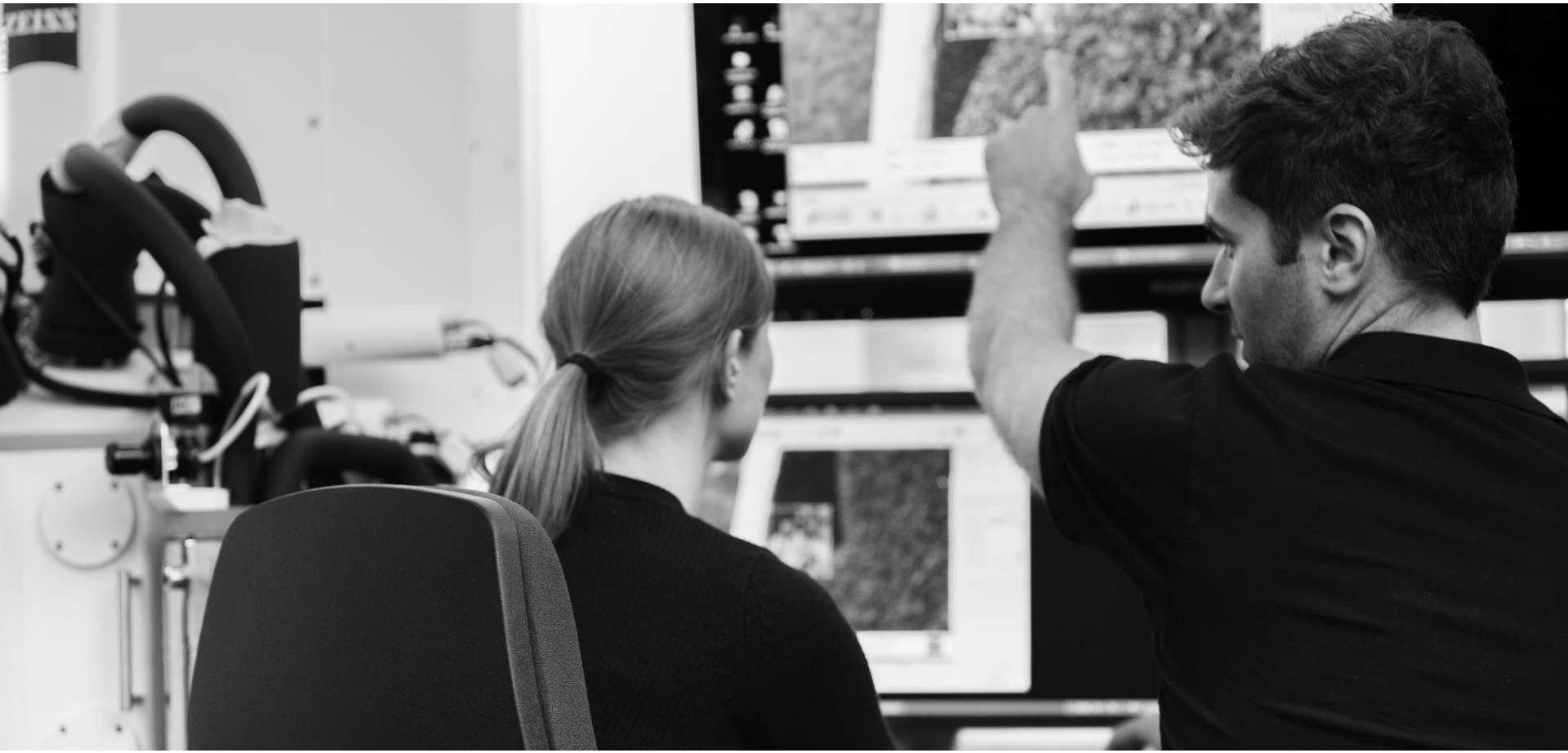
Nutshell – PALM Grouping

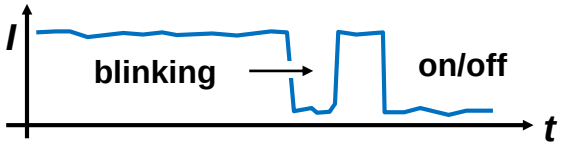


PALM Grouping

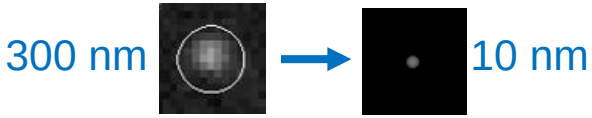
Scope and recommended previous knowledge.

Here we cover **grouping** as one of the tools in PALM / dSTORM process.
Previous knowledge on PALM / dSTORM and ELYRA is recommended.
In particular:

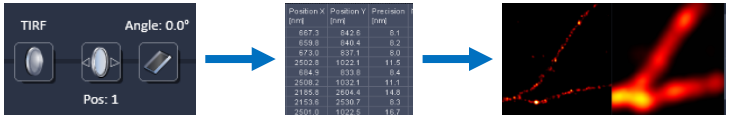
- single-molecule fluorescence behavior (on/off and blinking)



- positioning accuracy and the involved scales of length

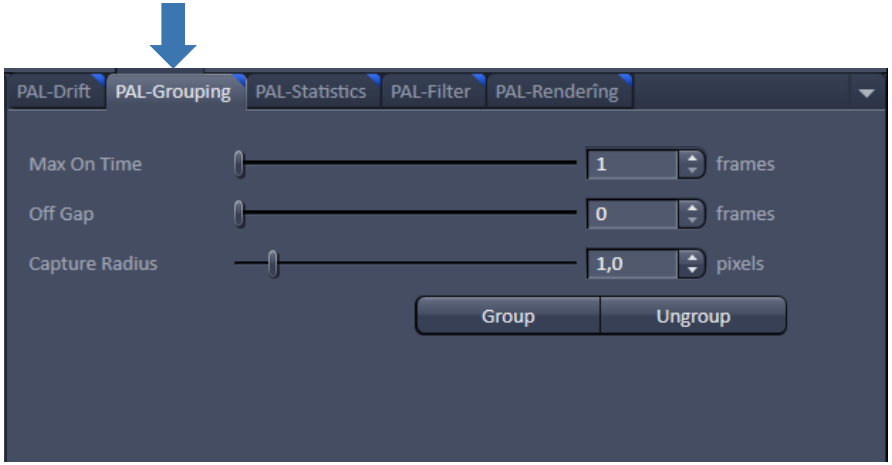


- basic knowledge of the PALM / dSTORM workflow in ELYRA



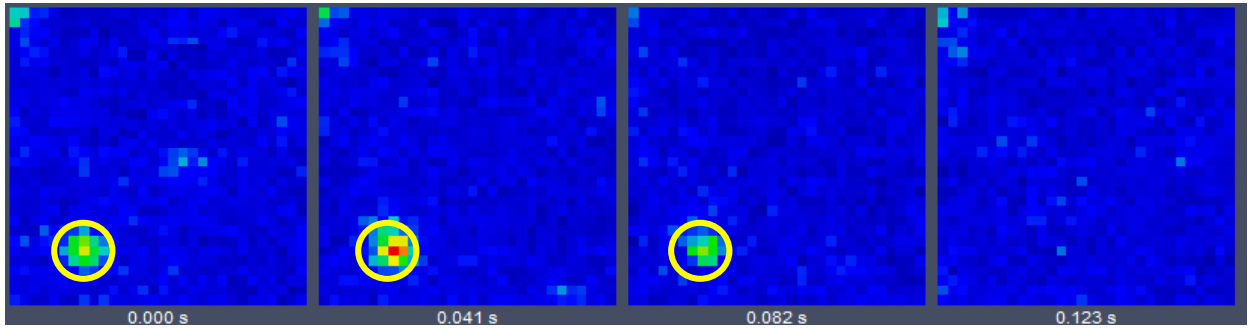
Overview

1. Why? What is PALM Grouping?
2. How does it work? Definitions
3. Additional Remarks



PALM Grouping
Why group?

