abbelight instrument

CAPTURE THE EVANESCENCE
Your upgradable nanoscope for single-molecule imaging
SAFe 180
From microscopy to nanoscopy

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<th>SAFe 180</th>
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<td>Large illumination TIRF/HiLo/EPI</td>
<td>✔️</td>
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<td>2D Single-molecule localization</td>
<td>✔️</td>
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<td>3D Single-molecule localization</td>
<td>-</td>
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Illumination on large field of view

200×200 μm²

2D STORM nanoscopy

HeLa - actin

Data analysis

SAFe Light

20 μm
With these instruments, researchers can perform any type of single-molecule localization imaging.

**SAFe 360**

Nanoscopy in 3 dimensions

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3D nanoscopy images

**3D STORM nanoscopy**

**Simultaneous multicolor**

Spectral demixing
instruments

Sample details on www.abbelight.com/gallery/
technology
From microscopy to nanoscopy

Revealing structures and dynamics at the nanoscale

Standard fluorescence microscopy techniques (widefield, confocal,…) operate in the resolution range of 200-300 nm laterally and 500-800 nm axially. However, biological structures and processes that occur at a lower scale require superior resolution.

Among recent techniques that break the diffraction limit, i.e. super-resolution techniques, nanoscopy retrieves structural or dynamic quantitative information with the highest resolution achievable in light microscopy.

Stefan Hell, Eric Betzig, and William Moerner were awarded the chemistry Nobel Prize in 2014 for their work on nanoscopy techniques.
**Single-molecule Localization Microscopy (SMLM)** is the nanoscopy technique that retrieves structural or dynamic quantitative information with the highest precision achievable.

**SMLM principle**

SMLM relies on the ability to randomly activate only a subset of fluorescent molecules in order to distinguish them spatially.

By repeating the process in consecutive image acquisitions, accumulated raw data are processed to detect single molecules with a nanometric precision (down to 10 nm).

Data quantification and analysis are then performed to resolve either structures or dynamics at the nanoscale level.

The uniqueness of SMLM is that it gives rise not only to highly resolved images, but also to the 3D coordinates of single molecules, opening up new avenues for spatial and temporal quantitative analysis.

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Tubulin AF647
Sample provided by C. Leterrier
NeuroCyto Lab, France
Localizing molecules in 2D

In order to reconstruct a nanoscopy image, each molecule is detected and localized by specialized algorithms.

To determine the x and y positions of each molecule, a commonly used localization algorithm is Gaussian fitting.

\[
\text{Localization precision} \approx \frac{\sigma}{\sqrt{N}}
\]

\(N=\text{number of photons}\)

\(\sigma=\text{standard deviation}\)

The localization precision is typically 10 nm.

Because images are now obtained at the nanoscale level, new challenges arise. Effects that were negligible at the microscopy level now need to be taken into account.

**Effect of labeling density**

- **High**
  - A → A → A

- **Low**
  - A → A → A

**Effect of drift**

- **High**
  - A → A → A

- **Low**
  - A → A → A

**Resolution** = \(2.35 \times (\text{Localization precision}) \ Θ (\text{labeling density, drift...})\)

*Epifluorescence image*  
*2D nanoscopy image (STORM)*

Sample provided by C. Guillaume

SKB3 - clathrin AF647
abelight
SMLM approaches… from structure to dynamics

Current SMLM approaches only differ in how the fluorophores activation-inactivation is induced. Among them, STORM, PALM and PAINT resolve spatial structures with nanometric precision, while SPT reveals temporal dynamic processes in living cells.

