# FandPLimitTool

For single-molecule localization and resolution

# **MUMDesignTool**

Designing the focal plane spacing for multifocal plane microscopy



# Ward Ober Lab

This document is a user's manual for the Fundamental and Practical Limit Tool (FandPLimitTool) and the MUMDesignTool. It explains how to calculate 2D and 3D localization accuracy limits as well as 2D and 3D resolution limits for single-molecule microscopy data. In addition, it illustrates how to design the focal plane spacing for a multifocal plane microscopy setup with up to 10 focal planes.

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Designing the focal plane spacing for multifocal plane microscopy

# User's Manual

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## Introduction

This document is a user's manual for the FandPLimitTool V1.2 and MUMDesignTool V1.0. It provides detailed instructions for performing five specific calculations which are representative of the types of calculations that can be performed with this application. Moreover, it provides detailed information about how to use the MUMDesignTool to design the focal plane spacing for a multifocal plane microscopy (MUM) setup [1].

Consider the following terminology which will be used multiple times throughout this document.

FLAM: fundamental localization accuracy measurePLAM: practical localization accuracy measureFREM: fundamental resolution measurePREM: practical resolution measure

#### What is new?

We have added several new features to the FandPLimitTool V1.2 which make it more powerful and faster than its previous version (V1.1). The new features are listed as follows and each option will be discussed in detail later.

- A new software module 'the MUMDesignTool' is incorporated with the package which provides tools to design the focal plane spacing for multifocal plane microscopy.
- New functionality that allows the calculation of the 3D resolution measure.
- A new function called "vary a parameter" is added to the software which allows users to calculate and plot the limits of accuracy for a range of different experimental parameters.
- New functionality that allows simulating single molecule model and data images.
- New functionality that allows determining limits of accuracy in the presence of stochastic signal amplification, i.e. when using an electron multiplying CCD (EMCCD) detector.
- The code is significantly optimized. The software therefore runs much faster than the previous version.
- Exporting tools are enhanced and user can now export the results, parameters, simulated model and data images and graphs as text, image and LaTex report files, respectively.
- Summary of all input parameters can be explored within the software.
- Enhanced user interface design.
- A console window and a log file to facilitate bug reporting.

#### Software overview

The FandPLimitTool allows users to determine the fundamental and practical limits of 2D and 3D localization accuracy and resolution for a variety of imaging conditions. The software has been

built using object oriented programming methodologies and has been designed for flexibility to accommodate new models and numerical calculation routines in the future.

Each calculation supported by this application requires a different set of parameters. The parameters are divided into two categories: *Required parameters*, and *Advanced parameters*.

Required parameters are those parameters that specify the imaging conditions under which the calculations are to be performed. Users must provide values for these parameters before a calculation can proceed. These parameters by default are visible to the user when software starts. They can also be accessed by clicking the Required Parameters option under the Parameters Menu or through its shortcut **Ctrl+U**. Although default values are always provided for all required parameters, users must modify the fields to suit their desired imaging conditions.

F Required Parameters 😂 🗖 🛙 🖾	🕞 FandPLimitTool 😂 🗔 🗉	x
2D Localization Accuracy - Airy Fundamental Limit         Fundamental inputs         Numerical aperture =         Numerical aperture =         1.45         Wavelength =         0.69         Photon detection rate =         0000         photons/s         Exposure time =         0.13         seconds	File       Export       View       Parameters       Tools       Help         Select a problem scenario:       2D       Localization Accuracy - Airy: Fundamental Limit         Perform calculations	

Advanced parameters, on the other hand, are optional inputs that govern the numerical routines involved in performing the required calculations. These parameters are optional; the default values supplied for these inputs will work for typical imaging conditions. Advanced parameters can be accessed by clicking the Advanced Parameters option under the Parameters Menu or through its shortcut **Ctrl+N**. For details about specifying values for advanced parameters, please refer to Section 1 to Section 5.

Clicking the Parameter Summary option under the View Menu or hitting **Ctrl+P** provides a summary of the required and advanced parameters in a plane text format. This helps to conveniently review and save the parameters as a text file. In addition, clicking the **Console** option under the View Menu shows a console window in which the details of calculations will be listed. This console window is also helpful for bug reporting purposes.

To calculate the limits of accuracy after specifying all desired parameters, click the Calculate Limits of Accuracy button. When the calculations are completed, a **Results window** will be brought into view displaying the results. The **Results window** can be accessed at any time by clicking the Results option under the View Menu or through its shortcut **Ctrl+R**.

Similarly, to calculate a model image after specifying all desired parameters press the Calculate Model Image. A model image is an image profile that is devoid of stochasticity and noise. To simulate realizations of this model image, press the Simulate Data Images button. Note that the simulated data images are corrupted by stochasticity and noise. For instance, depending on the

desired set of parameters, the image can be corrupted by extraneous noise sources such as background, readout or stochastic signal amplification.

Finally, clicking the MUMDesignTool option under the Tools Menu or hitting **Ctrl+M** calls the *MUMDesignTool* application which will be discussed in detail later in this tutorial.

#### Before you begin

Before following this tutorial, please make sure that the software has been correctly installed and started. Please refer to the *FandPLimitTool – Installation Guide* for instructions on installing and starting the application.

#### (WARNING) User defined functions

The **Advanced Inputs Window** has fields where the user is required to select which function to use to perform a certain part of the calculation. In the options available for such fields, the "User Defined" option is also often available. This option allows the user to specify the names of their own custom function for performing the associated part of the calculation.

However, in the executable version of the program, this option is not fully supported. Users cannot specify the names of their own functions for the user defined options as such functions will not have been compiled along with the original program to generate the executable and therefore will not be recognized by the executable version of the program.

#### 1. 2D-localization accuracy measure

This section provides an example of how to use the FandPLimitTool to calculate the accuracy with which the 2D location of an object can be estimated from its image (PLAM). The object is assumed to be in focus with respect to the objective lens and the image of the object is assumed to be an Airy profile.