PALM is based on the frame-by-frame localization of single emitter patterns.



Sometimes a pattern may persist over a couple of frames

Are these patterns coming from the same identical molecule?
If so, can I join the information from these positions?

PALM Grouping Tool

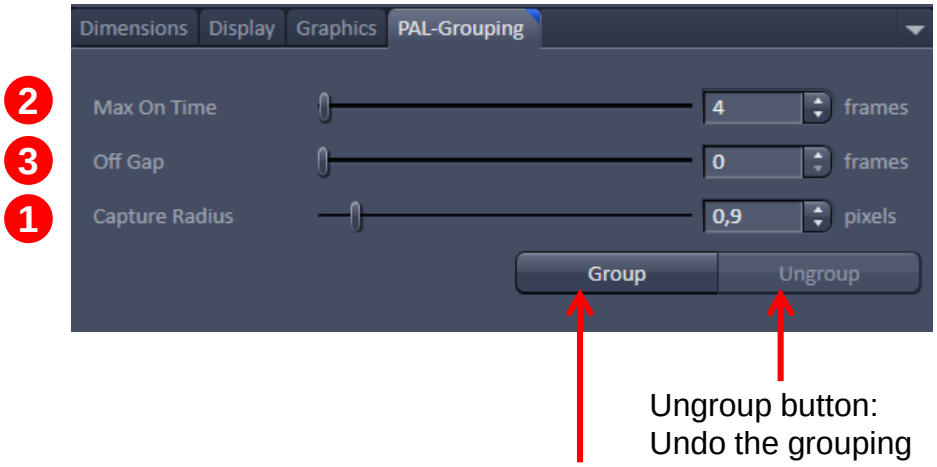
Overview

1. Why? What is PALM Grouping?
2. How does it work? Definitions
3. Additional Remarks

PALM Grouping – Three parameters

Capture radius, Max On Time and Off Gap.

Grouping is applied to the table of localized molecules, in order to group the molecules need to be near in space and time.



1. **Capture radius**: Starting from a given molecule position the SW will look for molecules within only that radius in the time sequence. (This radius is typically around 10 nm).

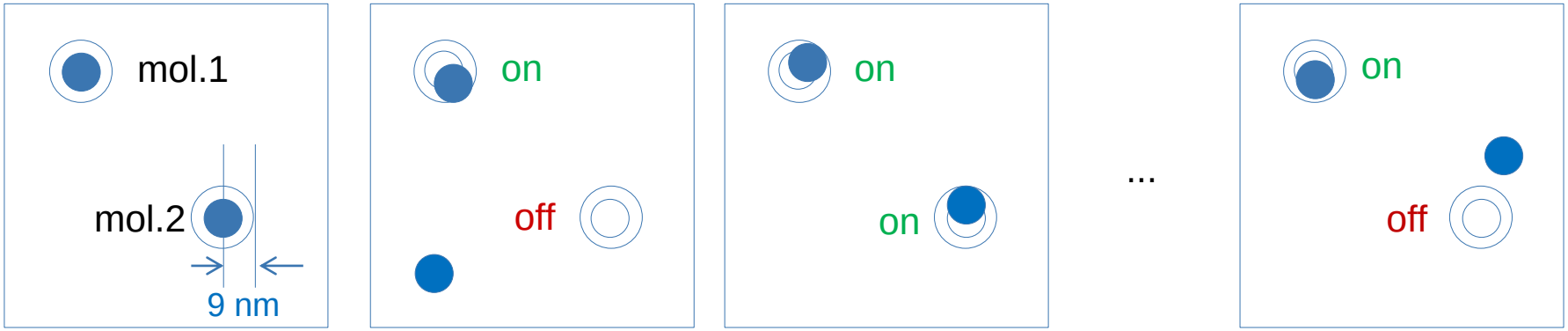
2. **Max On Time**: After step 1 the time sequence is analyzed. A molecule can be on for this number of subsequent frames. If it is on for longer than this value then it is completely erased.

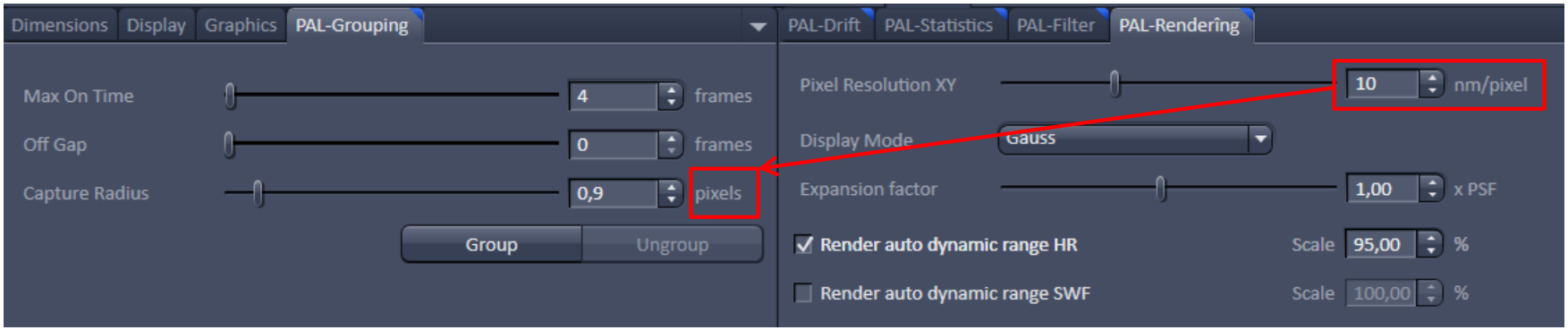
3. **Off Gap**: Allows for an off gap (due to e.g. blinking) of this maximum length. If equal or shorter then the sequence is joined. If longer then it is not joined.

! Data must be drift free or drift corrected (see also PALM-Drift)

PALM Grouping – Capture radius

First of all: Molecule has to be within a small radius.

