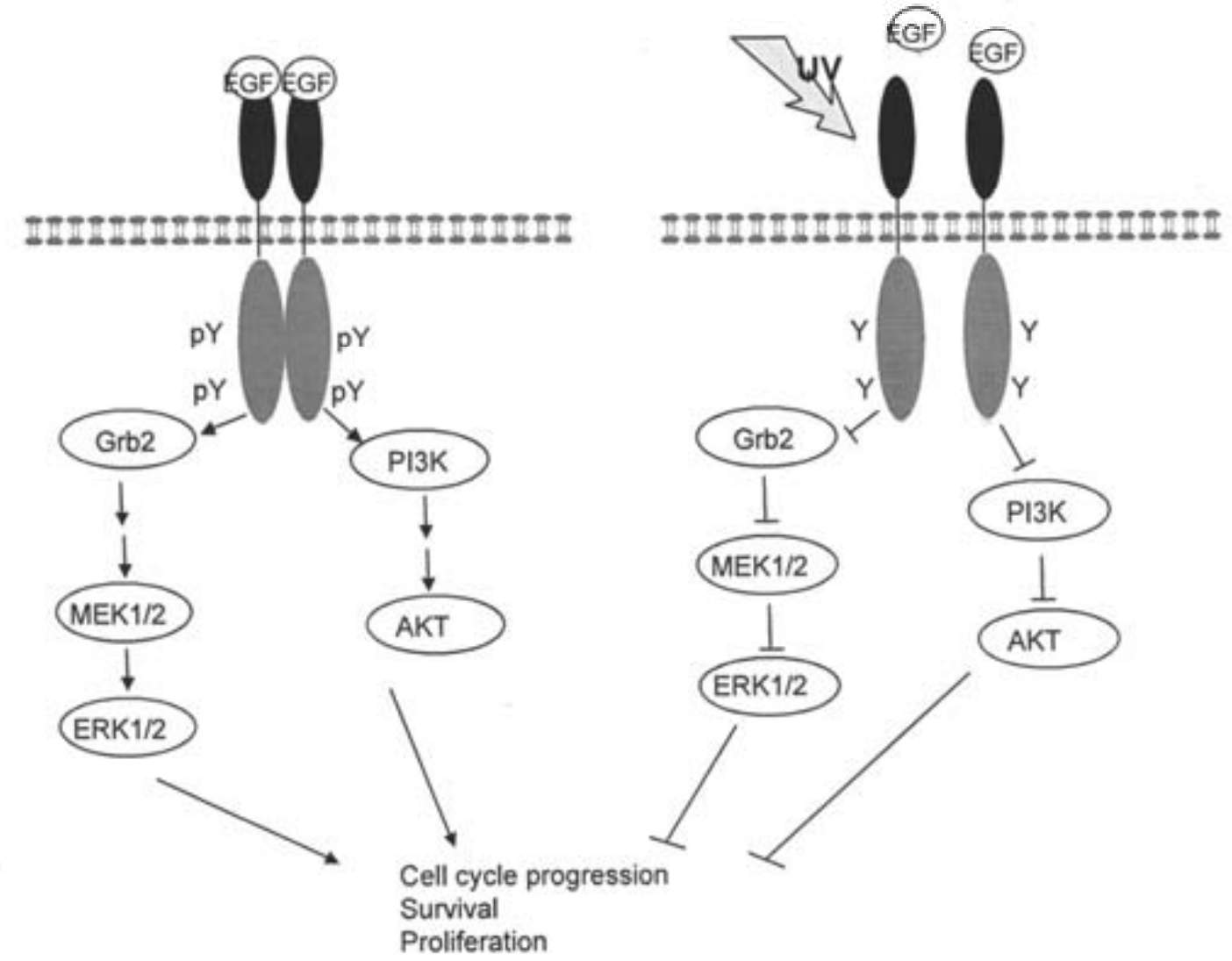


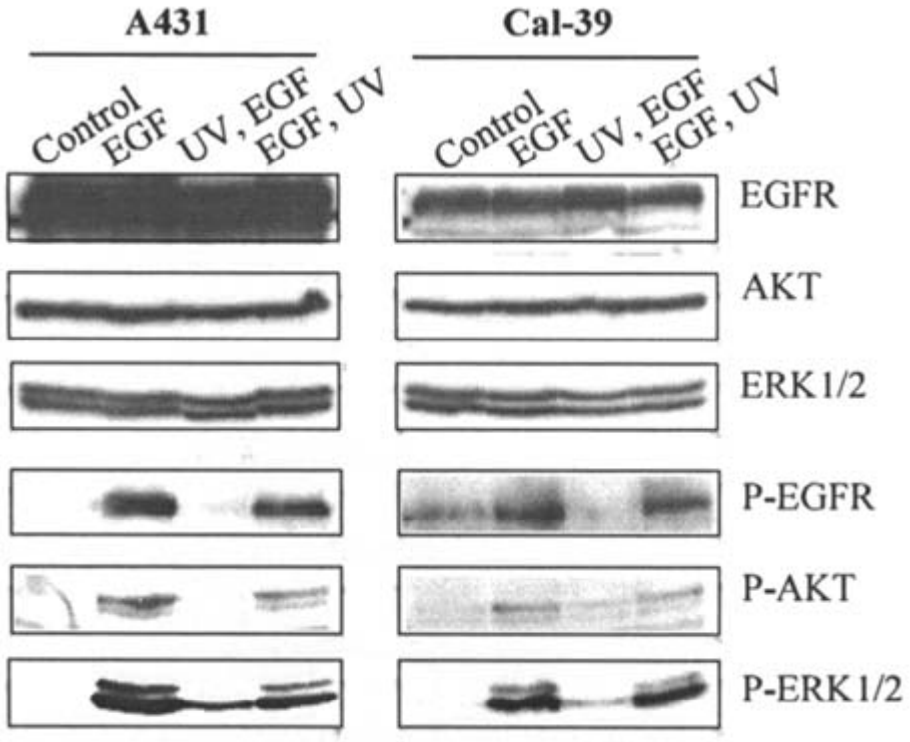
Bioinformatics studies show that **EGFR** (Protein tyrosine kinase receptor) is exceedingly rich in SS bridges and aromatic residues

Biophysical studies show that UV illumination of aromatic residues nearby disulphide bridges leads to the SS disruption.



