Super-Resolution Microscopy: Test Samples

Dan Metcalf

Biotechnology Group

National Physical Laboratory

NPL National Physical Laboratory

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

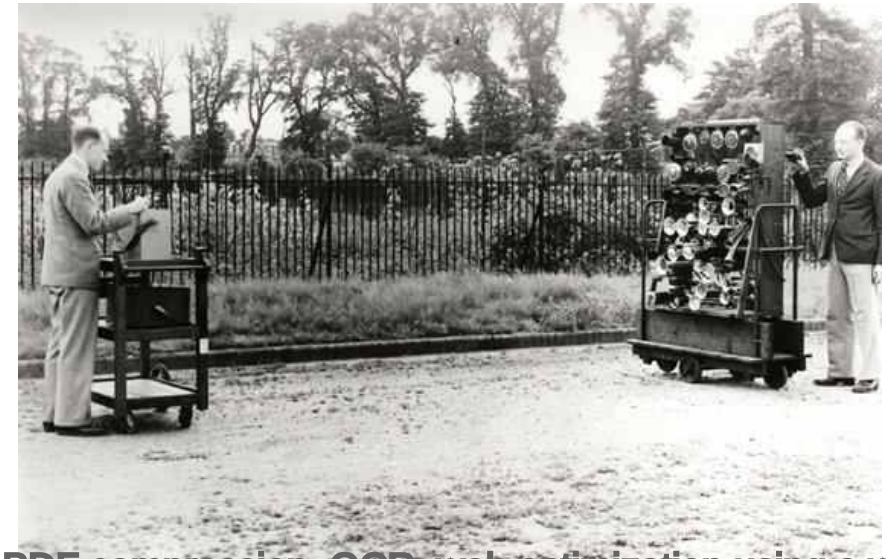
Outline

1. NPL > Biotechnology > Super-resolution
2. Localisation microscope hardware (*d*STORM)
3. Sample preparation (*d*STORM)

NPL National Physical Laboratory

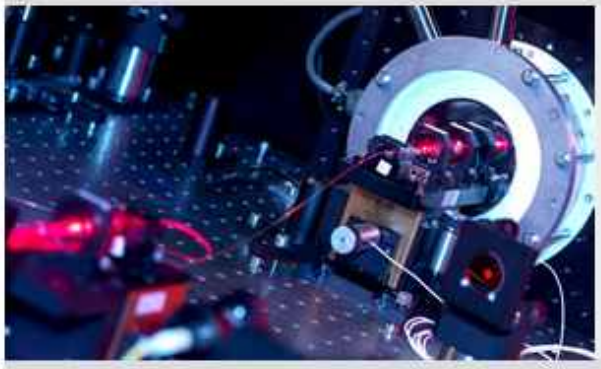
PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

History



PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

NPL



Science & Technology

Excellent science forms the core part of NPL's strategy and underpins all other activities. It is the foundation on which NPL operates and always will be the priority – driving innovation, supporting industry and improving quality of life, both for the UK and internationally.

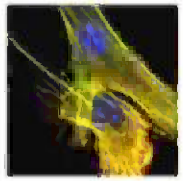
- Acoustics
- Advanced Materials
- Analytical Science
- Biotechnology
- Electromagnetics
- Engineering Measurements
- Environmental Measurement
- Ionising Radiation
- Mathematics & Scientific Computing
- Nanoscience
- Optical Radiation & Photonics
- Quantum Phenomena
- Time & Frequency

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Biotechnology Group

Bioengineering

- Prescriptive peptide design
- Membrane-active peptides (antimicrobial, fusogenic, cell-penetrating)
- Biomolecular self-assembly
- Drug delivery and transfection
- Tissue scaffolds and regenerative medicine
- Biomarker discovery and detection
- Nanoparticle engineering

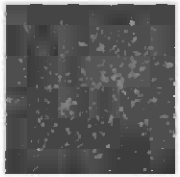


Biodiagnostics

- Point-of-care testing approaches
- Analyte multiplexing
- Alternative affinity reagents, e.g. aptamers, molecular imprinted polymers
- Standardisation, e.g. troponin I

Biophysics

- Nanoparticle-cell interactions
- Protein and membrane biophysics
- Protein misfolding and neurodegenerative diseases
- Biopharmaceutical stability



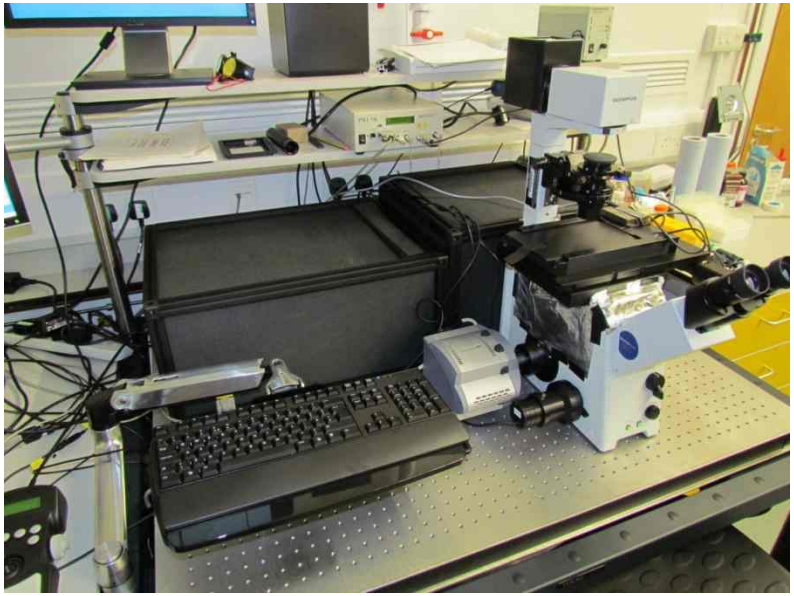
Biophotonics

- Optical wavefront modelling
- Adaptive optics in bioimaging
- Optical coherence tomography and tissue diagnostics
- Super-resolution fluorescence imaging of cells
- Point-of-care device instrumentation
- Single molecule imaging

For general enquiries, please click here to contact us

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Super-Resolution



Localisation Microscope

➔ Blinking or fluctuating signal

- dSTORM - dyes
- PALM - FPs (PA & PS)
- SOFI - dyes, FPs & QDots
- BaLM - any fluorescence*

**needs to bleach or blink*

Structured Illumination Microscope

➔ Continuous fluorescence

- Flexible fast multi-colour 3D design
- Live cell imaging

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Super-Resolution People

NPL National Physical Laboratory

Alex Knight
Super-resolution PRS, software
Miklos Erdelyi
Optics
Dan Metcalf
Cell biology, sample preparation, user
Kevin O'Holleran
Optics, software
Mike Shaw
Optics, software
Neelam Kumarswami
Biochemistry, sample preparation, user
Rebecca Edwards
Biology, sample preparation, user

Clemens Kaminski
Laser Analytics PI
Miklos Erdelyi
Optics
Eric Rees
Software, chemistry
Simon Jobst
Software
Gabi Schierle-Kaminski
Biology, sample preparation