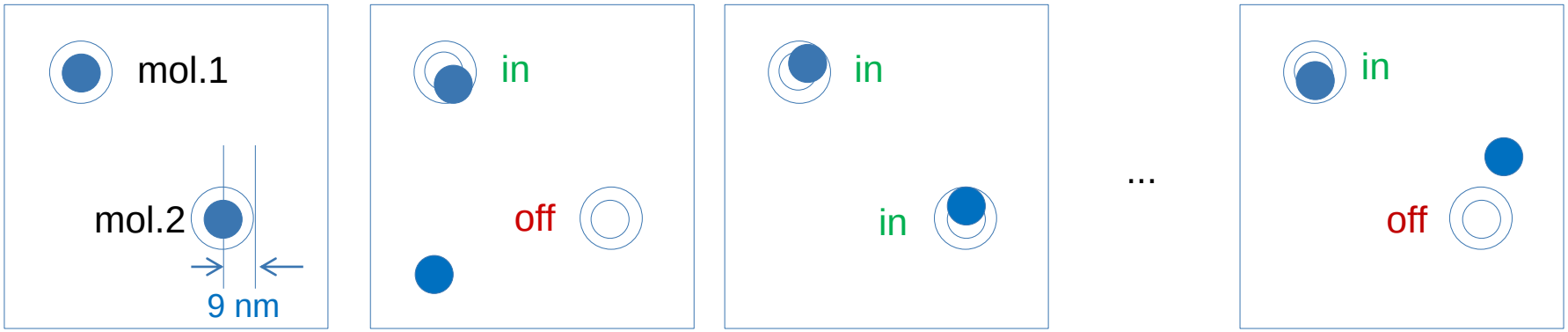




Attention! Radius is in units of **rendered image pixels** (set in the PAL-Render Tab)
Here the radius is 9 nm (if pixel resolution is set to 20 nm/pixel, then the radius will be 18 nm)
Attention! We assume that the data is drift free !!! (see PAL - Drift)

PALM Grouping – on/off times

Molecules within the radius have an on/off behaviour



Frame	1	2	3	4	5	6	7	8	9	10	11	...	N
Mol. 1	■	■	■	■	■	■	■	■					
Mol. 2	■		■		■	■				■			
Mol. 3		■				■	■	■	■				
...													
Mol. N				■	■	■		■	■	■			

■ in / on ■ not in / off

PALM Grouping – on/off times (consecutive)

On/off times are always in consecutive frames

Frame	1	2	3	4	5	6	7	8	9	10	11	...	N
Mol. 1				8									
Mol. 2	1	1	1	1	2			3		1			
Mol. 3		1	2				5						
...													
Mol. N					3		1		3				

in / on

not in / off

Numbers: Consecutive on and off times.

PALM Grouping – Max On Time (consecutive)

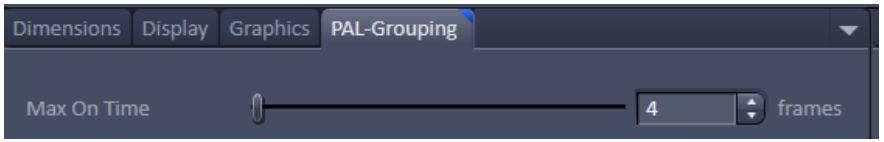
Max On Time sets an upper limit.
Removes molecules on for unreasonably long time.

Frame	1	2	3	4	5	6	7	8	9	10	11	...	N
Mol. 1				**8**						On for too long (remove)			
Mol. 2	1	1	1	1	2			3		1		OK	
Mol. 3		1	2				**5**			On for too long (remove)			
...													
Mol. N					3		1		3			OK	

in / on

not in / off

Numbers: Consecutive on and off times.



using these settings

Attention! It affects only consecutive on frames (i.e. green blocks).

Attention! The whole molecule sequence (the green block with the red number) is removed.

PALM Grouping – Max On Time (consecutive)

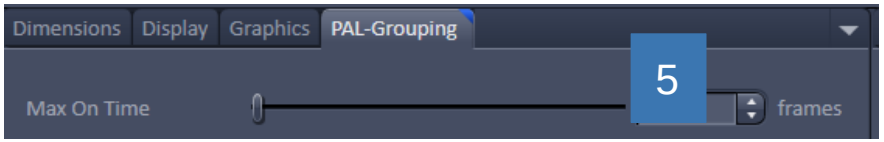
Max On Time sets an upper limit.
Removes molecules on for unreasonably long time.

Frame	1	2	3	4	5	6	7	8	9	10	11	...	N
Mol. 1				**8**						On for too long (remove)			
Mol. 2	1	1	1	1	2			3		1		OK	
Mol. 3		1	2				5					OK	
...													
Mol. N					3		1		3			OK	

in / on

not in / off

Numbers: Consecutive on and off times.



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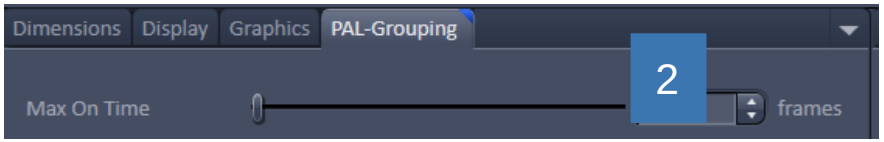
Removes molecules on for unreasonably long time.

Frame	1	2	3	4	5	6	7	8	9	10	11	...	N
Mol. 1				**8**						On for too long (remove)			
Mol. 2	1	1	1	1	2			3		1		OK	
Mol. 3		1	2				**5**			On for too long (remove)			
...													
Mol. N					**3**		1		**3**	On for too long (remove)			

in / on

not in / off

Numbers: Consecutive on and off times.



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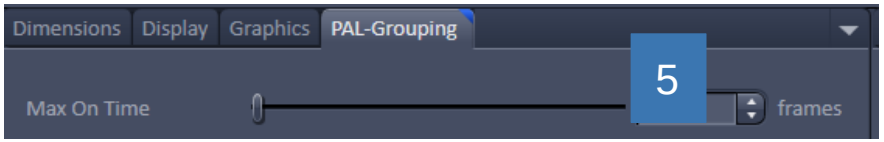
Removes molecules on for unreasonably long time.

Frame	1	2	3	4	5	6	7	8	9	10	11	...	N
Mol. 1				8						On for too long (remove)			
Mol. 2	1	1	1	1	2			3		1		OK	
Mol. 3		1	2				5					OK	
...													
Mol. N					3		1		3			OK	

in / on

not in / off

Numbers: Consecutive on and off times.



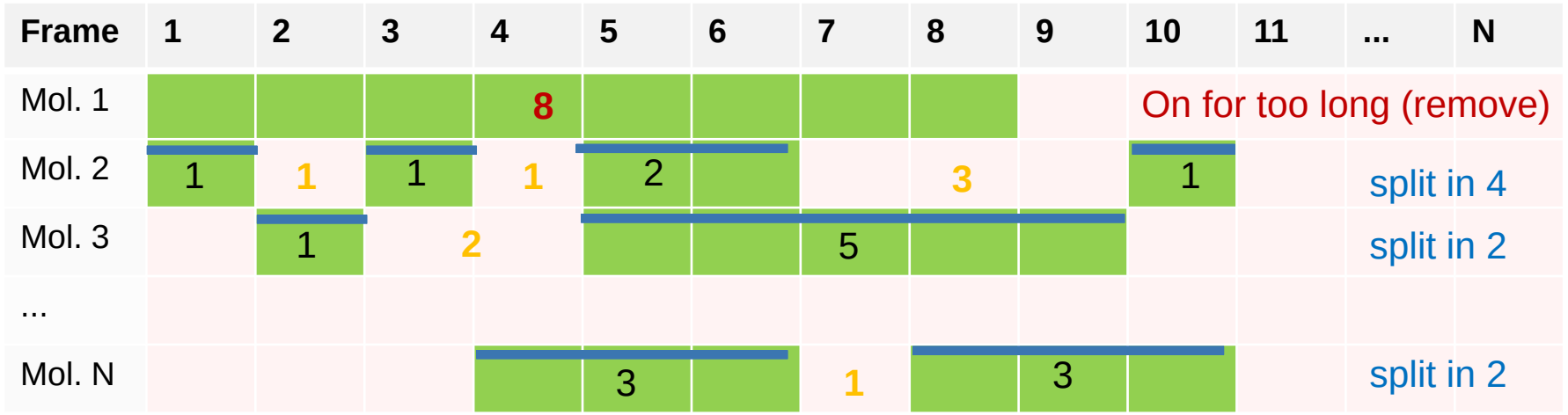
using these settings

Attention! It affects only consecutive on frames (i.e. green blocks).