Structures

- **STORM** (STochastic Optical Reconstruction Microscopy)
  - Standard organic fluorescent dyes (cyanines, rhodamines, oxazines...)
  - Specific imaging buffer (containing a reducer, which induces the transition to a dark state, and an oxygen scavenging system to stabilize this state before returning to the ground state)

- **PALM** (Photoactivated Localization Microscopy)
  - Photo-activatable or -convertible fluorescent proteins (mEos3.2, Dendra2, PA-mCherry, mMaple,...)
  - No specific buffer, live-cell compatible

- **PAINT** (Point Accumulation for Imaging in Nanoscale Topography)
  - Specific fluorophores that have the ability to emit fluorescence only upon binding to their biological target (ex: Nile Red, which fluoresces only when interacting with membranes)
  - No specific buffer, live-cell compatible

![Diagram of STORM, PALM, and PAINT processes](image)

Dynamics

- **sptSMLM** combines Single Particle Tracking with SMLM (PALM or STORM) to obtain spatially and temporally highly resolved diffusion maps of single molecules
DAISY technology

**DAISY** is the combination of two complementary strategies developed by abbelight and used to extract the “Z” position of a particle. (Cabrie et al. BioRxiv 2018)

The first approach is called **Magnified Astigmatism** and is inspired by the astigmatic PSF shaping published by Huang et al. (*Science* 2008) and commonly used in commercial systems.

The second approach exploits the near field information encoded in every single emitter, developed and published under the name **DONALD** by Bourg et al. (*Nature Photonics* 2015)

<table>
<thead>
<tr>
<th>3D method</th>
<th>Magnified astigmatism (in dual-view system)</th>
<th>DONALD</th>
<th>DAISY</th>
<th>Standard astigmatism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imaging depth</td>
<td>5-10 µm</td>
<td>0.5 µm</td>
<td>5-10 µm</td>
<td>5-10 µm</td>
</tr>
<tr>
<td>Capture range</td>
<td>1 µm</td>
<td>0.5 µm</td>
<td>1 µm</td>
<td>600 nm</td>
</tr>
<tr>
<td>Lateral loc. Precision*</td>
<td>10 nm</td>
<td>10 nm *</td>
<td>10 nm</td>
<td>10 nm</td>
</tr>
<tr>
<td>Lateral resolution**</td>
<td>23 nm</td>
<td>23 nm</td>
<td>23 nm</td>
<td>23 nm</td>
</tr>
<tr>
<td>Axial loc. Precision*</td>
<td>13 nm</td>
<td>13 nm *</td>
<td>13 nm</td>
<td>22 nm</td>
</tr>
<tr>
<td>Axial resolution**</td>
<td>&gt; 30 nm + focus &amp; drift dependence</td>
<td>30 nm</td>
<td>30 nm</td>
<td>&gt; 50 nm + focus &amp; drift dependence</td>
</tr>
<tr>
<td>Axial drift</td>
<td>Degrade axial resolution above</td>
<td>Not sensitive</td>
<td>Not sensitive</td>
<td>Degrade axial resolution above</td>
</tr>
</tbody>
</table>

* Mean value, for dSTORM imaging using AF647 and abbelight buffer
** Mean value, resolution = 2.35 × (Localization precision) × (labeling density, drift...)

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**COS7 actin – AF647**
Sample of N.Bourg

**abbelight**
Magnified Astigmatism

ASTIGMATIC PSF SHAPING is a very efficient way to extract the relative position of a single particle regarding the focus plane of the objective. Using an anastigmatic lens, a controlled aberration can be induced, measured, and related to the distance between the objective’s focal plane and the emitting particle.

The stronger the aberration is, the better the axial precision is. However, the lateral resolution is also degraded. Therefore, for conventional setups using a single camera, a compromise has to be found between astigmatism strength and X,Y localization precision.

Using a dual camera system, the astigmatic deformation can be enhanced while preserving the best lateral resolution. This is what we call Magnified Astigmatism.

Key features

+ Capture range of 1200 nm
+ Enhanced anastigmatic lens for better Z precision
+ No loss of lateral resolution
+ Two controls for false positive detections
  - Relative to the focal plane: sensitive to axial drift
  - Amplified chromatic aberrations

DONALD

Any single emitter is a dipole, emitting two components of fluorescence:

The far-field emission is a propagative wave, always collected in the low angles of the objective (UAF) and commonly used for any fluorescence microscopy technique.