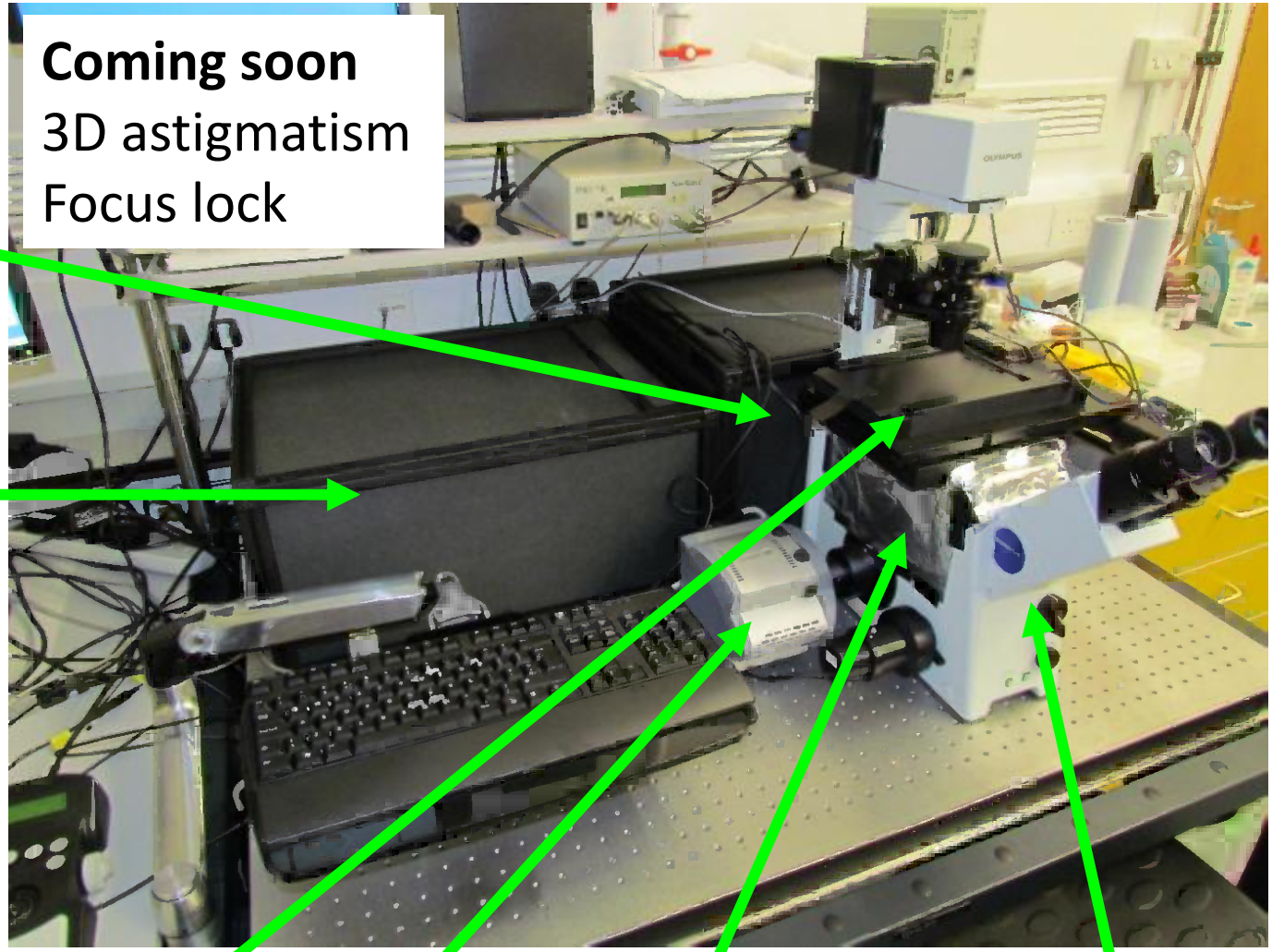
LMCB MRC Medical Research Council
MRC LABORATORY FOR MOLECULAR CELL BIOLOGY

National Measurement Office

EPSRC Pioneering research

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Localisation Microscope



TIRF mirror

TIRF, HILO, Epi illumination

Lasers & optics

405 nm (100 mW)

488 nm (150 mW)

642 nm (150 nm)

Super-continuum (450 nm – 650 nm)

Covered motorised piezo-Z stage

EMCCD

Foil

Inverted microscope

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Test Sample Preparation

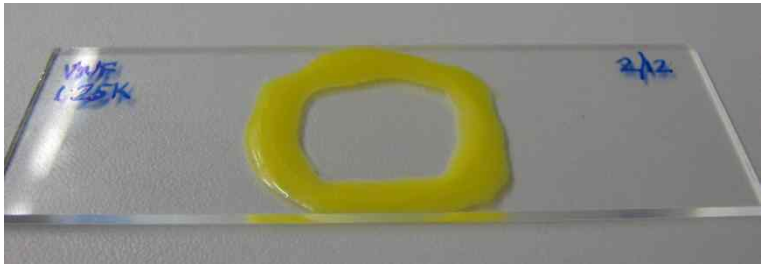
- Choose a test sample
  - Epidermal growth factor (EGF) in HeLa cells
  - Actin – *in vivo* & *in vitro*
  - Dextran – *in vitro*
- Choose a dye(s)
  - Alexa 647, Cy5
  - Atto 488, Alexa 488, Atto 520, Abberior CAGE 500
- Choose a labelling method
  - Immunolabelling - Fab, Fab(2), antibody
  - Direct conjugate – phalloidin, EGF etc
- Choose a format
  - LabTek chamber
  - Coverslips

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Choose a format…

LabTek chamber

Easy buffer exchange



Coverslip & cavity slide

Coverslip flexibility eg. correlative EM

Coverslip & ring chamber

Small coverslips

But grease = movement

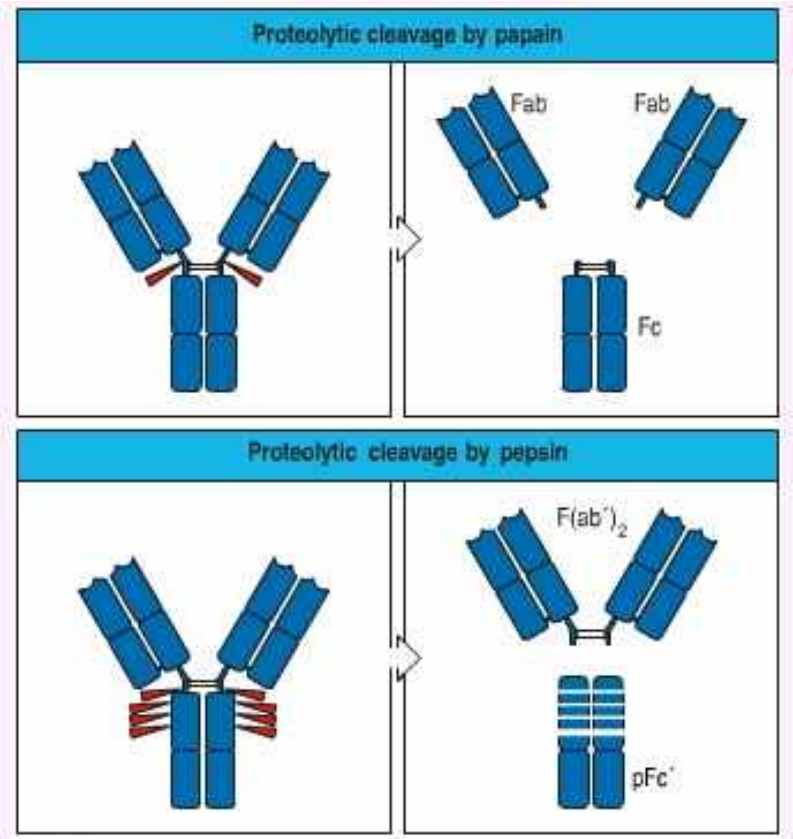
PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Choose a labelling method…

Antibody → $F(ab')_2$ → Fab → Small protein eg. EGF/phalloidin → Direct dye conjugation eg. actin-A647