Attention! The whole molecule sequence (the green block with the red number) is removed.

PALM Grouping – Off Gap (consecutive)

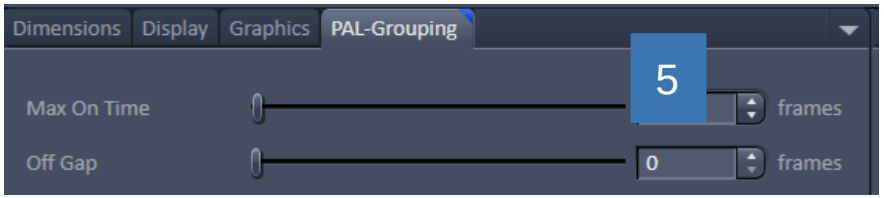
Molecules that remain off for too long are unreasonable



in / on

not in / off

Numbers: Consecutive on and off times.



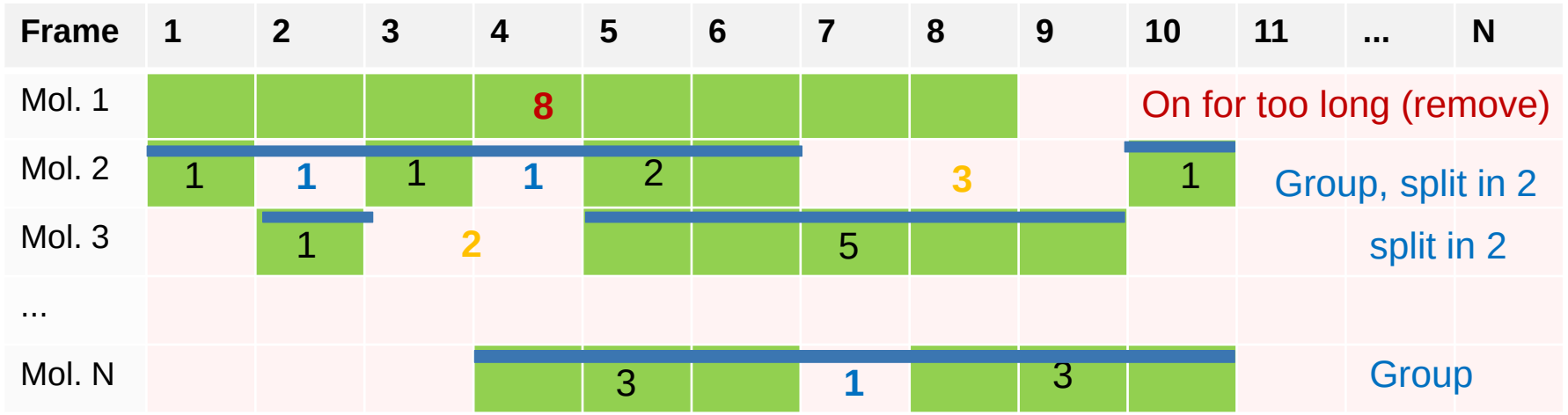
using these settings

Attention! It affects only consecutive frames.

Attention! The molecules are SPLIT (not removed).

PALM Grouping – Off Gap (consecutive)

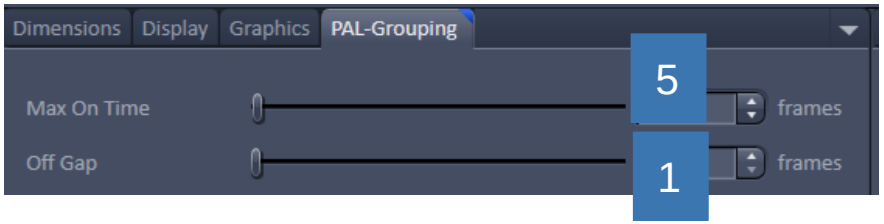
Molecules that remain off for too long are unreasonable



in / on

not in / off

Numbers: Max On Time / Consecutive on time.
Off Gap / Consecutive off gap



using these settings

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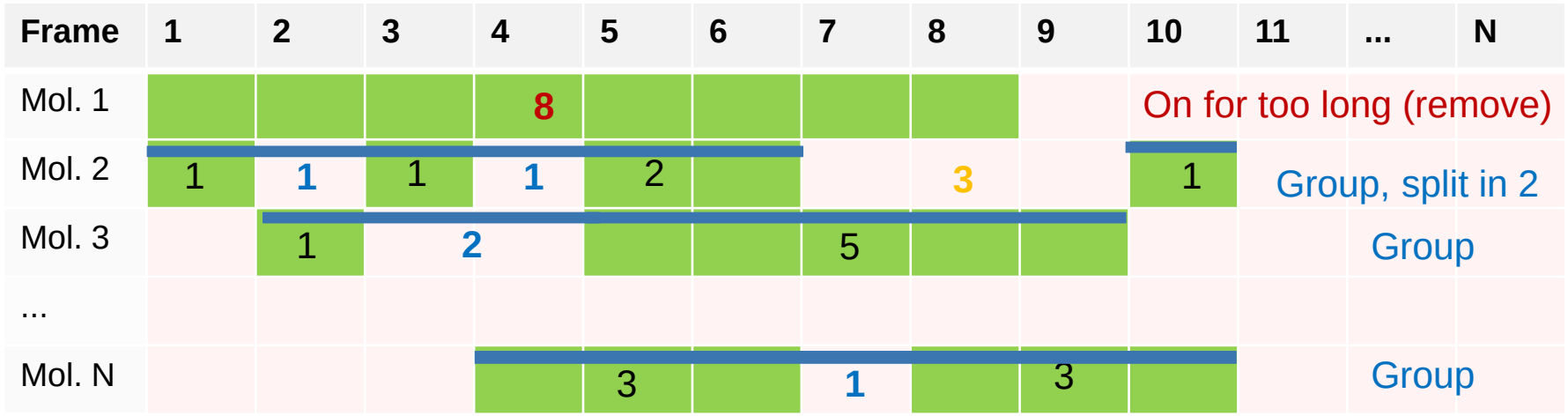
PALM Grouping – Off Gap (consecutive)

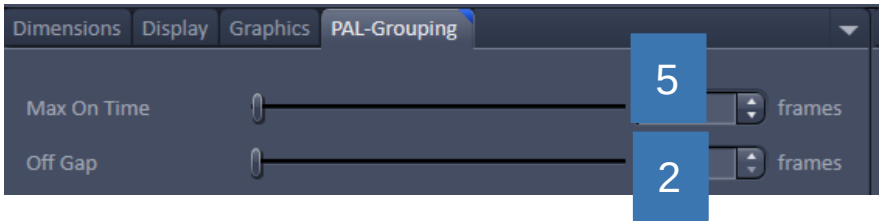
Molecules that remain off for too long are unreasonable



in / on

not in / off

Numbers: Max On Time / Consecutive on time.
Off Gap / Consecutive off gap



using these settings

Attention! It affects only consecutive frames.