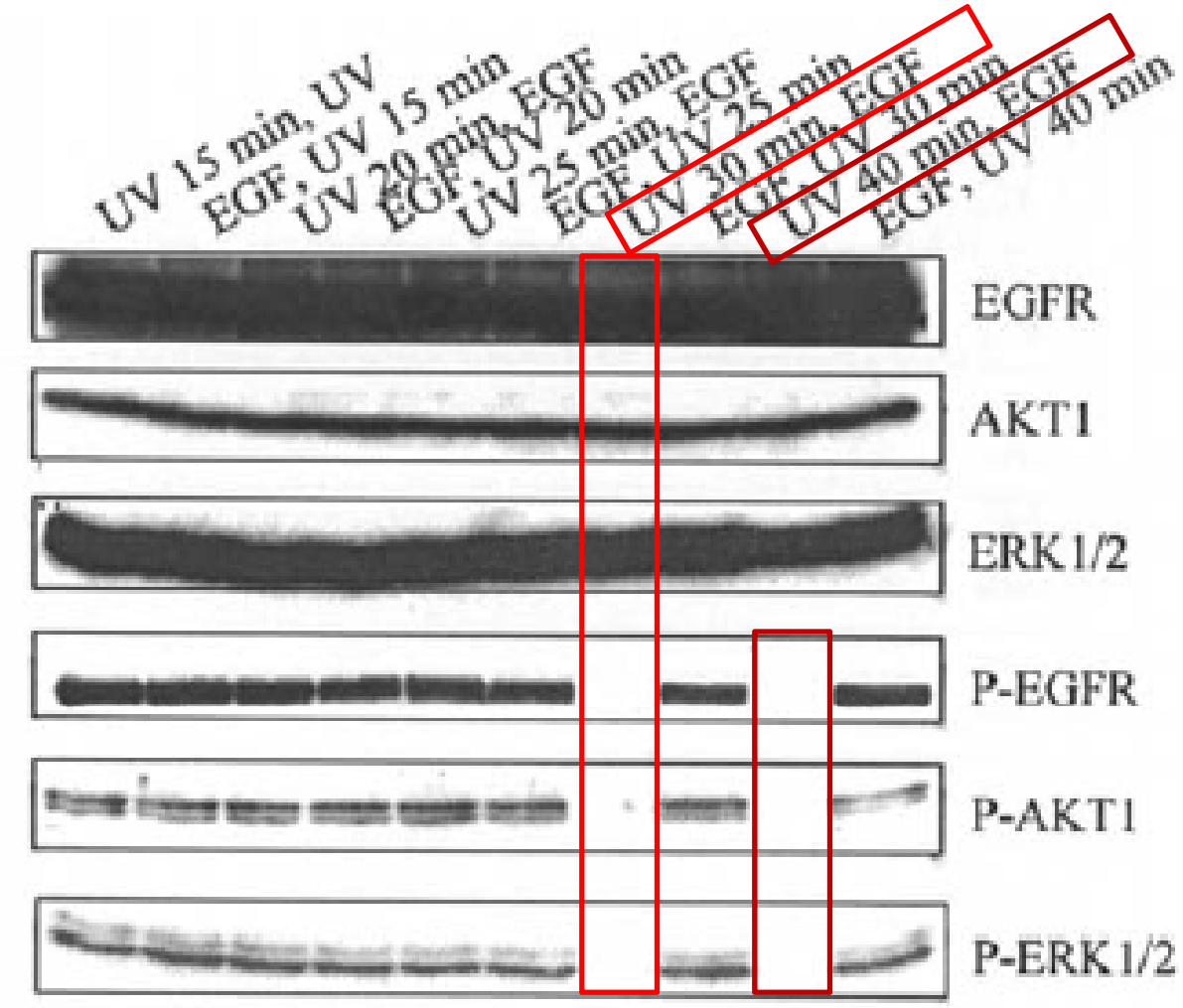
Overview of the cellular pathways affected by the laser-pulsed UV illumination of the EGF receptor leading to attenuation of the EGFR signaling cascade.





A431 cells (human epidermoid carcinoma cells)

Cal-39 cells (human vulva squamous cell carcinoma cells)



Primary antibodies:

