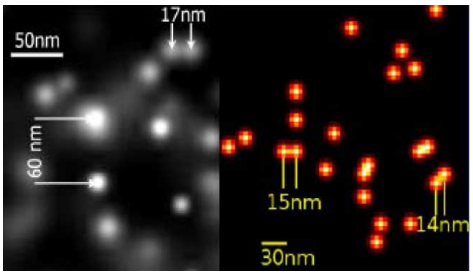
Technologie-Lizenz-Büro (TLB) der Baden-Württembergischen Hochschulen GmbH

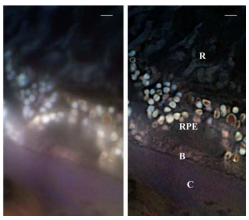


Work in your familiar GFP/YFP/RFP system – from the first experiment to the nanoimage

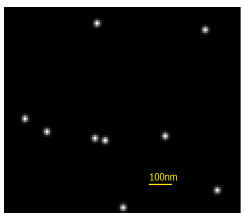
Bwcon business award winner: Inventor Prof Christoph Cremer

Next Level of Super Resolution Fluorescence Microscopy

- **Resolution: down to ~ 5nm in 2D (Dual Color 2CLM);**
  25 nm in 3D Dual Color (*conventional resolution: 200 nm 2D/600 nm 3D)*
- **3D Combination: localization microscopy SPDMphymod + structured illumination SMI**
- **Standard GFP, YFP, RFP (No photoactivation necessary)**
- **Standard fluorochromes (Alexa + Atto dyes, No switch required), Cy2, Cy3, fluorescein**
- **Works with living cells**
- **Wide Field microscope setup with large working distance**
- **Only 1 Laser excitation necessary, economical running**
- **Several super resolution microscope prototypes realized for different approaches**
- **High recording speed: complete 3D stack in 40 sec**
- **Fast image processing: nanoimage available after 2 min**
- **Imaging capability: mean distance between single molecules 6 nm**
- **Density of individually detected molecules ~ 2,8 · 10^4/µm²**

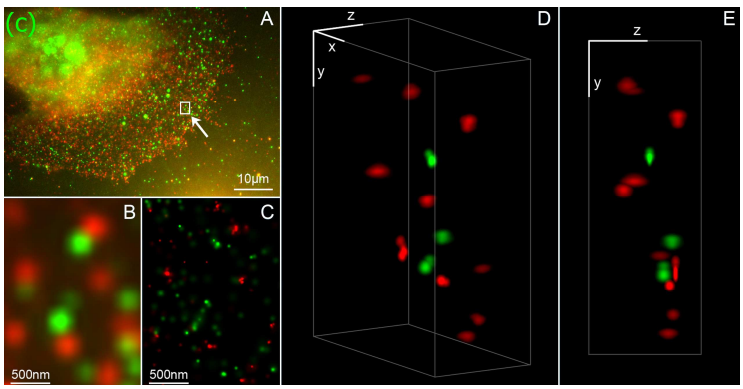


Macular degeneration diagnosis in human eye tissue (AMD)



Single GFP counting in live plant cell

Count single molecules – follow pharmaceuticals in the cell



Breast cancer: 3D Dual color super resolution microscopy of Her-2, Her-3 & cluster analysis

New Milestone in Optical Nanoscopy:

Green Fluorescent Protein (GFP) – High 2D & 3 D Resolution Imaging of Cellular Nanostructures

This light optical nanoscopy approach has the potential to revolutionize the entire molecular biology, medical and pharmaceutical research and allows the development of new strategies for the prevention, risk reduction and therapy of diseases.

From Professor Cremer's developments aimed to surpass the limits of optical resolution (~ 200 nm) postulated in 1873 by Abbe led to the invention of the world's fastest nanomicroscope based on the localization of single molecules (**SPDM**$_{Phymod}$) which allows the wide field investigation of supramolecular complexes under conditions suitable even for living cells.

Professor Cremer realized different prototypes for the different super resolution microscopes he developed (**SMI** structured illumination 2D, 3D, **SPDM** localization microscopy, **3D LIMON** complex labeling, **LSI-TIRF** total internal reflection interferometer with laterally structured illumination).