Attention! The molecules are SPLIT (not removed).

Overview

1 Why? What is PALM Grouping?

2 How does it work? Definitions

3 Additional Remarks

PALM Grouping
Additional remarks.

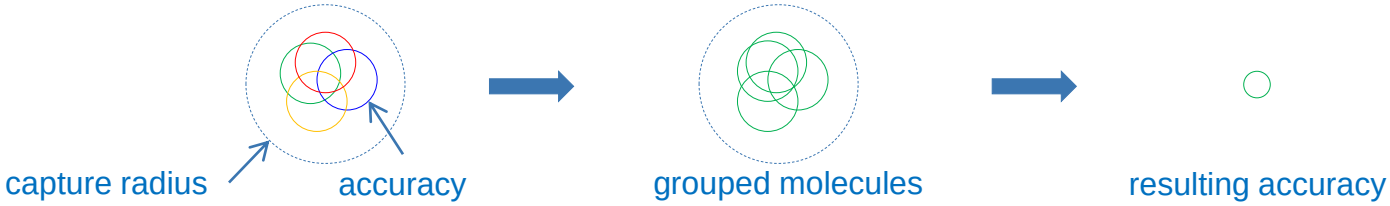
After understanding how **grouping** works, a few questions remain open:

- How important is this step? When do I have to use it?
- How do I set the values for Capture Radius, Max On Time and Off Gap?

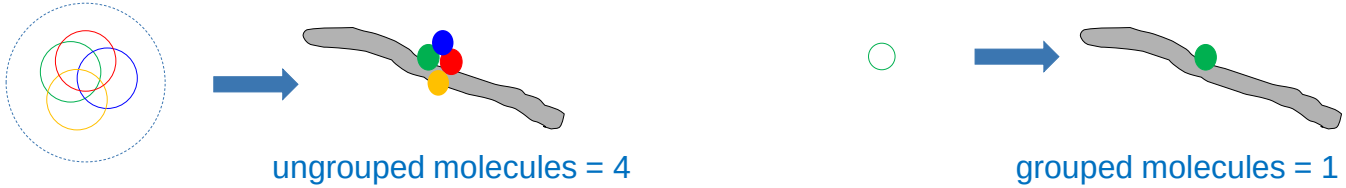
PALM Grouping

Importance and influence of grouping on the data.

(1) Grouping affects the positioning accuracy. A large group results in better precision



(2) To count molecules (e.g. proteins at a binding site) grouping must be used to avoid double counting the same molecule



(3) If the number of molecules is of no relevance, only the structure matters, then grouping may be disregarded.

PALM Grouping
Additional remarks.

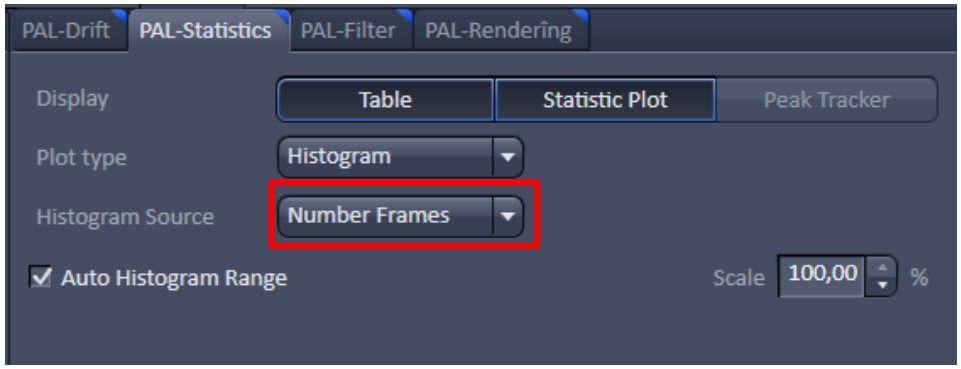
After understanding how **grouping** works, a few questions remain open:

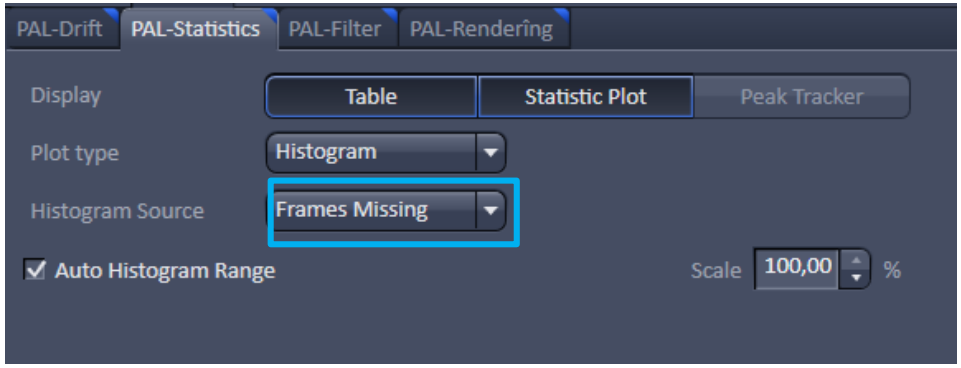
- How important is this step? When do I have to use it?
- How do I set the values for Capture Radius, Max On Time and Off Gap?

PALM Grouping – And Statistics

Not easy to find on / off times.
Given in Statistics is sum total.

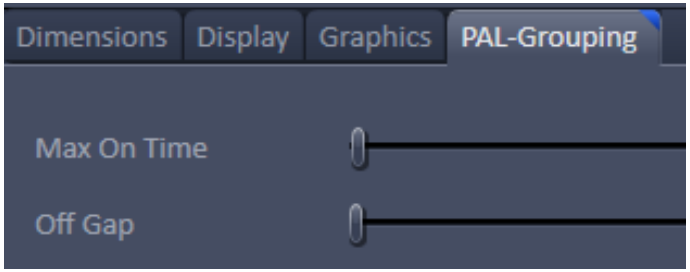
Index	First Frame	Number Frames	Frames Missing	Position X [nm]	Position Y [nm]	Pre [nm
4001	3895	2	0	756.3	906.9	
4002	3911	2	1	1665.2	1867.9	
4003	3929	2	0	2438.7	2830.3	
4004	3929	4	2	931.4	949.8	
4005	3932	2	1	2158.5	2617.8	
4006	3933	3	1	2162.7	2602.6	
4007	3941	2	0	498.4	793.5	
4008	3943	2	2	1809.6	909.3	





Max On Time ≠ Number Frames

Off Gap ≠ Frames Missing



It is not possible to infer from the statistics what the typical on and off time for the molecules is. This is so because the numbers given are the sum total of all on times and all off times. For an example see next slide.