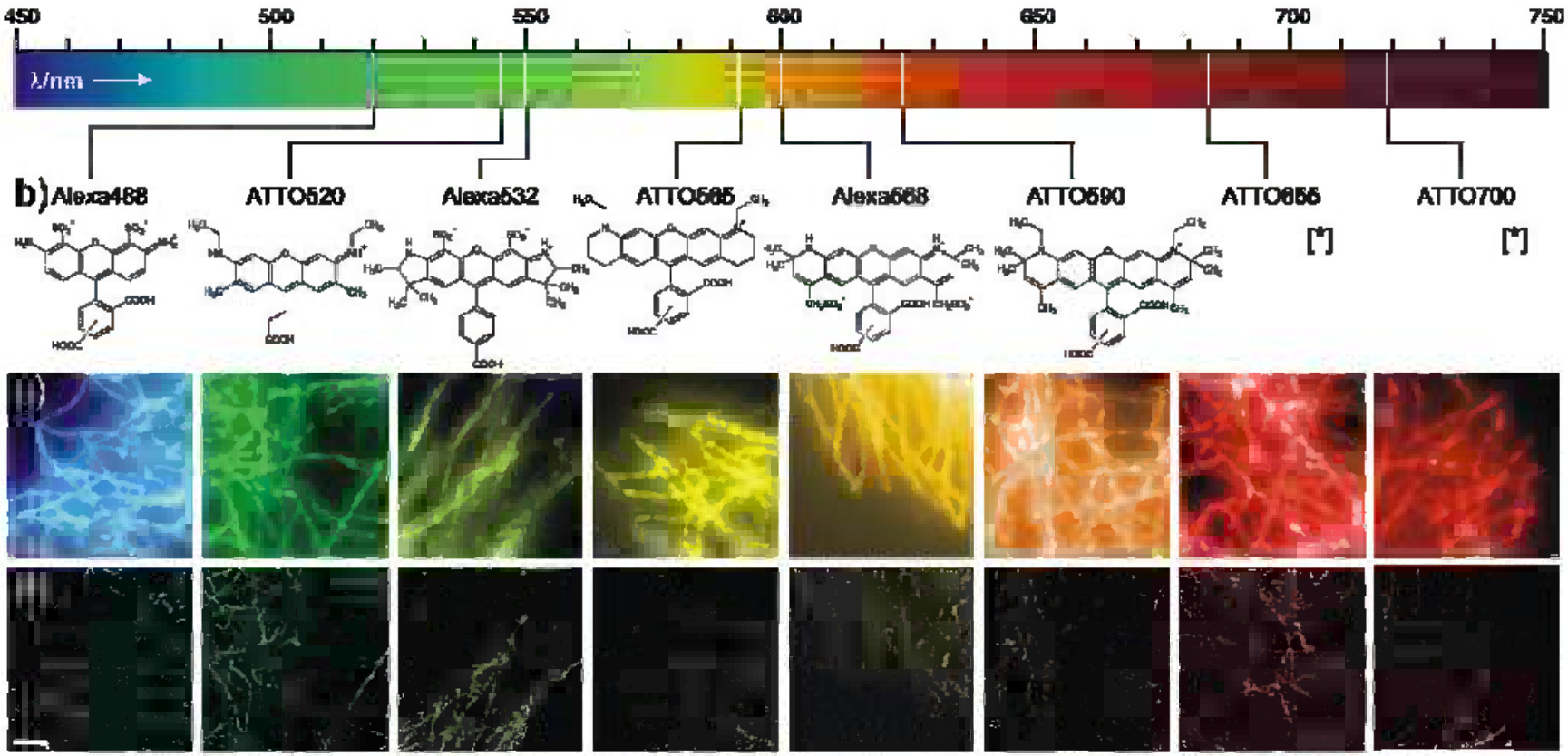
~20 nm = 1 & 2 antibodies

~ 1 nm = direct dye conjugation



PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Choose a dye…



Different dyes:

- Different chemistries
- Different buffers/mounting conditions
- Different acquisition parameters

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging

Graham T Dempsey[1,6], Joshua C Vaughan[2,3,6], Kok Hao Chen[3,6], Mark Bates[4] & Xiaowei Zhuang[2,3,5]

Table 1 | Summary of switching properties of the 26 dyes tested in this study

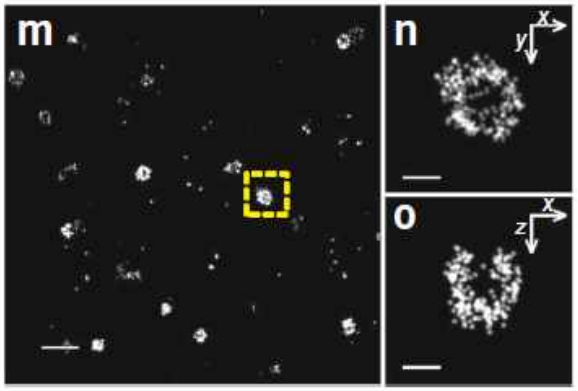
					Detected photons per switching event		Equilibrium on-off duty cycle (400–600 s)		Survival fraction after illumination for 400 s		Number of switching cycles (mean)	
Dye	Excitation maximum (nm)[a]	Emission maximum (nm)[a]	Extinction (M^{-1} cm^{-1})[b]	Quantum yield[c]	MEA	βME	MEA	βME	MEA	βME	MEA	βME
Blue-absorbing												
Atto 488	501	523	90,000	0.8	1,341	1,110	0.00065	0.0022	0.98	0.99	11	49
Alexa Fluor 488	495	519	71,000	0.92	1,193	427	0.00055	0.0017	0.94	1	16	139
Atto 520	516	538	110,000	0.9	1,231	868	0.0015	0.00061	0.92	0.86	9	17
Fluorescein	494	518	70,000	0.79	1,493	776	0.00032	0.00034	0.51	0.83	4	15
FITC	494	518	70,000	0.8	639	1,086	0.00041	0.00031	0.75	0.9	17	16
Cy2	489	506	150,000	0.12	6,241	4,583	0.00012	0.00045	0.12	0.19	0.4	0.7
Yellow-absorbing												
Cy3B	559	570	130,000	0.67	1,365	2,057	0.0003	0.0004	1	0.89	8	5
Alexa Fluor 568	578	603	91,300	0.69	2,826	1,686	0.00058	0.0027	0.58	0.99	7	52
TAMRA	546	575	90,430	0.2	4,884	2,025	0.0017	0.0049	0.85	0.99	10	59
Cy3	550	570	150,000	0.15	11,022	8,158	0.0001	0.0003	0.17	0.55	0.5	1.6
Cy3.5	581	596	150,000	0.15	4,968	8,028	0.0017	0.0005	0.89	0.61	5.7	3.3
Atto 565	563	592	120,000	0.9	19,714	13,294	0.00058	0.00037	0.17	0.26	4	5
Red-absorbing												
Alexa Fluor 647	650	665	239,000	0.33	3,823	5,202	0.0005	0.0012	0.83	0.73	14	26
Cy5	649	670	250,000	0.28	4,254	5,873	0.0004	0.0007	0.75	0.83	10	17
Atto 647	645	669	120,000	0.2	1,526	944	0.0021	0.0016	0.46	0.84	10	24
Atto 647N	644	669	150,000	0.65	3,254	4,433	0.0012	0.0035	0.24	0.65	9	39
Dyomics 654	654	675	220,000	–	3,653	3,014	0.0011	0.0018	0.79	0.64	20	19
Atto 655	663	684	125,000	0.3	1,105	657	0.0006	0.0011	0.65	0.78	17	22
Atto 680	680	700	125,000	0.3	1,656	987	0.0019	0.0024	0.65	0.91	8	27
Cy5.5	675	694	250,000	0.28	5,831	6,337	0.0069	0.0073	0.87	0.85	16	25
NIR-absorbing												
DyLight 750	752	778	220,000	–	712	749	0.0006	0.0002	0.55	0.58	5	6
Cy7	747	776	200,000	0.28	852	997	0.0003	0.0004	0.48	0.49	5	2.6
Alexa Fluor 750	749	775	240,000	0.12	437	703	0.00006	0.0001	0.36	0.68	1.5	6
Atto 740	740	764	120,000	0.1	779	463	0.00047	0.0014	0.31	0.96	3	14
Alexa Fluor 790	785	810	260,000	–	591	740	0.00049	0.0014	0.54	0.62	5	2.7
IRDye 800 CW	778	794	240,000	–	2,753	2,540	0.0018	0.038	0.6	1	3	127

Excitation wavelength, dichroic mirrors and emission filters used for characterization and imaging for each spectral range were 488 nm, T495LP (Chroma) and ET535/50m (Chroma) for blue-absorbing dyes; 561 nm, Di01-R561 (Semrock) and FF01-617/73-25 (Semrock) for yellow-absorbing dyes; 647 nm, Z660DCXRU (Chroma) and ET700/75m (Chroma) for red-absorbing dyes; 752 nm, Q770DCXR (Chroma) and HQ800/60m (Chroma) for NIR-absorbing dyes, respectively. Dye-switching properties are reported in the presence of GLOX and 10 mM MEA as well as GLOX and 140 mM βME.