The calculation takes into account the stochastic nature of the photon detection processes and assumes the photons detected from the object to be Poisson distributed. In addition, the calculation also takes into account extraneous noise sources - in particular the background component which is modeled as additive Poisson noise, the readout noise of the detector which is modeled as independent, additive Gaussian noise and the stochastic signal amplification noise which is present in EMCCD detectors [3].

It is assumed that the pixels are rectangular in shape with no dead space between any two pixels. Further, the region of interest (ROI) containing the image of the object is assumed to be a rectangular array of pixels. The object is assumed to be in the center of this rectangular pixel array and the location of the object is specified with respect to the object space. Refer to [2] for details on determining the 2D localization accuracy in single molecule microscopy.

#### **1.1. Summary of imaging conditions**

The following table summarizes the imaging conditions under which the calculations are performed.

Property	Value
Numerical aperture	1.45
Wavelength	0.69 μm
Photon detection rate	10,000 photons/second
Exposure time	0.13 seconds
Magnification	100 X
Location of object in ROI	0.585 μm, 0.585 μm
Pixel size	13 μm × 13 μm
ROI Size	9 pixels × 9 pixels
Background level (Poisson rate)	1000 photons/pixel/second
Gaussian noise mean	0 e <sup>-</sup>
Gaussian noise standard deviation	8 e <sup>-</sup> rms
Use EM	unchecked
EM gain	100
EM model	Geometric

Table 1.1.1: 2D Localization Accuracy - Summary of imaging conditions

## 1.2. Providing required parameters

**Step 1**: Select the problem scenario of "2D Localization Accuracy - Airy: Pixelated with Poisson + Gaussian Noise" from the Calculate pull-down menu in the main application window.

🔚 Required Parameters 🔅 🗖 🗉 🖾	
2D Localization Accuracy - Airy Pixelated with Poisson + Gaussian Noise	
Fundamental inputs	
Numerical aperture = 1.45	
Wavelength = 0.69 microns	
Photon detection rate = 10000 photons/s	
Exposure time = 0.13 seconds	
Additional inputs	
Mercification - 100	2D Localization Accuracy - Airy: Fundamental Limit
imagninication = 100	2D Localization Accuracy - Airy: Pixelated with Poisson Noise
Location parameters	2D Localization Accuracy - Airy: Pixelated with Poisson + Gaussian Noise
x0 = 0.325 microns Auto-center	2D Localization Accuracy - Gaussian Symmetric Prolated with Prisson Noise
y0 = 0.325 microns	2D Localization Accuracy - Gaussian Symmetric: Pixelated with Poisson + Gaussian Noise
Divelation narameters	2D Localization Accuracy - Gaussian Asymmetric: Fundamental Limit
Pixel size (beight wigth) = [13, 13] microps X microps	2D Localization Accuracy - Gaussian Asymmetric: Pixelated with Poisson Noise
Pixel size (reight, width) = [13, 13] microitis X microitis	2D Localization Accuracy - Gaussian Asymmetric: Pixelated with Poisson + Gaussian Noise
ROI size [height, width] = [5,5] no. of pixels X no. of pixels	3D Localization Accuracy - Born-Wolf Model: Fundamental Limit
- Extraneous noise source(s)	3D Localization Accuracy - Born-Wolf Model: Pixelated with Poisson Noise
	3D Localization Accuracy - Born-Wolf Model: Pixelated with Poisson + Gaussian Noise
Background level (Poisson rate) = 1000 photons/pixel/s	2D Resolution Accuracy - Gaussian Symmetric Would: Proteated with Poisson House (Symmetric Parameterization) 2D Resolution Accuracy - Gaussian Symmetric Model: Divided With Poisson Acquesian Noise (Symmetric Parameterization)
Readout (Gaussian) noise parameters	2D resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson Noise (Aymmetric Parameterization)
Standard deviation = 8 e-/bixel	Page 2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson + Gaussian Noise (Asymmetric Parameterization)
	2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson Noise (Cartesian Parameterization)
Electron multiplication parameters	2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson + Gaussian Noise (Cartesian Parameterization)
Use EM EM gain = 2.0082	2D Resolution Accuracy - Airy Model: Fundamental Limit (Symmetric Parameterization)
EM model = Geometric -	2D Resolution Accuracy - Airy Model: Pixelated with Poisson Noise (Symmetric Parameterization)
	2D Localization Accuracy - Airy: Pixelated with Poisson + Gaussian Noise
Parameters to be estimated	- Perform calculations
✓ x0 ✓ Alpha* ✓ Background level	
y0	Calculate Limits of Accuracy Calculate Model Image Simulate Data Images
*Alpha = 2 x pi x numerical aperture / wavelength, pi = 3.1416 (approx.) Vary a Parameter	

Figure 1.2.1: Selecting the 2D Localization Accuracy calculate type

**Step 2**: The required parameters window, which is visible by default, will be updated accordingly. If this window is not visible, access it by clicking the Required Parameters option under the Parameters Menu in the main application window.

💁 Required Parameters 🔅 🗖 🗉 🔀	
2D Localization Accuracy - Airy Pixelated with Poisson + Gaussian Noise	
- Fundamental inputs	
Numerical aperture = 1.45	
Wavelength = 0.69 microns	
Photon detection rate = 10000 photons/s	
Exposure time = 0.13 seconds	
- Additional inputs	
Magnification = 100	
v0 = 0.325 microns (Lute center)	
v0 = 0.325 microns	
Diversities assessments	
Pixel size [height_width] = [13, 13] microps X microps	
ROI size [height, width] = [5,5] no. of pixels X no. of pixels	
Extraneous noise source(s)	
Background level (Poisson rate) = 1000 photons/pixel/s	
Readout (Gaussian) noise parameters	
Standard deviation = 8 e-/pixel	📴 FandPLimitTool
Electron multiplication parameters	File Export View Parameters Tools Help
Use EM EM gain = 2.0082	Required Parameters Ctrl+U
EM model = Geometric -	Select a problem s Advanced Parameters Ctrl+N
	2D Localization Accuracy - Arry, Pixelated with Poisson + Gaussian Noise
Parameters to be estimated	Perform calculations
V x0 V Alpha* V Background level	
V V Photon detection rate	Calculate Limits of Accuracy Calculate Model Image Simulate Data Images
*Alpha = 2 x pi x numerical aperture / wavelength, pi = 3.1416 (approx.) Vary a Parameter	

Figure 1.2.2: Required parameters window (2D Localization)

**Step 3**: In the - Fundamental inputs - section of the window, enter the values for the various fields as follows: *Numerical aperture = 1.45*, *Wavelength = 0.69*, *Photon detection rate = 10000*, and *Exposure time = 0.13*.