PALM Grouping – And Statistics

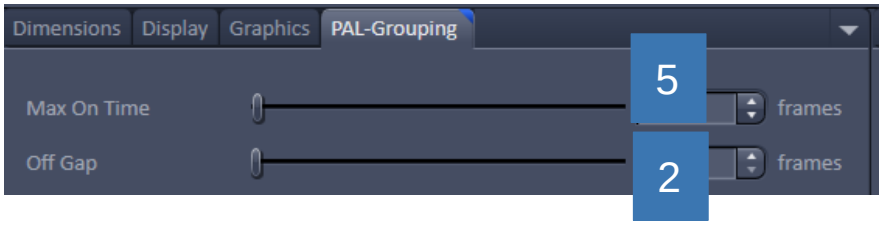
Not easy to find on / off times. Given is sum total.

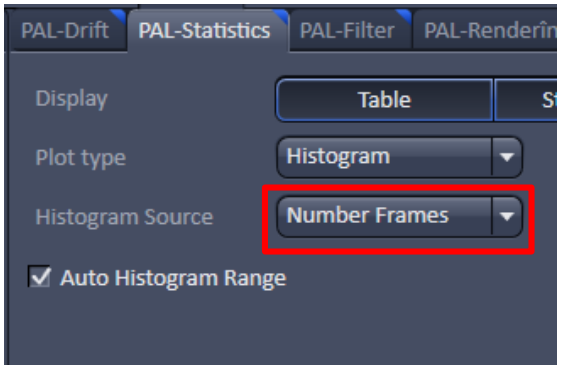
Frame	1	2	3	4	5	6	7	8	9	10	11	...	N
Mol. 1				8							On for too long (remove)		
Mol. 2	1	1	1	1	2			3		1	N Frames: 4 and 1		
Mol. 3		1		2			5				N Frames: 6		
...													
Mol. N					3		1		3		N Frames: 6		

in / on

not in / off

SUM TOTAL of On Times





PALM Grouping – And Statistics

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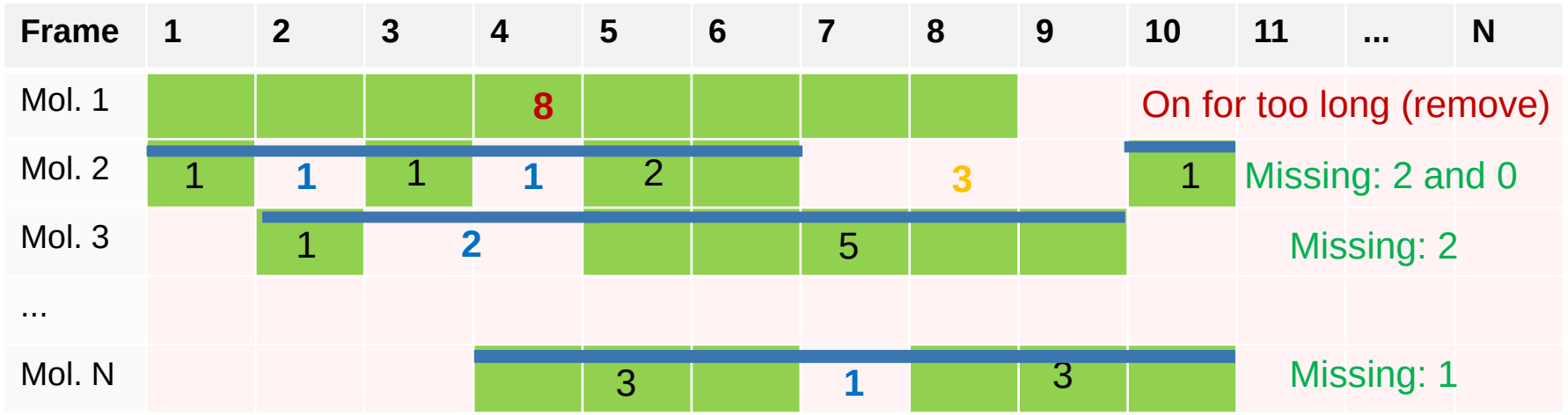
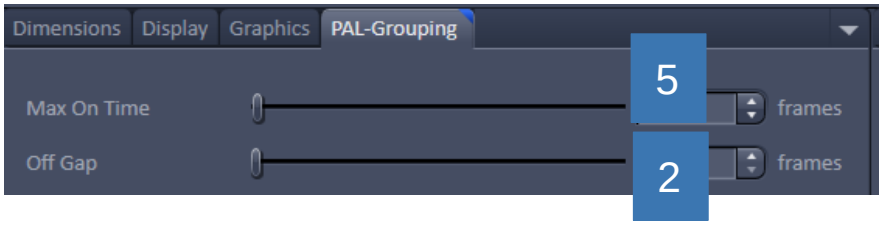
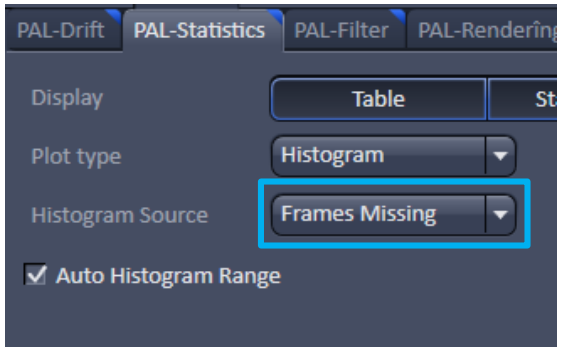






PALM Grouping – And Statistics

Not easy to find on / off times.
Given in Statistics is sum total.

So how to find on/off times?

(1) With control specimens: With diluted molecules, so that each molecule can be addressed individually (virtually no spatial overlap) then characterizing on / off times with external software (Origin, MatLab, XL, etc.)

(2) Try and error: Starting from restrictive settings (small on times, no off gap), gradually increasing on times and then gradually allowing for longer off gaps – then characterizing the histograms (using external software, Origin, MatLab, XL, etc.)

We make it visible.