The Vertico-SMI prototype (see image) for example, is the only nanoscope world-wide capable of recording 3D data of whole cells in less than one minute. Such a high resolution image is produced by a computer from several thousand single images.

The following combination of characteristics makes the Cremer Nanoscopes unique:

- **Wide field of view up to 5000 µm^2 (e.g. several cells; update to 125 000 µm^2 possible to allow analysis of tissue sections)**
- **Highest resolution: 5 nm in 2D, 25 nm in 3D, using visible laser light**
- **Extremely fast compared to the field of view: 40 s for complete 3D images (several thousand individual frames)**
- **Common, well established fluorescent proteins such as GFP, YFP, RFP and standard fluorochromes like Cy2, Cy3, Alexa and Atto dyes, diagnostic dye fluorescein**
- **Co-localisation of 2 - 3 dyes**
- **Up to several million individual molecules detectable in a single field of view (2,8 • 10^4/µm²)**
- **Cells or small animals expressing GFP and many other standard fluorophores can be immediately investigated**
- ***In vivo* nanoimages of cell agglomerations possible + protein cluster analysis**
- **Attomolar sensitivity: Detection of substances in attomolar concentrations**

Biotechnological and medical applications (selection only)

- **Age**-related neurobiological and ophtalmological degenerations
- **Kardiology**: Analysis of ion channels
- **Cancer research**: Analysis of membrane receptor induced cell death
- **Cancer** relevant genome instabilities due to environmental factors
- **Viruses**: binding of viruses to cell surfaces or intracellular spatial distribution
- **Bacteria:** e.g. development of new antibiotics, quality management, very fast detection of pathogens like MRSA or (EHEC) mutants
- **Stem cells:** reprogramming of aging stem cells to achieve renewal of tissue
- **Pharmaceuticals:** Screening and cellular molecular distribution of active substances
- **Personalized medicine:** individual tumor characterisation for therapy validation
- **High through-put system:** integration possible
- **3 D-Analysis** of genome nanostructure and biomolecular complexes supporting essential functions/epigenetics

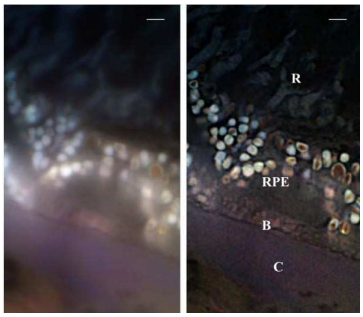
Technical applications (selection only)

- **Materials research**: e.g. analysis of damage on the nanoscale
- **Semi conductor industry:** quality control and research
- **Environmental research**: detection of substances in attomolar concentration
- **Food industry & agribusiness**: quality management + detection of toxins & allergens

The individual methods are under constant development. Several cooperative projects in the biomedical field are underway and further projects are currently being assessed or await final approval.

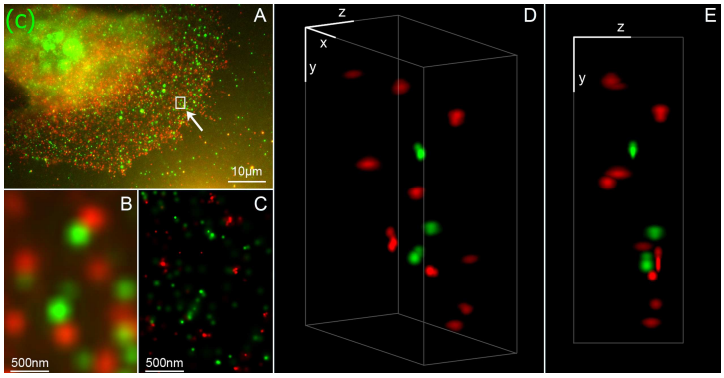
Pharmaceutical & Medical use

AMD age-related macular degeneration



AMD is a medical condition which usually affects older adults and results in a loss of vision in the center of the visual field (the macula) because of damage to the retina Macular degeneration.
Sections from human eye tissue were analyzed with Structured Illumination Microscopy (**SMI**) using a specially designed Super Resolution Microscope setup (**LSI-TIRF**). This SMI technique allowed to acquire light-optical images of autofluorophore distributions in the tissue with previously unmatched optical resolution. Use of three different excitation wavelengths (488, 568 and 647 nm) enables to gather spectral information about the autofluorescence signal.