Although it is often forgotten, dyes also have a near-field emission, which can also be collected in the objective - if the dye is close enough to the coverslip - but in the high angles of the objective (SAF).

Since UAF is constant and SAF decays exponentially, a simple ratio of intensities, for each dye, determines its absolute distance to the coverslip.

Besides its simplicity, the strength of this photophysical measurement is its insensitivity to axial drift or aberration.

Key features

+ Absolute measurement: insensitive to drift
+ No loss of lateral resolution
+ No chromatic aberration
+ Compatible with PSF shaping methods
  - Loss of axial precision above 300 nm
  - Capture range limited to 600 nm above coverslip

Far field emission
Propagative wave
Long range
Always collected in the objective

Near field emission
Evanescent wave
Short range
Can be collected in the vicinity of the coverslip

UAF
Under-critical Angle Fluorescence

SAF
Super-critical Angle Fluorescence

Detection (camera)

Dye (Sample)
Coverslip
Back focal plane (objective)

Intensity [a.u.]

axial distance to coverslip [1/λ]
DAISY technology

DAISY

The combination of DONALD and Magnified Astigmatism. The absolute, drift-free, aberration-free property of DONALD is used to correct the Magnified Astigmatic localization from drift and aberrations, and therefore achieve the true theoretical performances of Magnified Astigmatism.

Absolute VS Relative measurement

DRIFT-FREE
Since the theoretical localization precision is almost the same between DONALD and Magnified Astigmatism, the final resolution of the image will be different because of the axial drift. Even with the best focus control system, small oscillations and large drift of the focal plane position can occur, directly impacting the resolution of the final image.

STATISTICAL ANALYSIS
An absolute axial measurement enables straightforward statistical 3D multicolor analysis, since the reference is always the coverslip surface. It is now possible to compare thousands of acquisitions, on different samples, for different proteins, and easily compile the data*.

* Bouissou et al. ACSnano 2017

<table>
<thead>
<tr>
<th>DONALD &amp; DAISY (absolute)</th>
<th>MAGNIFIED ASTIGMATISM (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 frames</td>
<td>5 000 frames</td>
</tr>
<tr>
<td>( \sigma_z ) 10 nm</td>
<td>( \sigma_z ) 12 nm</td>
</tr>
<tr>
<td>FWHM 23.5 nm</td>
<td>FWHM 28.2 nm (axial drift)</td>
</tr>
<tr>
<td>5 000 frames</td>
<td></td>
</tr>
<tr>
<td>( \sigma_z ) 15 nm</td>
<td>( \sigma_z ) 60 nm</td>
</tr>
<tr>
<td>FWHM 35.2 nm</td>
<td>FWHM 141 nm (axial drift)</td>
</tr>
</tbody>
</table>

0 75 nm

0 250 nm
SAFe 360 optical path

The SAFe 360 module has been designed to integrate and use both Magnified Astigmatism and DONALD at the same time, and therefore DAISY. The optical cubes and specific lenses can be easily replaced to tune the system depending on the user’s needs.

1. Fluorescence from the microscope
2. Beam splitter 50/50
3. Camera 1 for 2D and SAF detection
4. SAF physical filtering for DONALD
5. Strong cylindrical lens to induce magnified astigmatism
6. Camera 2 for 3D astigmatic detection

Mouse hippocampal neuron
Sample provided by C. Leterrier
NeuroCyto Lab, France

SKB3 Clathrin – AF647
Sample provided by C. Guillaume
abbelight
abbelight **SAFe Light** technology offers the **largest field of view** in nanoscopy and requires **lower laser power**.