PALM Grouping – Extreme examples

Special cases that are unlikely to exist

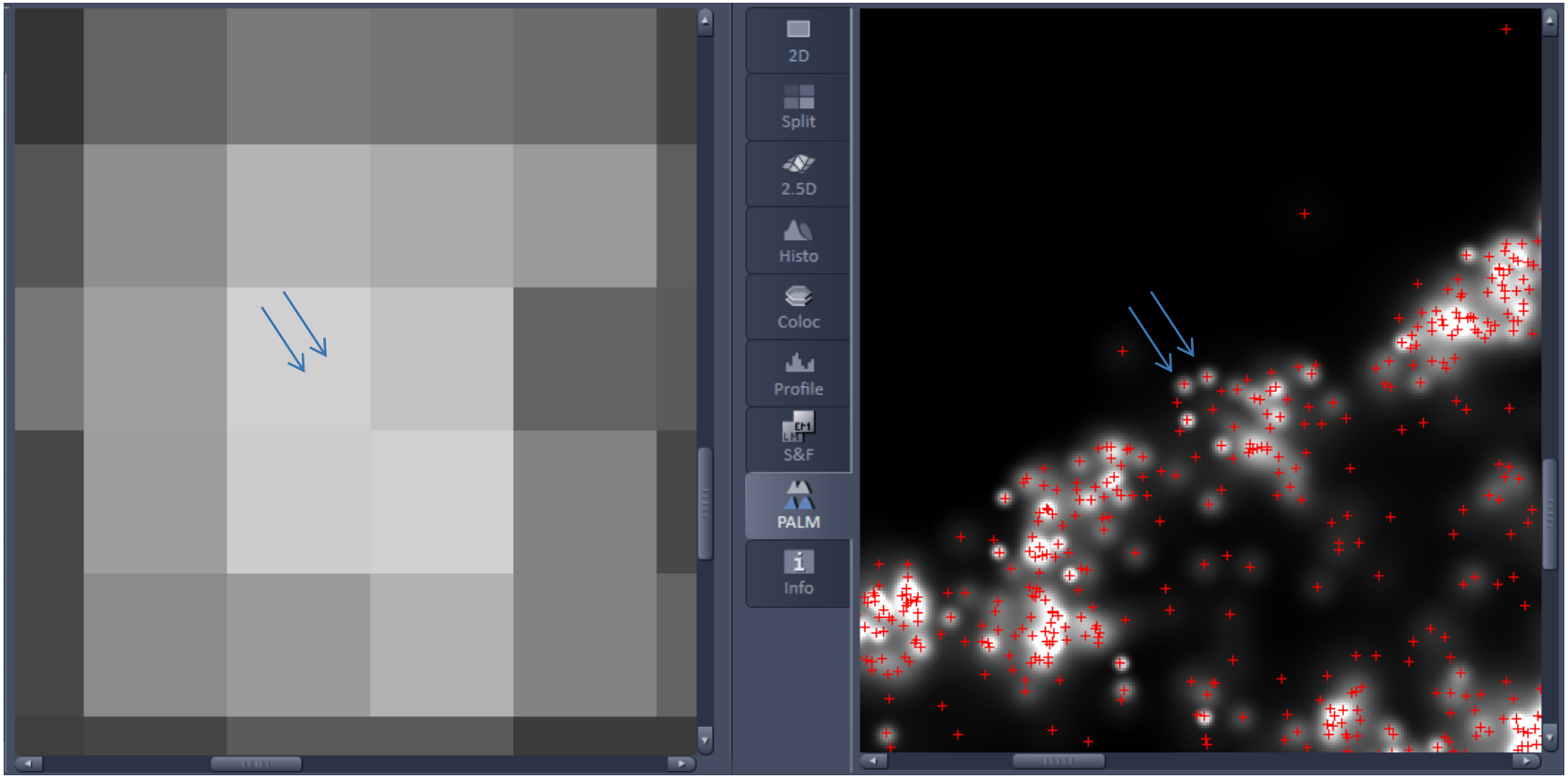
Frame	1	2	3	4	5	6	7	8	9	10	11	...	N
...													
...													
Mol. X	2		1	2		1	2		1	2			
...													
...													

A molecule could be on for 2 frames then blink for 1 frame (like above) indefinetly.
In this case there is no maximum number of frames, no maximum number of frames missing.

The statistics don't stop at Max On Time
The statistics don't stop at Off Gap

PALM Grouping – Capture radius

Going back to theory... Just a bit



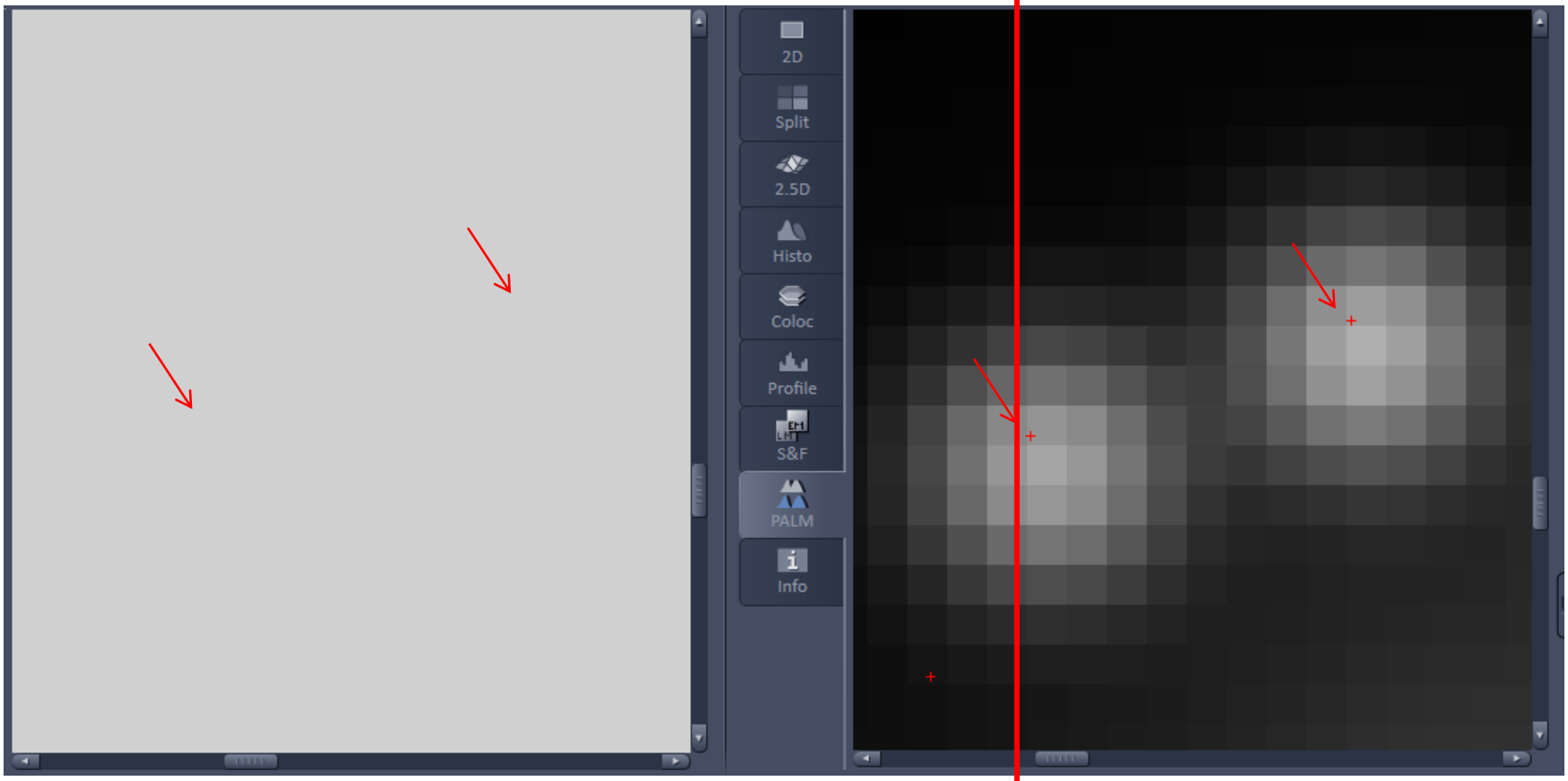
Camera Image PALM Image Same Zoom

Each camera pixel is 100 nm in size. The positioning accuracy is much better than that!

