
**New Milestone in Optical Nanoscopy**

# Green Fluorescent Protein (GFP) – High 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. This Vertico-SMI, as it is known, 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 Nanoscope 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: 10 nm in 2D, 40 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**
- **Co-localisation of two dyes of the GFP family**
- **Up to several million individual molecules can be detected in a single field of view**
- **Cells or small animals expressing GFP and many other standard fluorophores can be immediately investigated**
- ***In vivo* nanoimages of cell agglomerations possible**
- **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
- **Stem cells:** reprogramming of aging stem cells to achieve renewal of tissue
- **Pharmaceuticals:** Screening and cellular molecular distribution of active substances
- **High through-put system:** integration possible
- **3 D-Analysis** of genome nanostructure and biomolecular complexes supporting essential functions

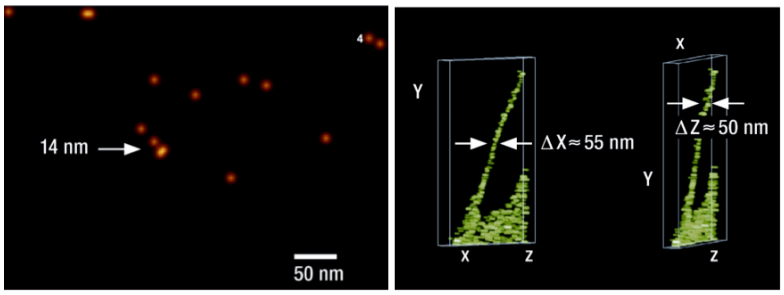
**Technical applications (selection only)**

- **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

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.

## Prototype of Vertico-SMI: The first optical nanoscope suitable for routine applications that is sufficiently fast to allow the observation of living cells

- **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 10 nanometers in 2D.
- In combination with localization microscopy SPDM (Spectral Precision Distance Microscopy) using physically modifiable fluorochromes (Phymod) it is possible to record nanoscopic images identifying the positions of thousands to millions of molecules with a 3D resolution of 40 nm.



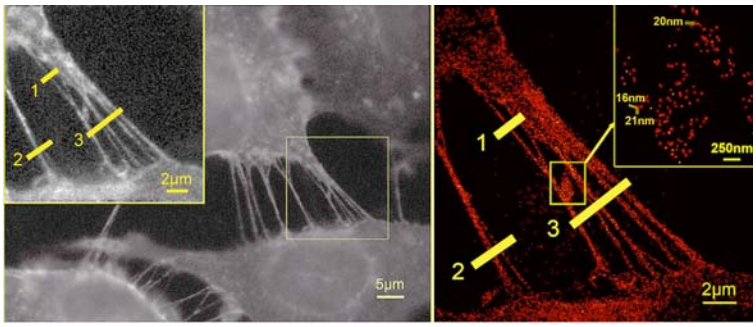

- **Unique resolution:**
  Molecules with a separation distance of 14 nm are clearly identifiable (cancer cell, left illustration). The 3D image of green fluorescent membrane protein was achieved by combining SMI and SPDM. The smallest measurable 3D distance between molecules is in this instance ~ 50 nm (~ 1/10 wavelength; illustration above on the right).

**Advantages compared with similar nanoscopy methods:**

- The US developed *PALM* und *SIM/OMX* techniques also use wide field localization of single molecules (PALM) and structured illumination (SIM/OMX) microscopy techniques but in separate instruments; they do not exhibit such a high overall recording speed as the Vertico-SMI, a significant problem to analyse e,g. 3D images of living cells with high molecular density .
- The *STORM* technology developed at Harvard is fast but requires a pH value that is damaging to living cells.
- Focusing nanoscopic methods such as *STED* and *ISOSTED* achieve fast image acquisition of small areas but would require too much time to acquire an image with a large field of view because many small areas would have to be recorded at the nanoscopic level first.

## Finally possible: Counting of molecules in extreme wide-field images using common fluorescence molecules of the GFP group

- 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 and fluorescein dyes.
- 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 1000/µm$^2$ – at present, 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.
- Establishing the reactivity of proteins and genes through localization of individual molecules (e.g. control of effectiveness of medical drugs on the molecular scale in single cells).
- 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),Lemmer *et al*.,Journal of Microscopy,in press




- Size of measurable area: Future developments by the research team aim at images of areas up to 350 µm x 350 µm (125 000 µm$^2$) through the use of more powerful laser sources and further improvements in optics, detection, and software.