Target screening & evaluation in pharmaceutical industry



3D **LIMON** (combination of localization microscopy **SPDM** + structured illumination **SMI**) offers the option to label the target molecule with a variety of fluorescent markers of the same type in order to highlight several different areas. Hidden proteins or nucleic acids can made visible without destroying the complex (state of the art: virtual computer simulation models or expensive nuclear magnetic resonance methods to visualize 3D complexes). 3D molecular complexes so-called biomolecular machines (BMM) are responsible for basic functions in the body cells and therefor targets of drugs can thus be studied in vivo.
Example for drug target validation: image: 3D nanoscopy of breast cancer with Her3 and Her2, target of the breast cancer drug Herceptin. In pharmaceutical research, target identification and personalized medicine the super resolution microscopy method LIMON will play an important role in the future

USP – unique selling points

All basic super resolution microscopy methods in 1 patent portfolio:
- **SPDM** localisation microscopy
- **SPDMphymod** localisation microscopy for blinking dyes
- **SMI** structured illumination 2D + 3D (1 or 2 objective lenses)
- **3D LIMON** through combination of localization microscopy + structured illumination
- **3D LIMON complex labeling** for partially hidden complex partners
- **LSI-TIRF**, total internal reflection interferometer with laterally structured illumination
- *Combination of methods possible*

Application patents in the same portfolio:
- **COMBO-FISH** (Fluorescence in situ hybridization) for native intact cell nuclei & pharmaceutical applications (development of personalized therapy in breast cancer)
- Computer simulation to shorten experiments
- High throughput target screening
- *Combination with all super resolution microscopy methods possible*

GFP use of the most common fluorescent protein
- related methods work with specially constructed photo-switchable or photo-activatable fluorescent dyes -> restricted market
- other fluorescent dyes like Alexa + Atto dyes, Cy2, Cy3, fluorescein
- co -localisation with 2-3 fluorophores

Technical simplicity
- simple, economical and universally applicable for the sample preparation
- robust setup, no need for a confocal stand as a basis
- low efforts for service and supervision
- the Vertico SMI prototype runs since 2008 in a routine manner, even used by students
- 1 single laser wavelength of suitable intensity is sufficient - in contradiction special photoswitchable/photoactivatable fluorophores for other SRM techniques need two laser wavelengths

Market for optical nanoscopes

Simplicity

In relation to the optical performance and the vast range of applications, the nanoscopy technologies developed in the Cremer laboratory are extremely economical, the production and maintenance costs of basic versions being far below that of other high end optical microscope systems

Pharmaceutical and molecular biology applications

Research institutes An enormous demand exists worldwide here undertaking biomolecular, medical and pharmaceutical research.
The current state of technology is to work with confocal fluorescence microscopes which however do not provide optical resolution at the molecular level. With just about every middle or large size research institute in these fields owning a confocal fluorescence microscope these institutes are potential customers for an optical nanoscope.

Pharmaceuticals industry: screening of targets/active ingredients. In addition, by counting the individual molecules and their intra- and extracellular spatial distribution with molecular optical resolution, it is possible to show how many of the active ingredient molecules actually reach their target location

Diagnosis: attomolar detection of substances/proteins in the range of 10^{-18}

Hospitals, smaller laboratories, surgeries, forensic institutes: Diagnostics using simplified versions of the nanoscope

Automated high-throughput screening equipment: The nanoscope is fully integratable. Investigations can be undertaken in microtitre plates with 96 or 384 wells. Beside cell nuclei and certain cell areas, it will be also possible to examine whole cells or cell structures, e.g. parts of transparent zebra fish embryos.

Material/Computer science applications

Material research laboratories for example to analyze nanodamage. To this end, fluorochromes can be introduced into fissures to assist the analysis of tiny cracks. The light optical nanoscope investigation is in principle suitable for use with any material on which fluorochromes can be applied or which itself fluoresces (autofluorescence).

Semiconductor industry: quality control and research

Environmental research: detection of substances in attomolar concentration

Patent portfolio: 11 Patent Families

All basic patents have been granted in the USA and Europe, resp. Germany. The patent portfolio covers microscopy, use of standard fluorescent dyes, genome markers, native FISH technologies without prior denaturation, high through-put systems and computer simulation. R & D in the labs of the main inventor leads to further patent applications.