- 150x150 μm² field of view with 300 mW laser power
- More intensity on the sample with lower laser power
- Illumination adaptable to the sample
- TIRF, HiLo or EPI illumination modes
- 16-times more quantitative data
- Homogeneous illumination, no interference patterns in the image

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COS7 cells – Tubulin AF647
Sample provided by N. Bourg
abbelight

COS7 cells – mitochondria AF647
Sample provided by N. Bourg
abbelight
<table>
<thead>
<tr>
<th></th>
<th><strong>EPI</strong></th>
<th><strong>HiLo</strong></th>
<th><strong>TIRF</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name</strong></td>
<td>Epifluorescence</td>
<td>Highly inclined and laminated optical</td>
<td>Total internal reflection fluorescence</td>
</tr>
<tr>
<td><strong>Type of illumination</strong></td>
<td>In-depth illumination, higher background</td>
<td>Limited background, not restricted to coverslip</td>
<td>Illumination close to the coverslip, removal of in-depth background</td>
</tr>
<tr>
<td><strong>Examples of biological structures</strong></td>
<td>Structures far from the coverslip: nucleus, thick cells, tissues…</td>
<td>Slightly in-depth samples</td>
<td>Structures close to the coverslip: membranes, cytoskeleton, in vitro surfaces…</td>
</tr>
</tbody>
</table>

Rat hippocampal neuron
Sample provided by C. Leterrier
NeuroCyto Lab, France
Simultaneous multicolor

**Multicolor imaging** is a powerful way to assess colocalization between different biological structures.

- Method 1: acquire different colors **sequentially**
- Method 2: use dichroic cubes to allow **simultaneous multicolor nanoscopy**.

Both these methods are possible with SAFe 360. However, they both require excitation with several lasers and compatibility of imaging buffers, and they can lead to chromatic aberrations.
abbelight implemented a technology to perform simultaneous multicolor single-molecule imaging with only one excitation laser: **spectral demixing** (Winterflood et al. Bophys 2015).

This method is **compatible** with a vast array of cell lines and standard commercial fluorophores. For example:

**Spectral demixing principle**
- Excitation with only 1 laser (640 nm)
- Same blinking efficiency
- Detection in **2D** or **3D** on the 2 cameras
- For each localization:
  - Measurement of the **intensity ratio** between the 2 cameras
  - Determination of the wavelength

**Sample provided by C. Guillaume**

![Spectral demixing principle](image)

**Intensity ratio:**
- 75%-25% → AF 647
- 50%-50% → CF 660
- 25%-75% → CF 680

**COS7 cells**
- EdU - AF 647
- Tub - CF 660
- TOM20 - CF 680

Before demixing

After demixing
Abbelight’s NEO SAFe software provides a user-friendly all-in-one workspace for acquisition, processing, and analysis of nanoscopy data.

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<th>Feature</th>
<th>NEO SAFe software</th>
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<tr>
<td>Control of instrument</td>
<td>Laser power and illumination angle (EPI, HiLo, TIRF)</td>
</tr>
<tr>
<td></td>
<td>Cameras</td>
</tr>
<tr>
<td>Control of acquisition parameters</td>
<td>Region of interest size</td>
</tr>
<tr>
<td></td>
<td>Exposure time (down to 10 ms per frame)</td>
</tr>
<tr>
<td></td>
<td>Frame number</td>
</tr>
<tr>
<td>Live reconstruction of nanoscopy data</td>
<td>Choice of localization parameters:</td>
</tr>
<tr>
<td></td>
<td>- Localization algorithms (center of mass, Gaussian fitting, phasor)</td>
</tr>
<tr>
<td></td>
<td>- Intensity threshold</td>
</tr>
<tr>
<td></td>
<td>- Background subtraction method</td>
</tr>
<tr>
<td></td>
<td>Live visualization and reporting</td>
</tr>
<tr>
<td>Live drift correction</td>
<td>Cross-correlation</td>
</tr>
<tr>
<td>Decision-making tools to guide acquisition</td>
<td>Real-time SQUIRREL algorithm, Culley et al. 2017</td>
</tr>
</tbody>
</table>
Nanoscopy data, unlike standard microscopy images, are coordinate-based rather than pixel-based, opening up new avenues for in-depth data analysis.