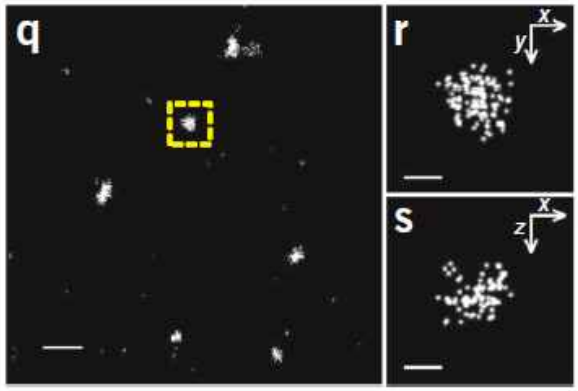
PDF compression, OCR web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Localisation precision

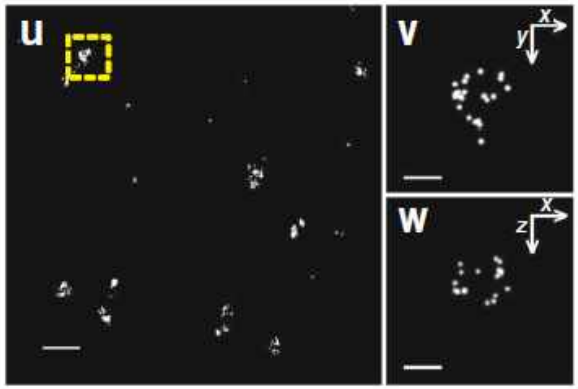
Alexa 647

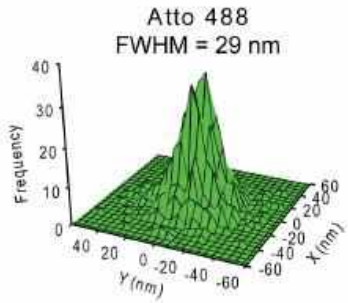


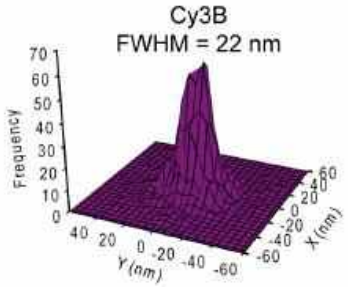
Atto 655

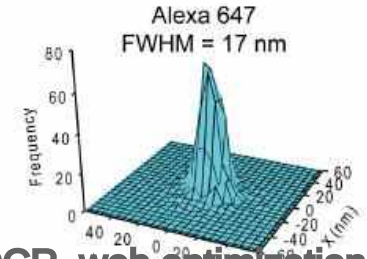


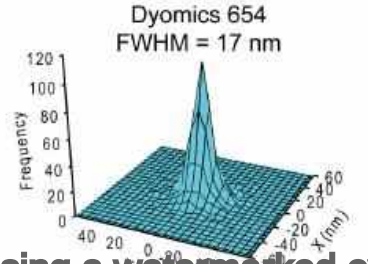
Cy 5.5

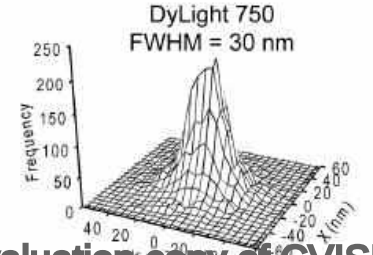












PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Dye issues

- Thiol, concentration, oxygen scavenger?
  - Contradictory protocols published
- Degree of labelling issue
  - Zhuang, Sauer, Hell custom labelled DOL = 1
  - Commerical = 3-8
- Current sample analysis work
  - In vitro samples
  - Software metrics (data quality)
  - Commercial & custom-made dye conjugates (Alexa 488, Atto 488, Atto 520)

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Test sample: EGF in HeLa cells

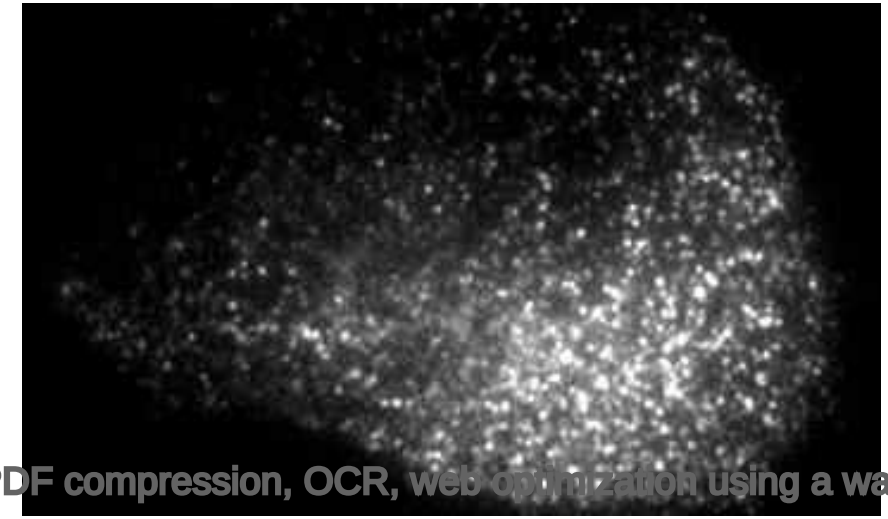
Seed HeLa cells

Staining protocol

1. Fix in 4% formaldehyde for 7 min
2. PBS wash x 2
3. Stain with EGF-Alexa 647
4. PBS wash x 2

EGF labelling

Cell surface receptors ➔ clathrin coated pits

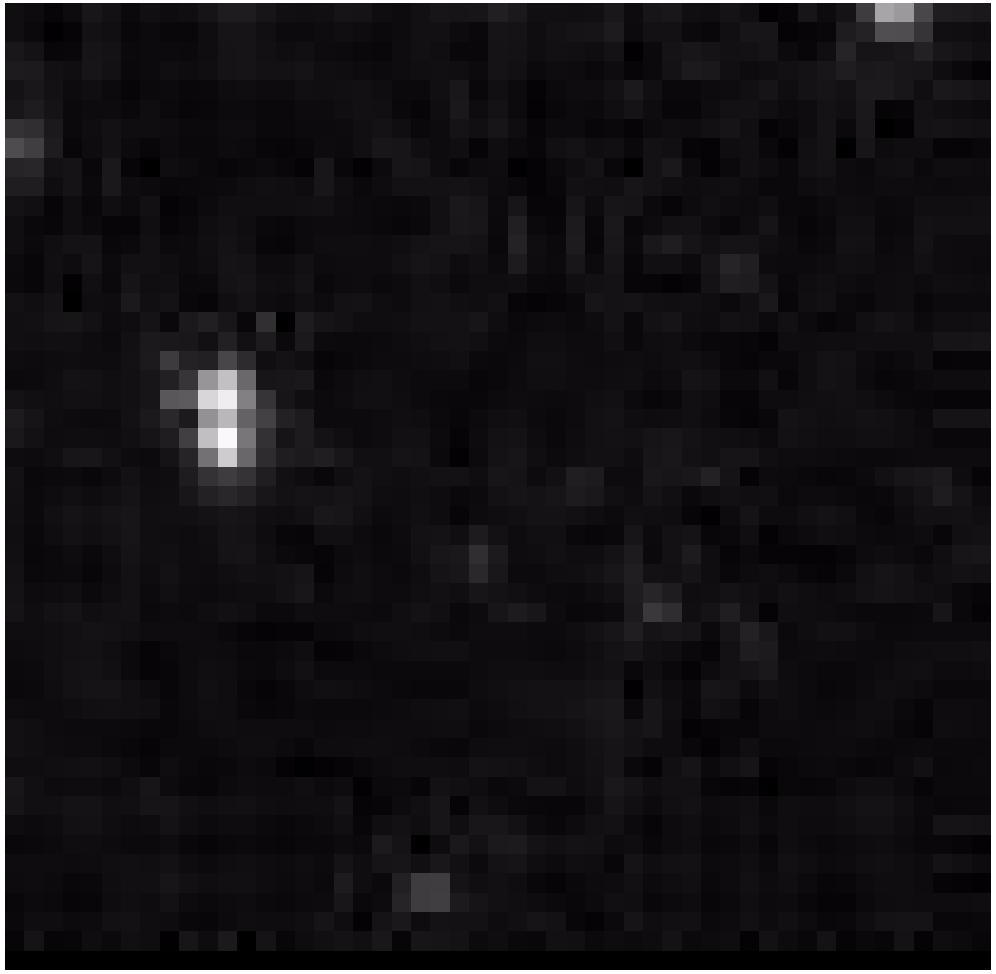


dSTORM imaging

1. Fill with buffer & seal
2. Find a cell of interest
3. Reference image(s)
4. Acquire dSTORM raw data
5. Process to reconstruct super-resolution image