**Step 4**: In the - Additional inputs - section of the window, enter the values for the various fields as follows: *Magnification* = 100,  $x_0 = 13 \times 4.5/100$ ,  $y_0 = 13 \times 4.5/100$ , *Pixel size* = [13, 13], and *ROI size* = [9, 9].

**Remark 1**: The center of a 9 *pixels* × 9 *pixels* pixel array where the dimensions of each pixel is  $13 \ \mu m \times 13 \ \mu m$ , is  $(4.5 \times 13) \ \mu m \times (4.5 \times 13) \ \mu m$ . To convert this location coordinates to the object space, the coordinate values are divided by the magnification. Hence the location coordinates are specified as  $13 \times 4.5/100 \ \mu m \times 13 \times 4.5/100 \ \mu m$ .

**Step 5**: In the - Extraneous noise sources - section of the window, enter the values for the various fields as follows: *Background level = 1000* and *Standard deviation = 8*.

**Remark 2**: In case that the localization accuracy is required for an EMCCD detector, the *Use EM* checkbox should be checked and the desired value for the electron multiplication gain should entered in the *EM gain* field. The default electron multiplication model is Geometric. However, different electron multiplication models, e.g. High gain and Gaussian, can be chosen from the *EM model* combo box based on the requirements. By holding the mouse pointer on the *EM model* combo box, a brief description of each option is shown on a tool tip text. For more information about different electron multiplication models consult [3].

Extraneous noise source(s)		
Background level (Poisson rate) = 1000 photon	s/pixel/s	
Readout (Gaussian) noise parameters		
Standard deviation = 8 e-/pixe	1	
Electron multiplication parameters		
✓ Use EM EM gain = 2.0082		
EM model = Geometric	-	
Geometric		
Parameters to be estimated High gain	"Geometric": standard geometric electron multiplication	
🔽 🛛 🔍 Alpha* Gaussian	"High gain": exponential electron multiplication,	
	(Use only with high EM gain)	
V V Photon detection rate	"Gaussian": Gaussian approximation of output of electron multiplication,	
	(Use only when signal is high)	
*Alpha = 2 x pi x numerical aperture / wavelength, pi = 3.1416 (approx.)	rameter	

Figure 1.2.3: Electron multiplication parameters

**Step 6**: In the - Parameters to be estimated - section of the window, ensure that all check- boxes for all parameters are checked.

Step 7: To improve screen clarity, close the Required parameters window.

**Remark 3**: Closing the window is not in any way required by the application for the calculations to be performed and is only a suggestion in order to reduce the number of open windows and hence improve screen clarity. You can access the window again at any time by clicking the Required Parameters option under the Parameters Menu in the main application window.

#### 1.3. Providing advanced parameters

**NOTE**: All inputs in the Advanced Parameters window are optional. The default values provided have been found to work adequately for typical imaging conditions.

**Step 1**: Access the advanced parameters window by clicking the Advanced Parameters option under the Parameters Menu in the main window.

Advanced Parameters	
2D Localization Accuracy - Airy Pixelated with Poisson + Gaussian Noise	
- Model profile calculations	
Pixel integration method : Trapezoidal   Additional para	ameters
Background type : Constant	ameters
Fisher information matrix calculations         Single integration method :         Trapezoidal         Double integration method :         Trapezoidal         Madditional para         Limits of summation [lower limit, upper limit] =         Limits of integration [lower limit, upper limit] =	ameters ameters fault
FandPLimitTool	⇔ 🗖 🗖 🔀
File Export View Parameters Tools Help	
Select a problem       Required Parameters       Ctrl+U         Advanced Parameters       Ctrl+N         2D Localization Accuracy - xary: Prixelated with Poisson + Gaussian Noise         Perform calculations         Calculate Limits of Accuracy         Calculate Model Image	▼ Simulate Data Images

Figure 1.3.1: Advanced parameters window (2D Localization)

**Step 2**: In the - Model profile calculations - section of window, access the Pixel integration method pull-down menu and select "Trapezoidal".

**Step 3:** Click the Additional parameters button next to the Pixel integration method pull-down menu. In the dialog box that appears, enter values for fields as follows: *XGridding = 13, YGridding = 13.* Click OK when done.

🔄 Advanced Parameters			🛃 Trapezo 💼 💷 👞
Pix	2D Localization Accuracy - Airy elated with Poisson + Gaussian No	ise	X Gridding
Model profile calculation	s		Y Gridding
Pixel integration method :	Trapezoidal	Additional parameters	13
Background type :	Constant	Additional parameters	OK Cancel
1			

Figure 1.3.2: Pixel Integration Trapezoidal options

**Step 4**: Access the Background type pull-down menu and select "Constant". There are no additional parameters to be accessed via the Additional parameters button for the "Constant" option.

**Step 5**: In the - Fisher information matrix calculations - section of the window, access the Single integration method and select "Trapezoidal".

**Step 6**: Click on the Additional parameters button next to the Single integration method pulldown menu. In the dialog box that appears, enter *Step size* = 0.05.

Fisher information matri:	< calculations			🚺 Trap	ezo 📼 🗉 💌
Single integration method :	Trapezoidal	- Ad	ditional parameters	Step size	•
Double integration method :	Trapezoidal	▼ Ad	ditional parameters	0.05	
	Noise factor calculation paramete	rs			OK Cancel
Limits of su	mmation [lower limit, upper limit] = 🛛	A	use_default 💌		
Limits of int	egration [lower limit, upper limit] = N	A	use_default 💌		

Figure 1.3.3: FIM Single Integration Trapezoidal options

**Step 7**: Access the Double integration method pull-down menu in the - Fisher information matrix calculations - section of the window and select "Trapezoidal".