Our Interest in you

We are interested in partners from industry & academia to further develop and commercialize our super resolution microscopy portfolio for new applications and different types of super resolution microscopes

Commercialisation
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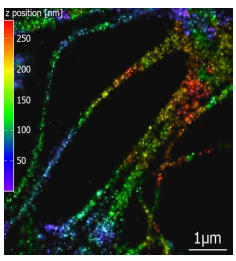


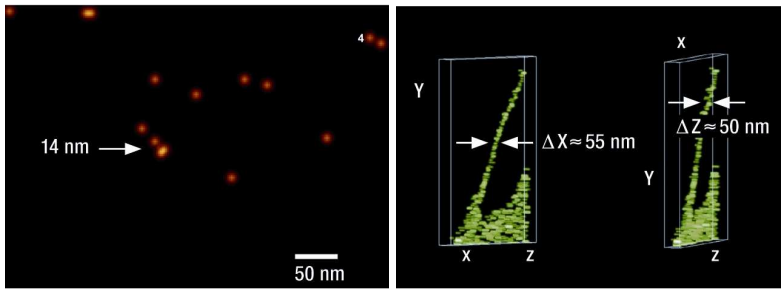
Topographic cell card: 3D cell-cell connection nanoimaging

Super Resolution Microscopy R & D

Prototype of Vertico-SMI: routine applications since 2008

- **Fast + wide + nano + in vivo:** with this combination Vertico SMI is leading the field
- **SMI** (Spatially Modulated Illumination) stands for a special way of laser optical structured illumination and **Vertico** stands for the vertical arrangement of the microscope axis which allows fixed and even living cells to be analyzed with an optical resolution of 5 nanometers in 2D.
- S**PDM** and **3D LIMON** super resolution microscopy can be processed as well
- The first optical nanoscope suitable for routine applications that is sufficiently fast to allow the observation of living cells.

Unique 2D & 3D resolution: Molecules with a separation distance of 6-14 nm are clearly identifiable in 2D (cancer cell, *left* illustration). The **3D LIMON** image of green fluorescent membrane protein GFP was achieved by combining **SMI** and **SPDMphymod**



Image/example: therefore it is possible to record nanoscopic images identifying the positions of thousands to millions of molecules with a 3D resolution up to 25 nm. The smallest measurable 3D distance between molecules is in this example of membrane protrusions is 50 nm (~ 1/10 wavelength; illustration above on the *right*).

Use of naturally GFP & Counting of molecules in extreme wide-field images

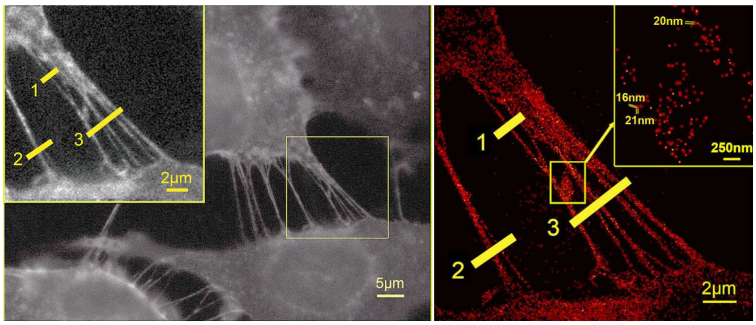
Naturally occurring GFP (green fluorescent protein from jellyfish)

- Possible to use conventional, well established and inexpensive fluorescent dyes from the GFP group, subject of a Nobel Prize in 2008, and its dye variants, to the well-known Alexa, Atto, fluorescein and Cy3/Cy5 dyes without any additional treatment.
- Fundamental to **SPDM$_{phymod}$** are blinking phenomena (flashes of fluorescence), induced by reversible bleaches (metastable dark states).
- Individual molecules of the same spectral emission color can be detected.

Counting individual molecules up to a density of 2,8 • 10^4/µm², this is possible in an area of up to 5000 µm^2 (can be extended to more ca. 125 000 µm^2).

- In a wide field of view, several to many million individual molecules of a given type can be localized using an appropriate instrumental update.