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

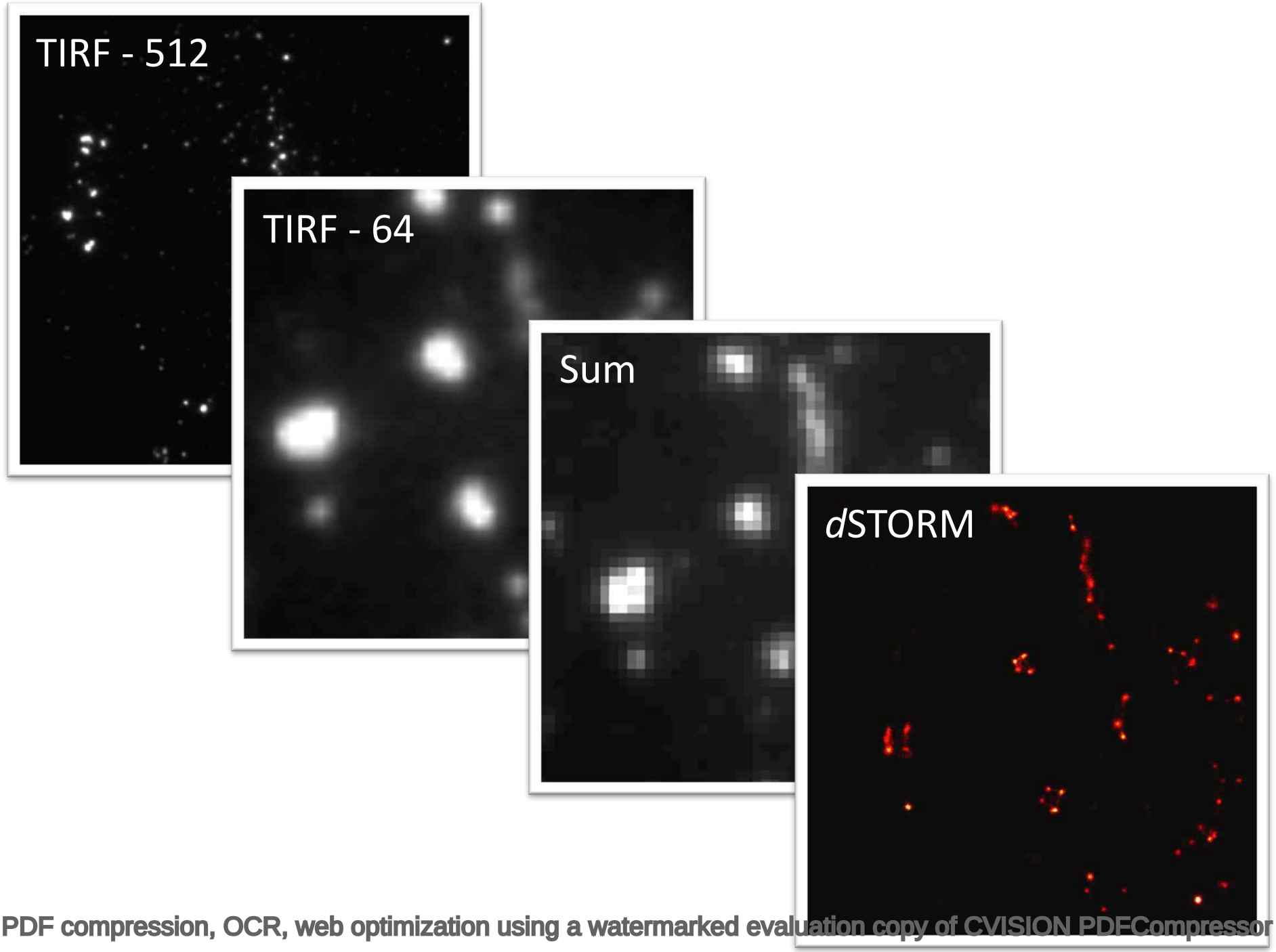
*d*STORM raw data



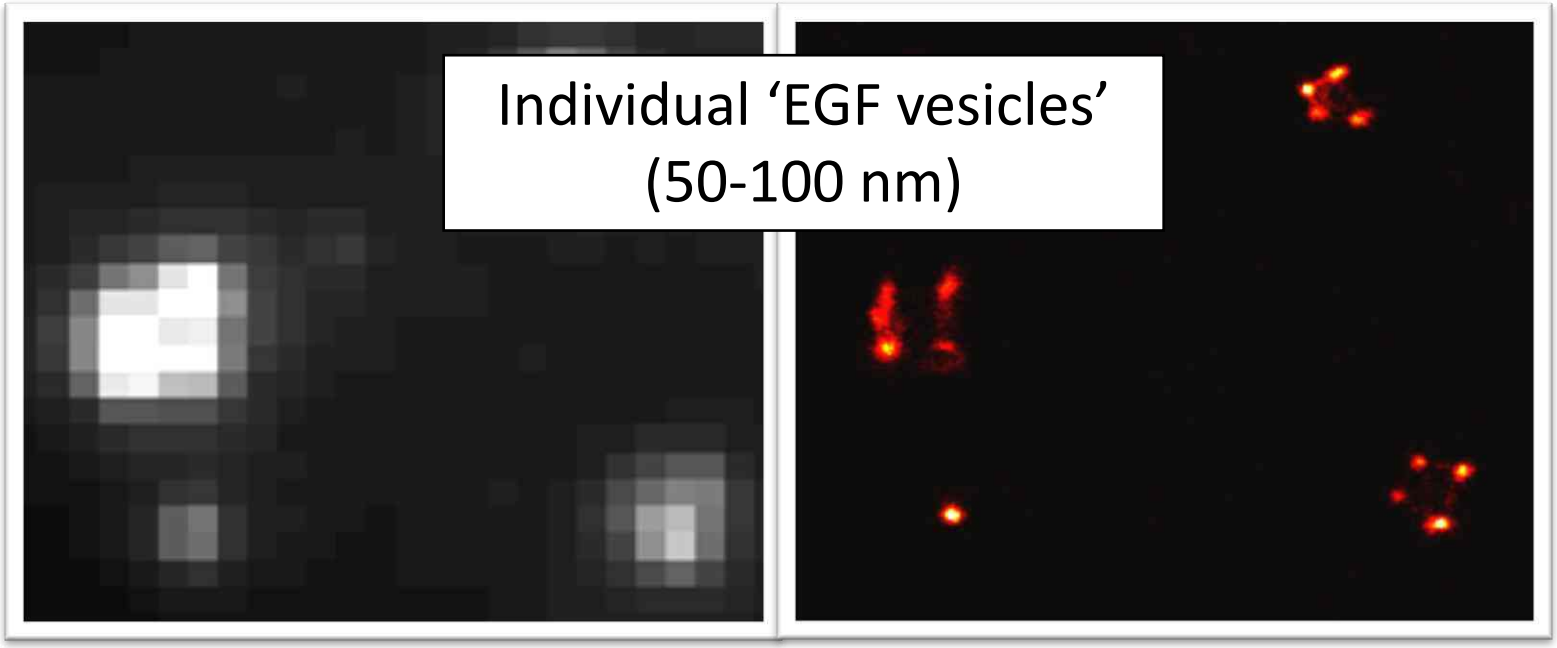
64 pixels = 10.24 µm

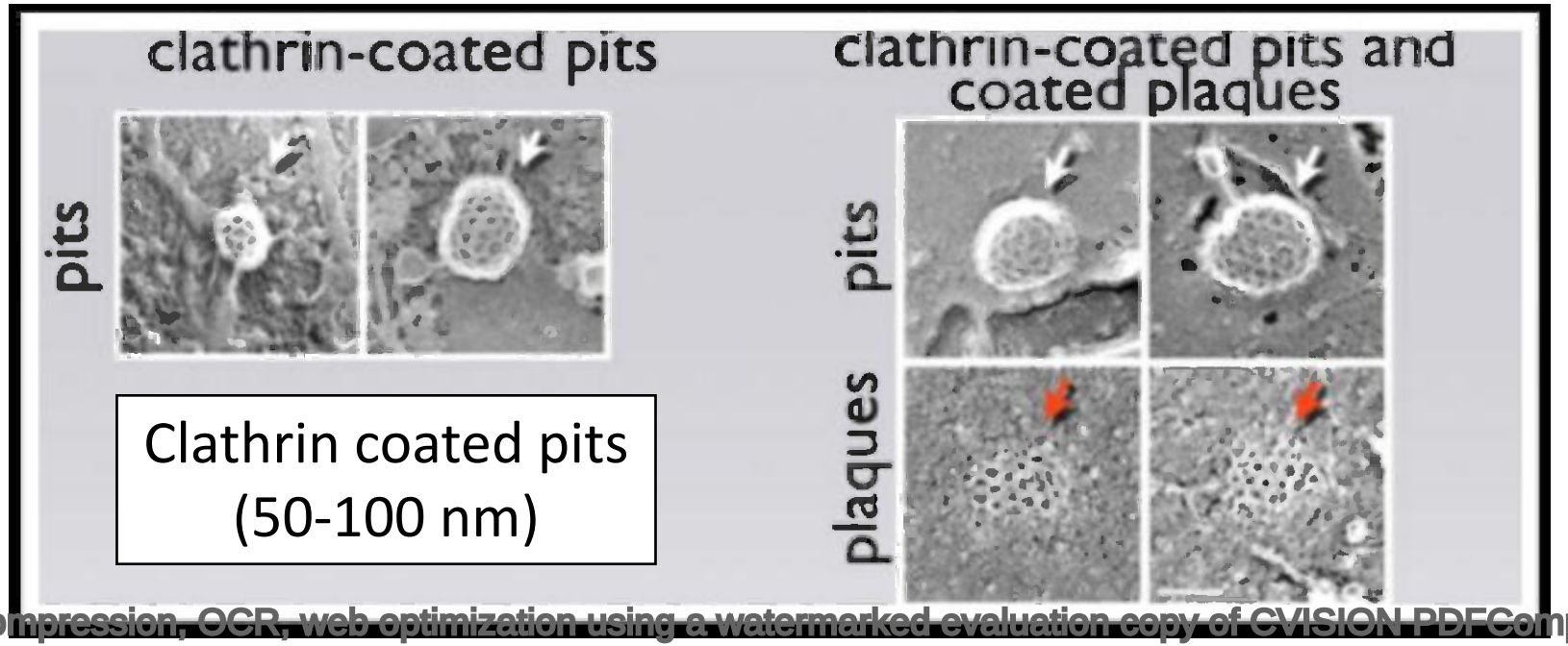
160 nm pixels

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor



PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

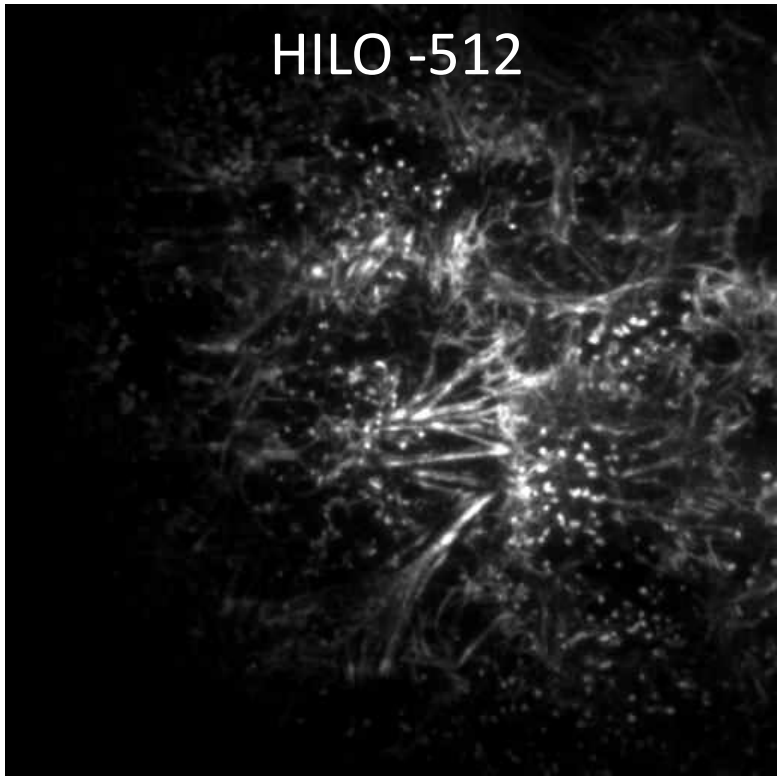


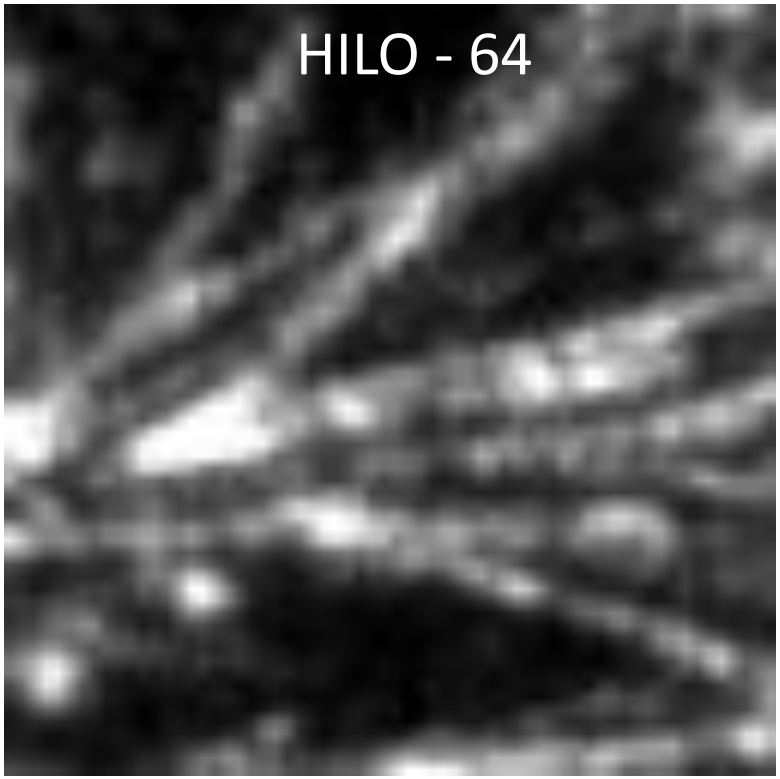


PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

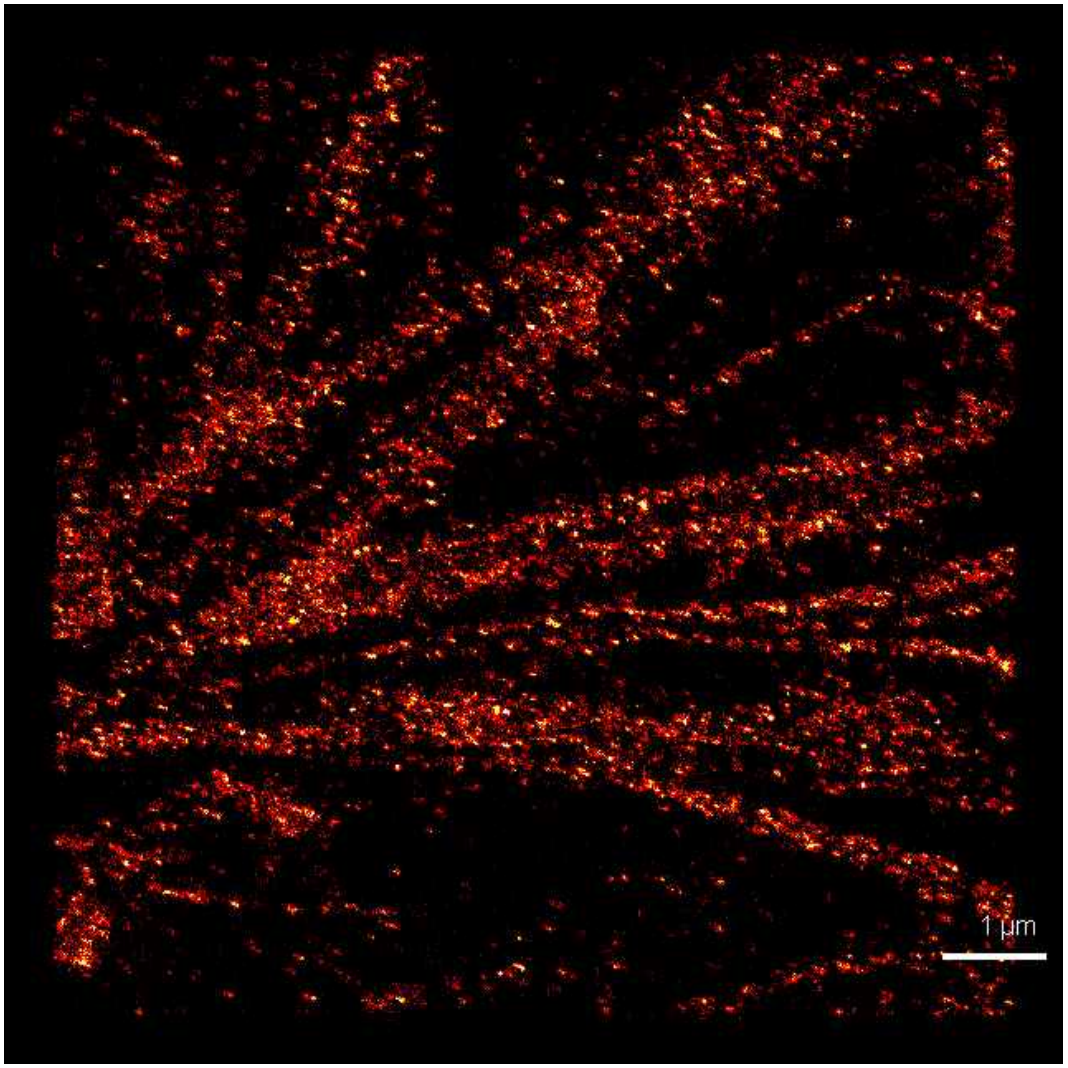
Test sample: Actin *in vivo*

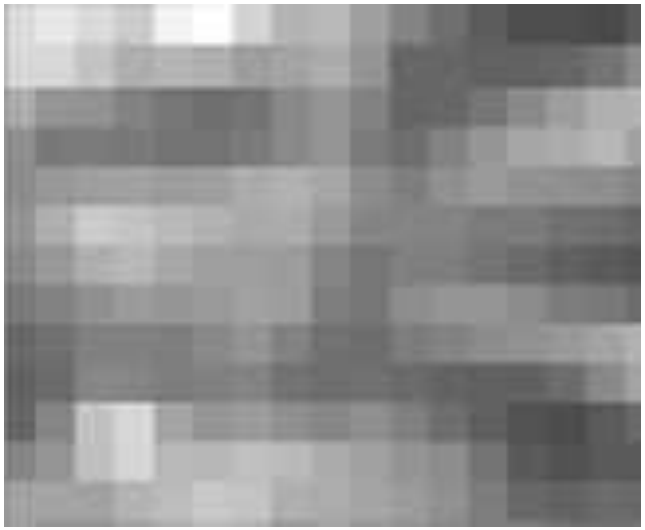
Phalloidin-Alexa 647 HeLa cells

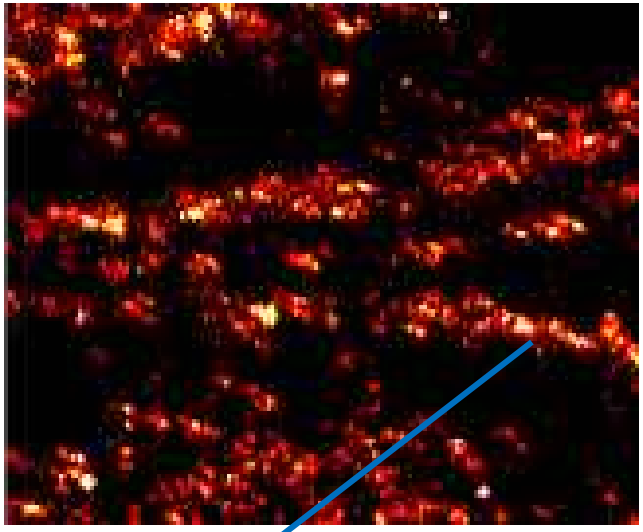




PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor





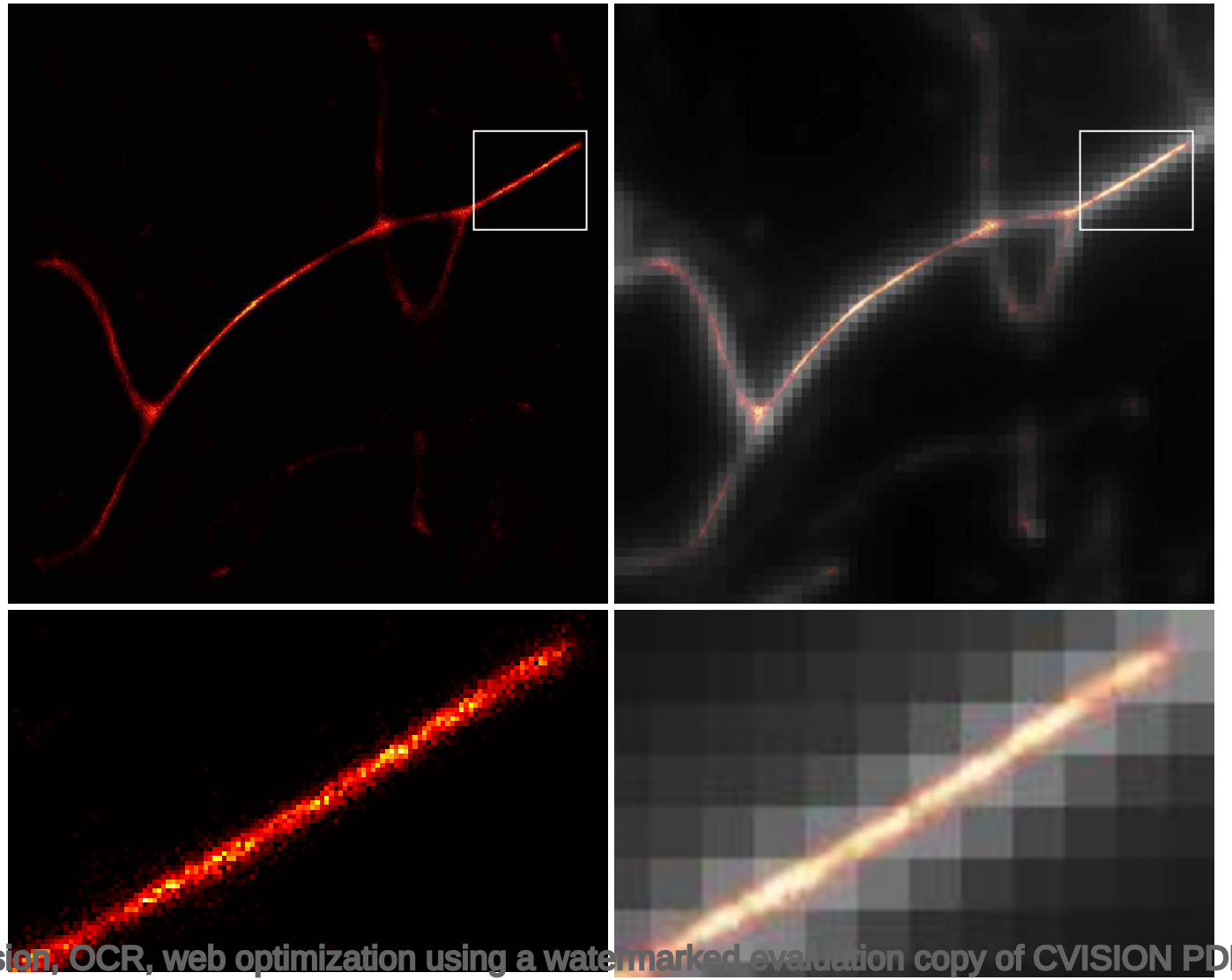


Discontinuous filaments

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