Fisher information matrix calculations	
Single integration method : Trapezoidal	🛃 Trapezo 😐 😐 🗾
Double integration method : Trapezoidal	X Gridding
Limits of summation [lower limit, upper limit] = N/A use_default Limits of integration (lower limit, upper limit) = N/A use_default	Y Gridding 13 OK Cancel

Figure 1.3.4: FIM Double Integration Trapezoidal options

**Step 8**: Click on the Additional parameters button next to the Double integration method pulldown menu. In the dialog box that appears, enter the values *XGridding* = 13 and *YGridding* = 13.

**Step 9**: The - Fisher information matrix calculations - section has a subsection titled - Noise factor calculation parameters - . Enter values *Limits of summation = [1, 3000]* and *Limits of integration = [-5000, 5000]* in this subsection.

**Remark 4**: The limits of integration and summation are initially set to default values which are automatically calculated based on the imaging conditions. It is possible specify these limits by changing the value of the combo box to the right of the limits of integration and summation fields.

Fisher information matrix	calculations					
Single integration method :	Trapezoidal	•	Additi	ional paramete	rs	
Double integration method :	Trapezoidal	•	Additi	ional paramete	rs	
	Noise factor calculation parameters					
Limits of summation [lower limit, upper limit] = [1, 3000] specify						
Limits of integration [lower limit, upper limit] = N/A specify						
use_default						

Figure 1.3.5: Noise factor calculation parameters

**Step 10**: To improve screen clarity, close the **Advanced parameters window**.

**Remark 5**: Closing the window is not in any way required by the application for the calculations to be performed and is only a suggestion in order to reduce the number of open windows and hence improve screen clarity. You can access the window again at any time by clicking the Advanced Parameters option under the Parameters Menu in the main application window.

#### **1.4.** Viewing and saving the parameter summary

**NOTE**: This is a new feature of the FandPlimitTool V1.2. Typically it is necessary to save the required and advanced parameters for later developments and to keep track of calculations. This new feature provides a summary of the required and advanced parameters in a plane text format.

**Step 1**: Access the required inputs summary window by hitting the Parameter Summary option under View Menu.

Localizat:	ion Accuracy 2D Airy P:	ixelated Poisson		
Gaussian Basic Inpu Numerical Wavelength Exposure t Magnificat Pixel Dime Photon Det Model para Background Readout NG 	tts: Aparture = 1.45 h = 0.69 microns time Interval = [0,0.12 ion = 100 msions = [13,13] micro tection Rate = 10000 pl mmeters = [0.325,0.32 i Noise = 1000 photons pise = 8 e-/p 	3] s = 5) s 10 tons/s 25,13.2038] 5/s/p 		
Pixel Inte Pixel Inte Save as text	eg. Func. = Trapezoio eg. Gridding = [13,13]	dal 🔹		
FandPLimitT	ool	11.1.	$\Leftrightarrow$	
Select a pri 2D Localizatio	Parameters Tools Parameter Summary Results Console calculations	Ctrl+P Ctrl+R Daussian Noise		
<ul> <li>Perform (</li> </ul>	aloulationo			

Figure 1.4.1: Viewing and saving the parameter summary (2D Localization)

**Step 2**: You will see the summary of the required and advanced parameters in a plane text format. This summary can be saved on the disc as a .txt file by hitting the Save as text button.

#### 1.5. Executing the task and viewing results

**Step 1**: In the main application window, click the Calculate Limits of Accuracy button. This will present a dialog box warning that the calculation may take a long time to complete. Click Yes to proceed. The Calculate button text will change to Calculating ... and the application will temporarily become unresponsive while the calculations are being performed. During the calculation, a console

window will be shown to inform the user about the different steps of the calculation. This console window will also display errors and warnings, if there is any, which is very useful for bug reporting purposes.

**NOTE**: The calculation could indeed take half an hour to complete depending on the hardware capabilities of the platform on which the application is being executed.

Warning	is calculation may take a long time to comp tinue?	ete. Are you sure
FandPLimitTool		
File Export View Parameters Tools	Help	
Select a problem scenario: 2D Localization Accuracy - Airy: Pixelated with Perform calculations	Poisson + Gaussian Noise Calculate Model Image	▼ Simulate Data Images
Console Poisson Gauss Mixture 2 o Poisson Gauss Mixture 1 o Poisson Gauss Mixture 1 o Poisson Gauss Mixture 1 o Poisson Gauss Mixture 2 Poisson Gauss Mixture 2 Poisson Gauss Mixture 2 Application initialized All rights reserved. Copyright (C) 2004-2014 V For single-molecule localiz FandPLimitTool V1.2 Initializing the application .	r 2. rf 2. rf 2. rf 2. rf 2. rf 2. rf 2. vard Ober Lab ration and resolution 	

Figure 1.5.1: Calculation and Final confirmation

**Step 2**: When the calculation is completed, the **Calculating** ... button text will change back to **Calculate Limits of Accuracy** and the **Results window** will be brought in focus. In addition, the console window will be hidden when the calculation is over. The console window can be accessed any time by selecting the **Console** option under the View Menu.

🜆 Results	-		
2D Localization Accuracy - Airy Pixelated with Poisson + Gaussian Noise			
Results : Limit of the	accuracy	of	
×0 =	6.349	nanometers	
y0 =	6.349	nanometers	
Alpha* =	0.000686	1/nanometers	
Photon detection rate =	746.5606	photons/s	
Background level =	30.2715	photons/pixel/s	
*Alpha = 2 x pi x numerical ap pi = 3.1416 (approx.)	oerture / w	ravelength	

Figure 1.5.2: 2D localization accuracy Results window

#### 2. Varying a parameter (multivalue mode)

Multivalue Mode is a new functionality designed for the calculation and visualization of the fundamental and practical limits on the 2D and 3D localization accuracy and resolution for a range of parameter values. This section provides an example of how to use the Multivalue Mode to calculate the 2D PLAM for a range of an input parameter. We assume that user has applied all the steps in Section 1 (2D-Localization Accuracy Limit).