PALM Grouping – Capture radius

Going back to theory... Just a bit

this is the camera pixel

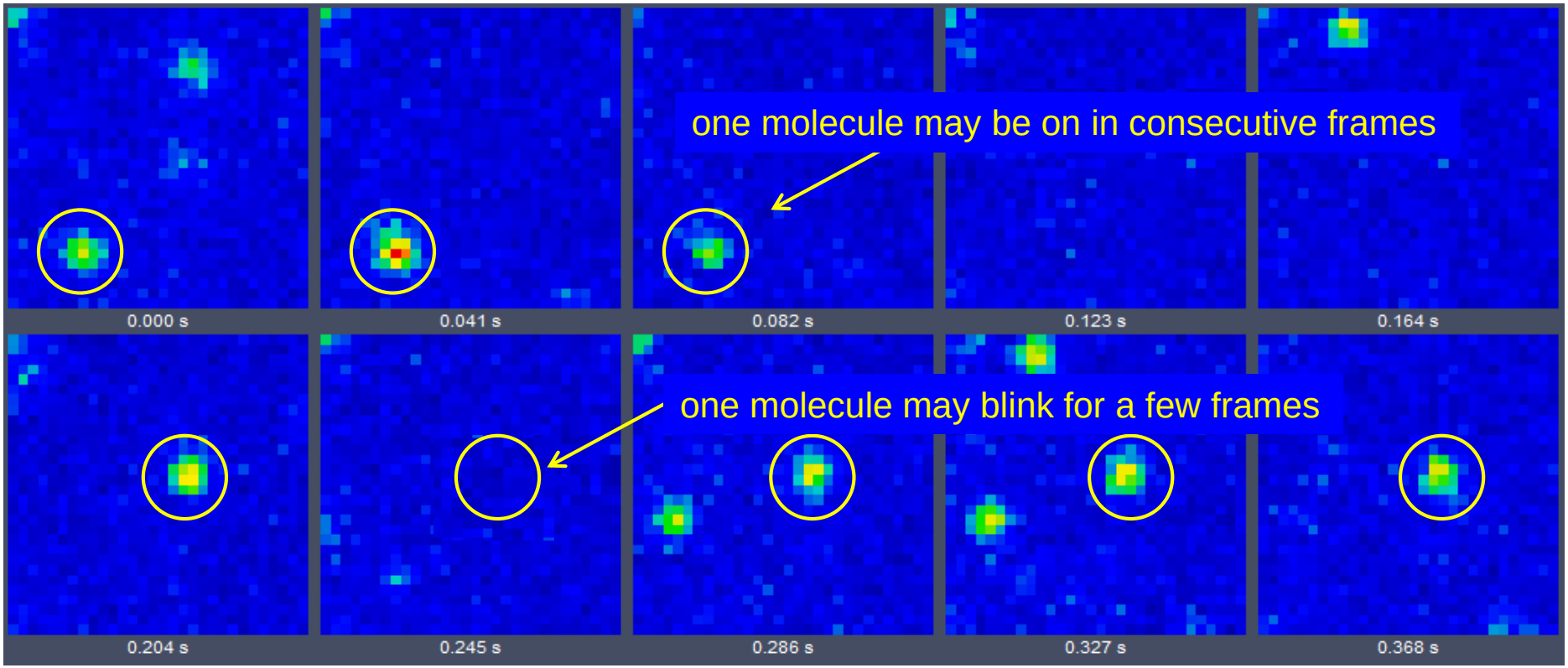


Camera Image PALM Image Same Zoom

Each camera pixel is 100 nm in size. The positioning accuracy is <u>much</u> better than that!

PALM / dSTORM (Localization Microscopy)

Molecules may persist over a few frames or blink.



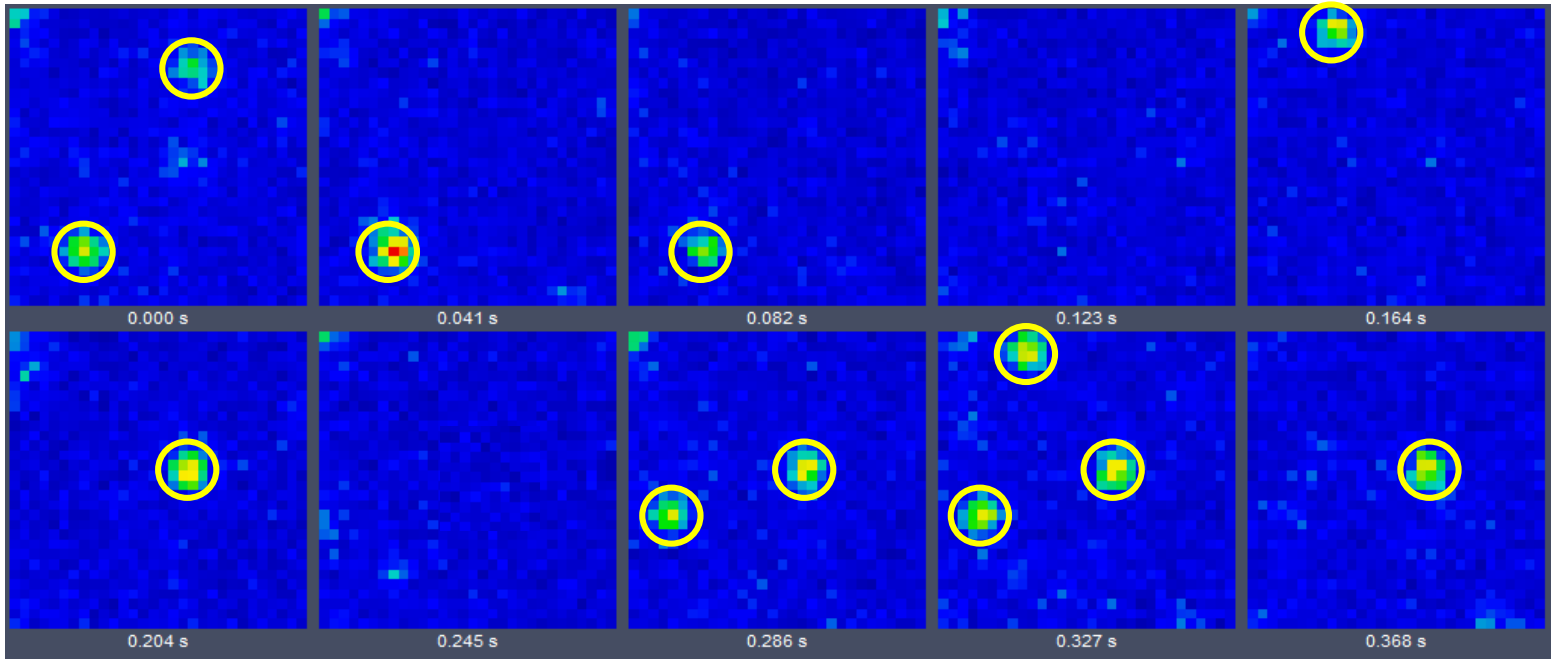
It is reasonable to assume that these highlighted patterns in each frame originate from one molecule. In that case, the retrieved positions can be assigned to one and the same molecule - These patterns can be **grouped**.

PALM Grouping

Why group?

I want to count molecules. To do that: I need to control grouping properly. Example:

12 molecules or 6?



PALM Grouping

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