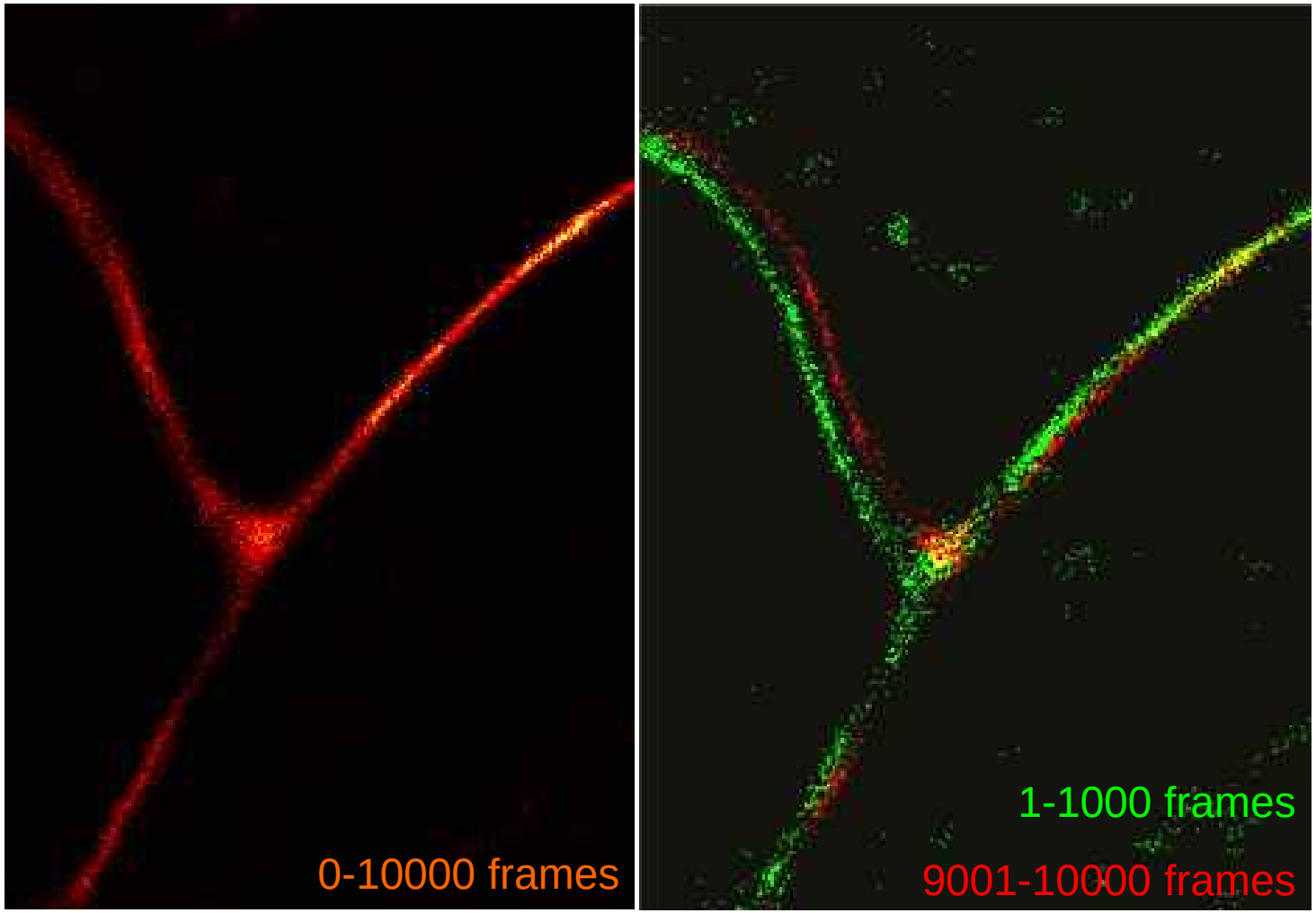
Test sample: Actin *in vitro*

Phalloidin-Alexa 647 labelling in vitro actin filaments



PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Drift Analysis



PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

Standards & References

- Dye testing
- Staining optimisation eg. immunolabelling protocols
- Microscope performance
- Localisation software performance
- Acquisition settings