#### 2.1. Providing inputs for the multivalue mode

**Step 1**: Access the multivalue mode window by toggling the Vary a Parameter button at the bottom of the Required Parameters window.

Extraneous noise source(s)	
Background level (Poisson rate) = 1000 photons/pixel/s	🔚 Multivalue Mode 🛛 😁 📼 💌
Readout (Gaussian) noise parameters	2D Localization Accuracy Airy
Standard deviation = 8 e-/pixel	Pixelated with Poisson + Gaussian Noise
Electron multiplication parameters	Muttivalue parameter
Use EM EM gain = 2.0082	Parameter: Photon detection rate
EM model = Geometric -	Values: [100 200 300 400 500 600
Parameters to be estimated	Units: photons/s
▼ ×0 ▼ Alpha* ▼ Background level	Example: 10:1:13 or [10 11 12 13]
V y0 V Photon detection rate	Auto-center image for each calculation
*Alpha = 2 x pi x numerical aperture / wavelength, pi = 3.1416 (approx.) Vary a Parameter	Results Plot

Figure 2.1.1: Multivalue Mode setting window

**Step 2**: In the – Multivalue parameter - section of window, access the Parameter pull-down menu and select "Photon detection rate".

**Step 3:** In the Values field, enter 100:100:1000. This will automatically define a vector containing [100 200 300 400 500 600 700 800 900 1000].

**Step 4:** check the Auto-center image for each calculation checkbox. This will automatically center the particle in the ROI.

**Step 5:** In the main application window, click the Calculate Limits of Accuracy button. This will present a dialog box warning that the calculation may take a long time to complete. Click Yes to proceed. The Calculate Limits of Accuracy button text will change to Calculating ... and the application will temporarily become unresponsive while the calculations are being performed. In addition the console window will become visible.

**NOTE**: The calculation could indeed take a couple of hours to complete depending on the number of data points and hardware capabilities of the platform on which the application is being executed.

**Step 6**: When the calculation is completed, the Calculating ... button text will change back to Calculate Limits of Accuracy.

**NOTE**: Steps 5 and 6 are explained in more details in Section 1.5 (Executing the task and viewing results).

#### 2.2. Plotting the limit of accuracy versus a parameter value

**Step 1:** In the **Multivalue Mode window**, click the **Plot** button. This will open the **Multivalue Plot Window** shown in the following figure.



Figure 2.2.1: Multivalue results and plot windows

**Step 2:** In the **Multivalue Plot window**, access the Select Plot pull-down menu and select "Limit of accuracy of x0 vs Photon detection rate". A plot of the limit of accuracy of x0 versus the photon detection rate will be shown as illustrated in the above figure.

**Step 3:** In the **Multivalue Mode window**, click the **Results** button. This will open the **Multivalue Results Window** shown in the above figure. The selected values for the parameter will then be shown in a list box. Clicking different lines of this list box will show the corresponding results in the **Results Window**.

#### 3. Simulating and visualizing model and data images

Another new feature of the FandPLimitTool V1.2 is that user can now simulate and visualize model and data images of a single molecule associated with a desired problem scenario and a specific set of parameters. A model image is in fact the image profile of a single molecule (or two single molecules in case of a resolution problem) in the absence of stochasticity and extraneous noise. A simulated data image, on the other hand, is a realization of a model image and is corrupted by stochasticity and extraneous noise sources such as background, readout and stochastic signal amplification. The following sections provide examples for calculating and visualizing model and data images, respectively. We assume that user has applied all the steps in Section 1 (2D-Localization Accuracy Limit).

#### 3.1. Calculate a model image

**NOTE**: For some of the problem scenarios (mostly fundamental limits), a model image may not be available. In such cases, the **Calculate Model Image** button will be grayed out and as such a model image cannot be calculated.



Figure 3.1.1: Unavailable model and data images

**Step 1**: If a model image is available for current problem scenario, in the main application window, click the Calculate Model Image button. The Calculate Model Image button text will change to Calculating Model Image ... and the application will temporarily become unresponsive while the calculations are being performed.

FandPLimitTool	
<u>File Export View Parameters T</u> ools <u>H</u> elp	
Select a problem scenario:	
2D Localization Accuracy - Airy: Pixelated with Poisson Noise	<b>•</b>
Perform calculations	
Calculate Limits of Accuracy Calculate Model Im	age Simulate Data Images
	Calculate a (deterministic) model image
FandPLimitTool	
FandPLimitTool File Export View Parameters Tools Help	<b>-</b>
FandPLimitTool File Export View Parameters Tools Help Select a problem scenario:	
FandPLimitTool File Export View Parameters Tools Help Select a problem scenario: 2D Localization Accuracy - Airy: Pixelated with Poisson Noise	
FandPLimitTool File Export View Parameters Tools Help Select a problem scenario: 2D Localization Accuracy - Airy: Pixelated with Poisson Noise Perform calculations	
FandPLimitTool File Export View Parameters Tools Help Select a problem scenario: 2D Localization Accuracy - Airy: Pixelated with Poisson Noise Perform calculations Calculate Limits of Accuracy Calculating Model Im	age Simulate Data Images

Figure 3.1.2: Calculate a model image

**Step 2**: When the calculation is completed, the Calculating Model Image ... button text will change back to Calculate Model Image and the display tool will be brought in focus.