Phospho-AKT (T308) (#9275 Cell Signaling) 1:500. Rabbit

AKT (610860 BD Transduction Laboratories) 1:500. Mouse

ERK1/2 (p42/p44) (# Cell Signaling) 1:1000. Rabbit

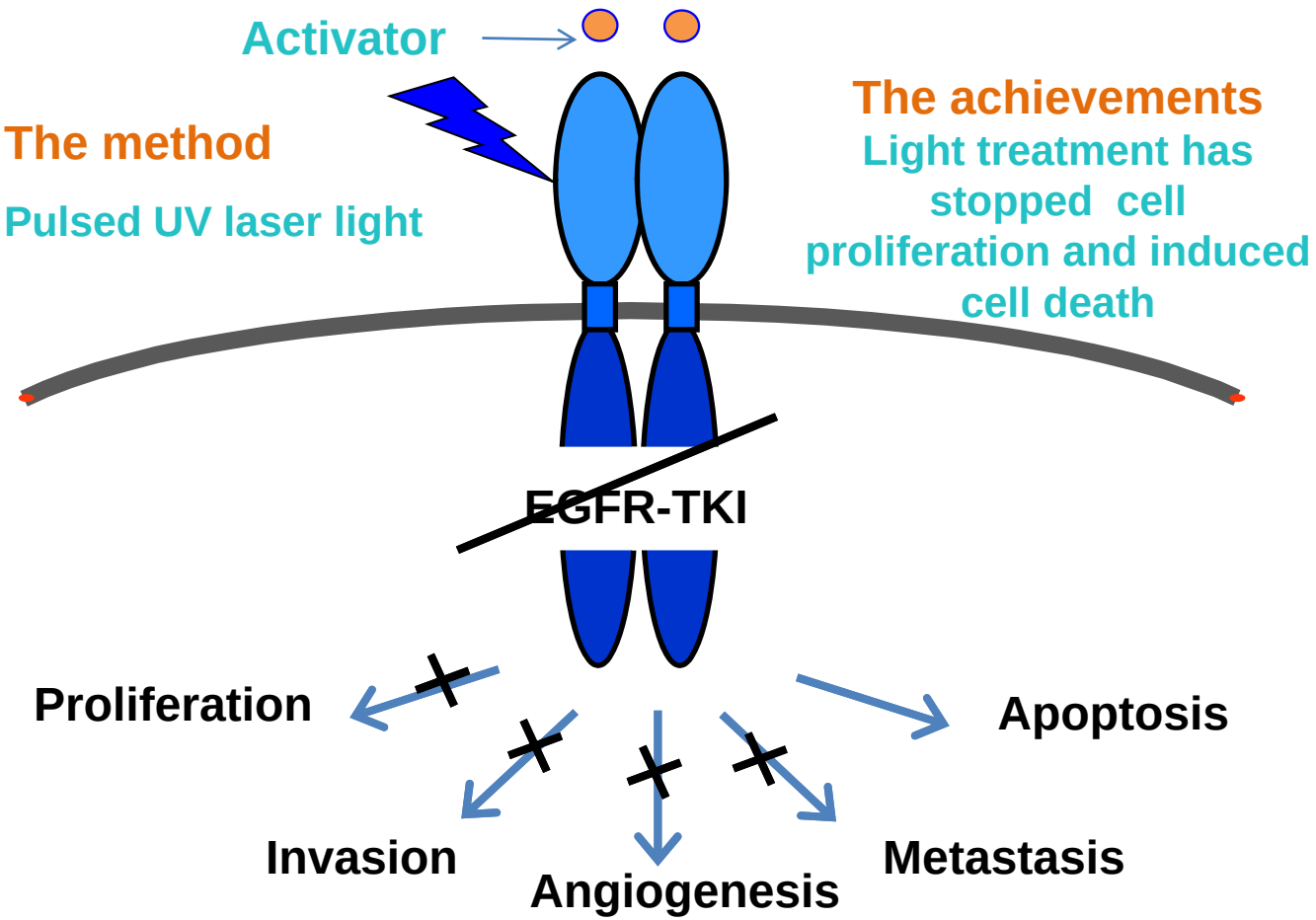
Phospho-ERK1/2 (p42/p44) (# Cell Signaling) 1:1000. Rabbit

EGFR (Santa Cruz) 1:200. Rabbit

Phospho-EGFR (Santa Cruz) 1:100. Goat

Anti-actin (Sigma) 1:10000. Mouse

NANOMEDICINE - Cellular modulation with Light



UV light blocks EGFR signalling in human cancer cell lines, Neves-Petersen and Olsen et al., **International Journal of Oncology. 2007 ; vol. 30, nr. 1, s. 181-185**

The EGFR family of receptors sensitizes cancer cells towards UV light. Petersen et al, **Optical Interactions with Tissue and Cells XIX. Proceedings of the SPIE, Volume 6854, pp. 68540L-68540L-10 (2008).**

Summary

New strategy to induce tumor cell death using UV light

- Autophosphorylation in the intracellular domain of the protein is activated by ligand binding to the extracellular domain.

- So, targeting the extracellular domain is one way to spot signalling and prevent unwanted reactions intracellularly.

- The extracellular domain of ERBB type of proteins are loaded with disulphide bridges nearby Trp and Tyr. Most likely illumination of these proteins with UV light will impair correct signaling and thereby lead to tumor cell death.

- Experiments show that UV illumination has stopped cell proliferation and induced cell death

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  - **Post Doc** Gnana Prakash
  - **Post Doc** Antonietta Parracino
  - **PhD** Ane Kold
  - **PhD** Manuel Correia
  - **Master Student** Sofia Figueiras

DK PT IN

DK PT I

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Institute of Physical Biology, University of South Bohemia, Nove Hrady, Czech Republic

AAU Physics Group - Jens Rafaelsen, AAU

University of Southern Denmark – Prof Olaf Issinger, Cancer research

PT

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Prof. Søren Gregersen, Dr. Per Bendix

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