- Data quality

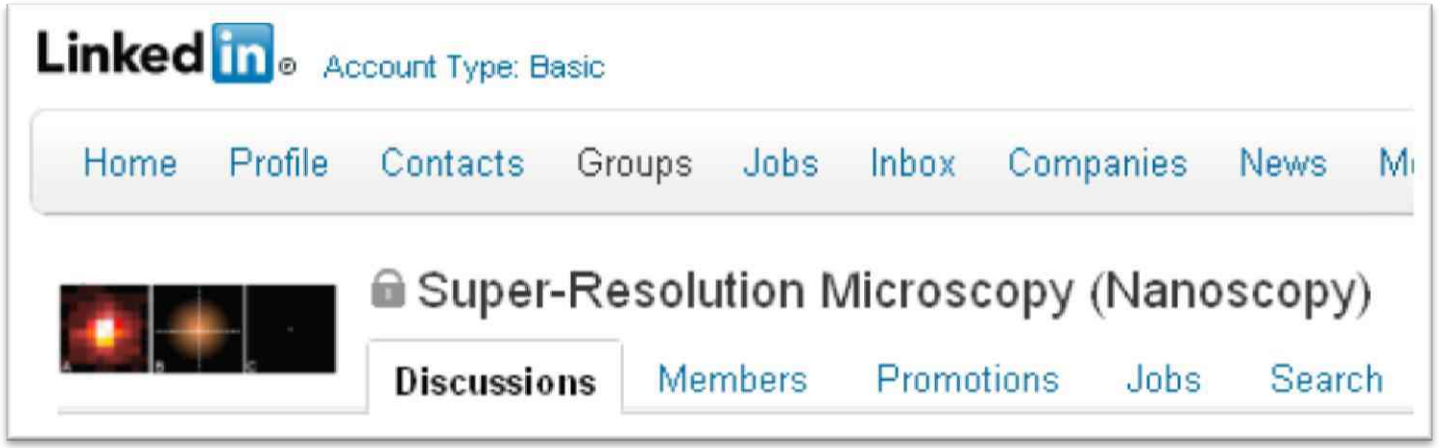
➔ Microscope comparisons (hardware & software)
➔ Microscope maintenance
➔ Training & controls

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor

daniel.metcalf@npl.co.uk

alex.knight@npl.co.uk

www.npl.co.uk/biotechnology



NPL National Physical Laboratory

PDF compression, OCR, web optimization using a watermarked evaluation copy of CVISION PDFCompressor