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

**Advantages with respect to comparable nanoscopy methods**:

- PALM, FPALM and related methods work with specially constructed photo-switchable or photo-activatable fluorescent dyes, in contrast to the standard fluorophores used in SPDM$_{Phymod}$
- Our method achieves single molecule resolution at a molecular density that is better by a factor of 30 than conventional light microscopy together with a spatial resolution that is 20 times better.
- A single laser wavelength of suitable intensity is sufficient for nanoimaging by SPDM$_{Phymod}$ ;on the other hand, two laser wavelengths are needed when special photoswitchable fluorescence molecules are used. Thus SPDM$_{Phymod}$ presents an essential technical simplification.
- SPDM$_{Phymod}$ is simple, economical and universally applicable for the sample preparation

**Significant advantage for researchers in biomedical fields***:*

- All of the gene constructs that have a GFP (or RFP or YPF) marker (worldwide several million applications) can now be investigated nanoscopically as easily as when using confocal fluorescence microscopy.
- There exist cultivatable cells in laboratories worldwide which produce green fluorescent proteins 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.

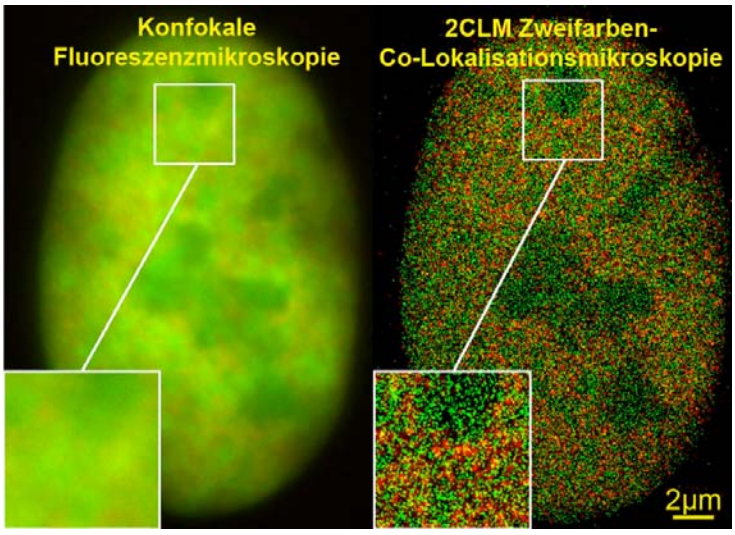
## Colocalization microscopy: Two proteins – two colours
## Where 2CLM is clearly better than the FRET technology

**120,000 individual molecules counted in a cell nucleus**

- Extending SPDM$_{Phymod}$ it is possible to detect two different fluorescent molecule types (this technology is referred to as 2CLM, 2 Color Localization Microscopy)
- In the example shown below, both protein types are labelled with commonly used fluorescence molecules; for each type, measurements are carried out at different fluorescence emission wavelengths
- Spatial molecular distribution and number of proteins allow conclusions regarding hot spots of interaction.

**Advantages with respect to comparable nanoscopy methods**:

- View of a nucleus of a bone cancer cell: using normal high resolution fluorescence microscopy, it is not possible to distinguish details of its structure (image on the left). Using the two Color Localization Microscopy 2CLM (image on the 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. Common fluorescence markers were used.
- 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.
- Due to its high optical single molecule resolution, 2CLM allows significantly more precise analyses of potential protein interactions than FRET-(Fluorescence Resonance Energy Transfer) technology, which is at present the preferred method for such investigations. This is of particular significance in studies of biomolecular machines (BMMs) within cells: Single BMMS can be analysed, including the number of molecules of a given type; distances between proteins in these BMMs often are substantially greater than those that can be analyzed by FRET (restricted to a maximum distance of only a few nm).



## 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

### Biomedical and molecular biology applications

- An enormous demand exists worldwide in **research institutes** 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 (cost approx. Euro 250k – 400 k), these institutes are potential customers for an optical nanoscope.
- **Pharmaceuticals industry:** screening of active ingredients. In addition, by counting the individual molecules and their intra- and extracellular spatial distribution with molecular optical resolution, it is possible to establish how many of the active ingredient molecules actually reach their target location
- **Diagnosis:** attomolar detection of substances/proteins
- **Hospitals, smaller laboratories and surgeries**: 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 is 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.
- **Semi conductor industry:** quality control and research
- **Environmental research**: detection of substances in attomolar concentration

## Patent portfolio

All basic patents have been granted in the USA and Europe, resp. Germany. The patent portfolio covers microscopy, fluorescent dye use, genome markers, high through-put systems and computer simulation.

## Price range and introduction to market

- Prototypes for trial purposes can be manufactured at the University of Heidelberg in the context of collaborative projects.
- Cost of materials per instrument (basic high resolution design): Euro 100k, excl. cost of the camera and laser sources. In comparison, the only optical nanoscope currently commercially available while significantly less powerful costs about Euro 1 million.
- In contrast to other nanoscope designs, our technology can be scaled down: Scaled down versions can be designed for special applications in diagnostic laboratories or surgeries (cost of materials from Euro 10k).
- With a suitable licensee established in the field of optical instrumentation, it would be possible to introduce this nanoscope to the market forthwith.

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