🛃 Window 1 x : 10 y : 7 Adj. Pixel :0 Orig. Pixel :100	Viewer Slider 1	
File Edit View Insert Tools Desktop Window Help 🏻 🏾		
1 🖆 🖬 🔌 🔖 🔍 🖤 🕲 🐙 🔏 - 🗔 🔲 📰 💷 💷	1 1	
		Details
		Open Tools
		Mode
		Normal
		Channel
		All
		Display
		Standard
		Multiplicity
	1 1	Single
		Options
		Parallel
		Free Dim
		Apply
		Save Overlay
		Open Player
		Open Zoom Tool
		Open Montage Tool
		More

Figure 3.1.3: Model image viewer

**Step 3**: In the viewer window, you can visualize the model image and change the visual effects.

**NOTE**: The viewer belongs to our previously developed Microscopy Image Analysis Tool (MIATool). For more information please see [4].

**Remark 6**: In case that the **Show Model** functionality is used together with the **Multivalue Mode**, the viewer will show a stack of images associated with the different values of the Multivalued parameter. In this case, user can visualize the different model images by scrolling the slider to the left in the **Viewer Slider window**, as shown below. The current value of the parameter (e.g. Magnification = 150) will also be shown in the viewer window (see below).



Figure 3.1.4: Model image viewer in Multivalue Mode

**Step 4**: To improve screen clarity, close the **Model Image Viewer** using the standard exit buttons on any of the viewer windows.

#### 3.2. Simulate data images

**NOTE**: For some of the problem scenarios (mostly fundamental limits), a data image may not be available. In such cases, the **Simulate Data Images** button will be grayed out and as such a data image cannot be simulated (this is shown in Figure 3.1.1).

**Step 1**: If a data image is available for current problem scenario, in the main application window, click the Simulate Data Images button. This will present a dialog box to enter the desired number of data images (i.e. realizations) of current model.

FandPLimitTool		
File Export View Parameters	Tools Help	
Select a problem scenario: 2D Localization Accuracy - Airy: Pixi	elated with Poisson + Gaussian Noise	•
Perform calculations —		
Calculate Limits of Accu	racy Calculate Model Image	Simulate Data Images
	Simulate a se in the presen	t of images (realizations) given a model and ce of stochasticity and extraneous noise.
	🛃 Number of Data Images 🛛 💼 📧	
	Please enter the number of data images (realizations): 35	]
	OK Cancel	]

Figure 3.2.1: Enter the number of data images

**Step 2:** Enter the desired number of data images. Click **OK** to proceed. The Simulate Data Images button text will change to Simulating X Data Images ..., where X is the number of desired images specified by user. The application will temporarily become unresponsive while the calculations are being performed.

FandPLimitTool	
File Export View Parameters Tools Help	
Select a problem scenario:	
2D Localization Accuracy - Airy: Pixelated with Poisson + Gaussian Noise	-
Perform calculations	
Calculate Limits of Accuracy Calculate Model Image Simulating 3	35 Data Images

Figure 3.2.2: Simulating data images

**Step 3**: When the calculation is completed, the Simulating X Data Images ... button text will change back to Simulate Data Images and the display tool will be brought in focus.

**Step 4**: In the viewer window, you can visualize the data images and change the visual effects. You can also visualize different realization by scrolling the slider to the right in the **Viewer Slider window**.

**NOTE**: The viewer belongs to our previously developed Microscopy Image Analysis Tool (MIATool). For more information please see [4].

**Remark 7**: In case that the **Simulate Data Images** functionality is used together with the **Multivalue Mode**, the viewer will show a stack of data images associated with the different values of the Multivalued parameter. In this case, user can visualize images that correspond to different model by scrolling the slider to

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the left in the **Viewer Slider window**, as shown below. On the other hand, different realizations (data images) of a specific model can be accessed using the slider to the right (see below).



Figure 3.2.3: Simulated image viewer in Multivalue Mode

### 4. Saving and loading the settings

User can conveniently save and load the software settings which include the current problem scenario, required and advanced parameters, etc., into and from the disk, respectively. This functionality is useful for retrieving the adjustments and results of a specific calculation after closing and reopening the software. We next briefly describe how to make use of this important functionality.

#### 4.1. Saving the settings

**Step 1**: To save the settings, click the Save Settings option under the File Menu or use the shortcut **Ctrl+S** (see below). This will present a dialog box to enter a file name. The file extension is .fim. Enter your desired name and click Save.

F. <sub>P</sub> F	andPLimitTool			×
File	Export View	Paramete	ers Tools Help	
	Load Settings	Ctrl +L		
	Save Settings	Ctrl+S		_
	Exit	Ctrl+Q	Pixelated with Poisson + Gaussian Noise	▼.
	Perform calcula	ations —		
	Calculate Li	imits of A	Ccuracy Calculate Model Image Simulate Data Images	

Figure 4.1.1: Saving and loading the settings

#### 4.2. Loading the settings

**Step 1**: To load the settings, click the Load Settings option under the File Menu or use the shortcut **Ctrl+L** (see Figure 4.1.1). This will present a dialog box to choose the desired file with extension .fim. Select a previously saved setting and press Open.

#### 5. Exporting results, parameters, images and report

The FandPLimitTool V1.2 provides very flexible tools for exporting the results of calculations, simulated images and even a report file. More specifically, user can export the results and parameters of a desired problem scenario, the simulated model and data images and a LeTex report that can be generated when performing a multivale calculation (i.e. when varying a parameter). The simulated model and data images can also be exported when varying a parameter. In such cases, a sequence of images will be exported. In this section, we discuss different steps of exporting various results.

#### 5.1. Exporting the results and parameters

**NOTE**: To export the desired outputs such as results, images and a report, appropriate calculations has to be performed prior to exporting. If the results, model and data images and a LeTex report are not available, the corresponding menu options under the **Export Menu** will be grayed out (see below). After the appropriate calculation is performed the corresponding menu option becomes accessible.

File	Export View Parameters Tools He	lp
Se 2D	Results and Parameters Model and Data Images LaTex Report	•
	All Ctrl+E Calculate Limits of Accuracy	Calculate Model Image Simulate Data Images

Figure 5.1.1: Exporting is not available

**Step 1**: Perform either a standard (see e.g. Section 1) or a multivalue (see Section 2) calculation for the limits of accuracy. Once the calculation is performed and the results are available within the software, the Results and Parameters option under the Export Menu becomes accessible.