NEO offers a variety of tools for nanoscopy data visualization and analysis.

<table>
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<th>Feature</th>
<th>NEO SAFe software</th>
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<tr>
<td>Visualization</td>
<td>3D visualization</td>
</tr>
<tr>
<td></td>
<td>Multicolor visualization</td>
</tr>
<tr>
<td></td>
<td>Possibility to export images in TIFF format compatible with commonly used softwares</td>
</tr>
<tr>
<td>Descriptive spatial statistics</td>
<td>Localization distribution</td>
</tr>
<tr>
<td></td>
<td>Measuring tools</td>
</tr>
<tr>
<td>Clustering analysis</td>
<td>K-Ripley function, DBSCAN, Voronoi tessellation</td>
</tr>
<tr>
<td></td>
<td>Centroid, density and volume measurements</td>
</tr>
<tr>
<td>Single-particle tracking</td>
<td>Track reconstruction, quantification of the number of tracks, track duration, diffusion coefficient…</td>
</tr>
<tr>
<td>Spectral demixing</td>
<td>Separation of far-red dyes for multicolor imaging</td>
</tr>
</tbody>
</table>
Clustering analysis

Among several analysis tools, Abbelight’s NEO SAFe software provides several methods to analyze clusters in a dataset.

Determining if a dataset is clustered

The K Ripley function evaluates whether a population of localizations is aggregated or not based on a neighborhood analysis.

The bell-shaped curve indicates the presence of aggregated datapoints and provides an estimate of the size of these aggregates.

Isolating clusters in a dataset: two methods

**DBSCAN** (Density Based Spatial Clustering of Applications with Noise) requires two input parameters: a distance \( \varepsilon \) and a minimum number of neighbors MinPts. For each localization in the dataset, the algorithm searches whether it has enough neighbors MinPts within the distance \( \varepsilon \). If yes, it considers the localization as part of a cluster, etc.

Ester et al. 1996

**Voronoi** partitions the image into polygons, where each polygon contains one, and only one, localization. The area of the polygon is indicative of the density of localizations: a dense region will have small polygons while a low-density region will have big polygons. The user can choose a density threshold, above which localizations are considered as part of a cluster.

Levet et al. 2015

Quantification

Once clusters are identified, the software can quantify: the number of clusters, their localization, their volume, their density, their radius of gyration…

COS7 cells – DNA replication sites
EdU-AlexaFluor647
Sample provided by C. Clément
abbelight
Single-particle tracking analysis (SPT)

To study the dynamics of single particles, NEO software can reconstruct trajectories from SPT raw data.

Reconstructing trajectories

The goal of an SPT algorithm is to connect the localizations from frame to frame. The algorithm takes all the tracks at frame $t$ and all the dots at frame $t+1$ and calculates the probability of assigning each track to each localization. Afterwards, it chooses the solution that maximizes the probability. These probabilities can be calculated based on a number of factors, including distance and motion speed. (Jaqaman et al., 2008, Ségé et al., 2008)

Quantification

After reconstruction of the tracks, the software can quantify: the number of tracks, their duration, their average intensity, their diffusion coefficient (based on Mean Square Displacement analysis)...

Sample provided by Dr. Chiaruttini, University of Geneva, Switzerland
Abbelight technologies have been applied to a number of biological fields - ranging from cytoskeleton architecture to chromatin dynamics - and in a great number of organisms – human cell lines, bacteria or yeast, drosophila tissue...

**Cell Biology**
- Cytoskeleton
- Metabolism
- Membrane proteins
- Transport
- Signaling

**Bacteriology**
- Antibiotic resistance
- Replication
- Membranes

**Immunology**
- Immune receptors

**Neuroscience**
- Axon nanoscale architecture

**Nucleus**
- Replication and transcription
- Chromatin
- Nuclear pores and nuclear envelope