Localisation & Counting importance: Establishing the reactivity of proteins and genes through localization of individual mole-cules (e.g. control of effective- ness of medical drugs on the molecular scale in single cells).



Image/example: Widefield images of membrane protrusions (here on the right 4300 µm^2) in nano resolution is possible (right: spatial resolution of two molecules 16 nm apart). In this section, 15,000 lck tyrosin kinase molecules were counted, labelled with the commonly used fluorescence protein YFP (Yellow Fluorescent Protein)

Size of measurable area In future not only images of cell structures and tissue sections but of whole animals (e.g. nematodes, zebra fish embryos) are anticipated.

Use of GFP - Benefit for researchers

Worldwide several million applications: all gene constructs having a GFP (or RFP or YPF) marker can now be investigated immediately nanoscopically as easily as when using confocal fluorescence microscopy.

Cultivatable cells exist in laboratories worldwide which produce green fluorescent proteins GFP to suit almost any biological or medical investigation.

Many transgenic animals exist which carry green fluorescent fusion proteins, from nematodes and fruit flies to vertebrates including zebra fish, mice and primates.

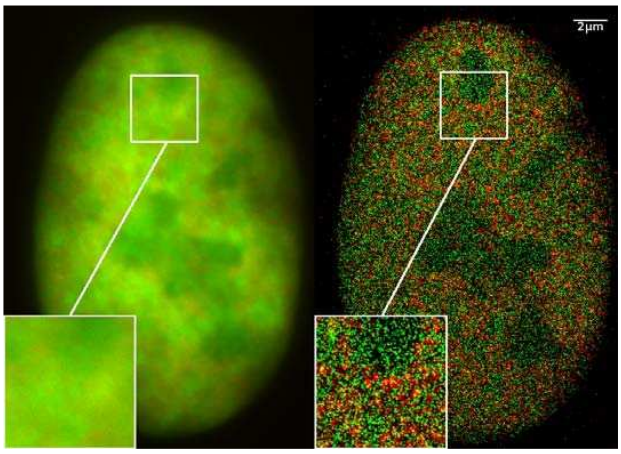
Thus there is a multitude of material for investigation readily available for use without any additional preparation simply as is normally done when employing a normal confocal fluorescence microscope.

Co-localization studies: precise & clearly better than the FRET technology

Protein interaction studies: Due to its high optical single molecule resolution, **SPDMphymod** allows significantly more precise analyses of potential protein interactions than FRET (Fluorescence Resonance Energy Transfer) technology (state of the art). This is of particular significance in studies of biomolecular machines (BMMs) within cells: Single BMMs can be analyzed, including the number of molecules of a given type (proteins, nucleic acids); distances between proteins in these BMMs often are substantially greater than those that can be analyzed by FRET

120,000 individual molecules counted in a cell nucleus (up to 2,8 • 10^4/µm²)

- Extending **SPDM*Phymod*** it is possible to detect 2 - 3 different standard fluorescent molecule types (this technology is referred to as **2CLM**, 2 Color Localization Microscopy)
- 2CLM is the only optical nanoscopy method that allows position based co-localization of single molecules at high density in a wide field of view using conventional fluorescent proteins such as GFP, YFP, RFP, or other conventional fluorochromes.



Image/example: View of a nucleus of a bone cancer cell: using normal fluorescence microscopy, it is not possible to distinguish details of its structure (*left*). Using 2CLM /**SPDMphymod** (*right*) it is possible to localize 70,000 histone molecules (red: RFP-H2A) and 50,000 chromatin remodeling proteins (green: GPF-Snf2H) - in a field of view of 470 µm^2 with an optical depth of 600 nm (each spot represents a single molecule, total 1,2x10^5).

Spatial molecular distribution and number of proteins allow conclusions regarding hot spots of interaction.

Autofluorescence of hidden structures

Label-free cells using SPDM: beside a substantial resolution improvement of autofluorescent structures, SPDM (localization microscopy) revealed cellular objects with are not detectable under conventional fluorescence imaging conditions

Procedure: Conventional wide-field fluorescence image have been taken: objects which are visible as dark spots in the bright-field image are missing in the fluorescence image

- Then **SPDM** was performed with an excitation of 568 nm and 488 nm -> Spherical objects are clearly recognizable at the positions where the dark spots were detected in the bright-field image.