**Step 2**: Select the Results and Parameters option under the Export Menu (see figure below). This will present a folder browser.

**Step 3**: Select a folder name in which you would like to save the results and parameters and click OK. The results and parameters will be saved as two independent text files in the specified folder.

Required Parameters	
2D Localization Accuracy - Airy Pixelated with Poisson Noise — Fundamental inputs————————————————————————————————————	Results 😂 🗆 🖾 🖾
Numerical aperture = 1.45 Wavelength = 0.69 microns Photon detection rate = 1000 photons/s	2D Localization Accuracy - Airy Pixelated with Poisson Noise — Results : Limit of the accuracy of————————————————————————————————————
Exposure time = 1 seconds	x0 = 6.3716 nanometers
Additional inputs           Magnification =         100	y0 = 6.3716 nanometers Alpha* = 0.000620-1/hanometers Photon detection rate = 60.07 photons/s Background level = 0.98603 photons/pixel/s *Alpha = 2 × pi × numerical aperture / wavelength pi = 3.1416 (approx.)
Pixel size [height, width] = [13, 13] microns X microns	🕞 FandPLimitTool 🐡 🗖 🖬 🌄
Extraneous noise source(s)       Background level (Poisson rate) = 100       phrameters to be estimated       Image: source with the set of the set	File     Export     View     Parameters     Tools     Help       Se     Results and Parameters     Model and Data Images     son Noise       2D     LaTex Report     all     Ctrl+E       Calculate Limits of Accuracy     Calculate Model Image     Simulate Data Images
*Alpha = 2 x pi x numerical aperture / wavelength, pi = 3.1416 (approx.) Vary a Parameter	

Figure 5.1.2: Exporting the results and parameters

**NOTE**: Depending on the type of calculation (i.e. singlevalue and multivalue), the exported results text file may include one value or multiple values for the limit of accuracy of each parameter. An example for the exported text file for a multivalue calculation is shown below.

					• 🕱
		<b>-</b> ↓	Search Exported	files	Q
Organize ▼ Include in library ▼ Share with ▼ New folder					0
Favorites Name	Date modified	Туре	Size		
📃 Desktop 🛛 📋 Parameters.txt	5/20/2014 2:30 PM	Text Document	1 KB		
🔒 Downloads 🗉 📄 Results.txt	5/20/2014 2:30 PM	Text Document	1 KB		
Recent Places					
🔄 Results.txt - Notepad 📃 📼 💌	🥘 Parameters.txt - N	otepad			
File Edit Format View Help	File Edit Format	View Help			
Photon detection rate,x0,y0,Photon detection rate 100.0.021005.0.021005.27.735	Localization A	ccuracy 2D Airy 	/ Fundamenta	l Limit	~
200,0.014853,0.014853,39.2232 300,0.012127,0.012127,48.0384 400,0.01503,0.010503,55.47 500,0.0093939,0.0093939,62.0174 600,0.0085754,0.0085754,67.9366 700,0.0079393,0.0079393,73.3799 800,0.0074265,0.0074265,78.4465 900,0.007018,0.0070018,83.205 1000,0.0066425,0.0066425,87.7058	Basic Inputs: Numerical Apar wavelength = Exposure time Magnification Photon Detecti Model paramete  Advanced Input	ture = 1.45 0.69 microns Interval = [0,0 = 100 on Rate = 1000 rs = [0.325,0 s:	0.13] s photons/s 0.325,13.203	8]	
	There are no a	dvanced inputs	for this ca	iculate type	•
	*				► ai

Figure 5.1.3: An example for the exported results and parameters

#### 5.2. Exporting the model and data images

**Step 1**: Exporting the model images is available upon performing at least one of the three different actions which are the calculation of the limits of accuracy (see e.g. Section 1), the calculation of a model image (see Section 3.1) and the simulation of data images (see Section 3.2). On the other hand, both model and data images can be exported only after simulating data images. Once one of the mentioned calculations is performed, the Model and Data Images option under the Export Menu becomes accessible.

**Step 2**: Select the Model and Data Images option under the Export Menu (see figure below). This will present a folder browser.

**Step 3**: Select a folder name in which you would like to save the simulated model and data images and click OK. The model and data images will be saved within two separate folders under the specified folder.

**NOTE**: Depending on the type of calculation (i.e. singlevalue or multivalue), the exported model and data image folders may contain a sequence of images.

🔚 Fa	ndPLimitTool				- • ×	Browse For Folder
File Se 2D	Export View Results and Model and LaTex Repo	Parameters Tools Help Parameters Data Images rt sson Noise				Select a folder to export the results, parameters, images, etc.
	All	Ctrl+E	Model Image	Simulate Da	ta Images	<ul> <li>&gt; □ Libraries</li> <li>&gt; □ Amir Tahmasbi</li> <li>= □ ○ Computer</li> <li>&gt; □ ○ Local Disk (C:)</li> <li>&gt; □ □ Losoft (\labsoft) (S:)</li> <li>&gt; □ □ filtransfernew (\labsoft) (T:)</li> </ul>
	<b>○ -  ▲</b> « M	y Documents 🕨 Exported files 🕨	<b>- 4</b> , S	earch Exported files		Eolder: Amir Tahmasbi Make New Folder OK Cancel
0	rganize 🔻 In	clude in library   Share with	New folder	Ture		h.
	Favorites     Eavorites     Desktop     Downloads     Recent Place:		5/20/2014 4:25 PM 5/20/2014 4:25 PM 5/20/2014 4:25 PM 5/20/2014 2:30 PM	File folder File folder File folder Text Document	JIZE 1 KB	
C	Libraries	Results.txt	5/20/2014 2:30 PM	Text Document	1 KB	

Figure 5.2.1: Exporting the model and data images

#### 5.3. Exporting a LeTex report

**NOTE**: A LeTex report can be generated only after performing a multivale calculation (i.e. calculating the limits of accuracy when varying a parameter).

**Step 1**: Perform a multivalue calculation for the limits of accuracy (see Section 2). Once the calculation is performed and the results are available within the software, the LeTex Report option under the Export Menu becomes accessible.

**Step 2**: Select the LeTex Report option under the Export Menu (see figure below). This will present a folder browser.



Figure 5.3.1: Exporting a LaTex report

**Step 3**: Select a folder in which you would like to save the LaTex report and click OK. A .tex file together with several .eps images will be saved within a folder named LaTex Report under the specified folder.

#### 5.4. Exporting everything simultaneously

**NOTE**: If all the required calculations are performed, the export All... option becomes available and user can export everything simultaneously.

Step 1: Select the All... option under the Export Menu. This will present a folder browser.

**Step 2**: Select a folder name in which you would like to export all the results including the limits of accuracy, the simulated model and data images and the LaTex report, and then click OK.

#### 6. Console window, log file and bug reporting

The FandPLimitTool V1.2 is equipped with a new console window and a log file. The console window, which can be accessed directly within the software, not only can provide users with the details of calculations in real-time, but also is very useful for identifying warning, errors and reporting bugs. The information that are shown in the console window are also stored in a log file.

#### 6.1. The console window

As mentioned earlier, during the long calculations a console window is automatically displayed to inform the user about different steps of the calculation. This console window can also display errors and warnings, if there is any, which is very useful for bug reporting purposes.

**Step 1**: To access the console window at any time, select the Console option under the View Menu.

**Step 2**: To hide the console window, simply close it using the standard close button. Note that closing the console window will not stop storing the information in the log file.

🔄 FandPLimit	Tool				$\Leftrightarrow$		x
File Export	View Parameters Tools	Help					
Select a pr 2D Localizati — Perform	Parameter Summary Results Console calculations	Ctrl+P Ctrl+R 38	aussian Noise				•
Calc	ulate Limits of Accuracy	Calcu	ulate Model Image	Simu	ulate Data Ima	ges	
Poisson Gaus Poisson Gaus Poisson Gaus Poisson Gaus Poisson Gaus Poisson Gaus Application init All rights reser Copyright (C) 2 For single-mo FandPLimitTo Initializing the	s Mixture 2 of 2. s Mixture 2 of 2. ialized! ved. 2004-2014 Ward Ober Lab ecule localization and resolut ol V1.2 application	on					

Figure 6.1.1: Console window

#### 6.2. The log file and bug reporting

The information that are displayed in the console window are also stored in a log file "fandp\_log.mia" in the following path:

C:\Users\{username}\AppData\Roaming\FandPLimitTool

Constant form	nuter 🕨 Local Disk (C4) 🕨 Users 🕨 amir	▶ AnnData ▶ Roaming ▶ Fandl	21 imitTool	▼ (▲ Search FandPl	imitTool	
Organize - Inclue	de in library	folder	Linitrool	· · · · · · · · · · · · · · · · · · ·	i= • E	
🔶 Favorites	A Name	Date modified	Туре	Size		
E Desktop Downloads Carcent Places Libraries Documents Music Fictures Videos	i fandp_log.mia	5/19/2014 5:21 PM	MIA File	1 KB		
1 item						

Figure 6.2.1: Log file

To report a bug (that leads to an error or warning), please send this log file to us. More information about the current versions of the FandPLimitTool and the MUMDesignTool, their homepages and licensing can be found in the corresponding **About windows** (e.g. see below).

	🖬 About FandPLimitTool 🔅 🗖 🛙 🖾
	FandPLimitTool V1.2         For single molecule localization and resolution         Copyright (C) 2004-2014 Ward Ober Lab         All rights reserved.         MATLAB         Copyright (C) 1984-2014 The MathWorks, Inc.         All rights reserved.         Martial         Copyright (C) 1984-2014 The MathWorks, Inc.         All rights reserved.         Martial         Copyright (C) 1984-2014 The MathWorks, Inc.         All rights reserved.         Martial         Copyright (C) 1984-2014 The MathWorks, Inc.         All rights reserved.         Martial         PadPLimitTool home page:         htp://www.wardoberlab.com/software/fandplimittool/         Please refer to license, pdf for conditions of distribution and use.         BrandPLimitTool Developement Team:         Amish V. Abraham,         - Jenry Chao,         - Sinpad Ram,         - Sinpad Ram,         - K
FandPLimitTool	
File Export View Parameters Tools Help	
Select a problem scenario:	out Ctrl+A
2D Localization Accuracy - Airy: Pixelated with Poisson +	Gaussian Noise
- Perform calculations	
Calculate Limits of Accuracy Ca	Iculate Model Image Simulate Data Images

Figure 6.2.2: About the FandPLimitTool

#### 7. The MUMDesignTool

This section describes how to use the MUMDesignTool, a new software module in the new version of the FandPLimitTool, to calculate and plot the Fisher information matrix (FIM) and the PLAM [1] along the z-axis. The MUMDesignTool is developed to help in designing the plane spacing for a multifocal plane microscopy (MUM) setup including up to 10 focal planes.

The MUMDesignTool provides two working modes. The *Rapid Mode* takes into account the stochastic nature of the photon detection processes and assumes that the observed data is Poisson distributed [1]. However, this mode does not take into consideration other extraneous noise sources such as the background, readout and stochastic signal amplification. This mode yields a fast approach for the calculation of the FIM and the PLAM, and allows changing the number of focal planes and their spacings in real-time. In addition, the *Precise Mode* takes into account the extraneous noise sources, such as the background noise which is independently Poisson distributed, the readout noise which is modeled as independent additive Gaussian noise [2], and in case of using an EMCCD detector, the stochastic electron multiplication noise that is modeled as a branching process [3].

It is assumed that the pixels are rectangular in shape with no dead space between any two pixels. Further, the ROIs containing the images of the object are assumed to be rectangular arrays of pixels. The object is assumed to be in the center of this rectangular pixel array and the location of the object is specified with respect to the object space. Refer to [1] for more details regarding the design of focal plane spacing for a MUM setup.

#### 7.1. Summary of simulation conditions

The following table summarizes the imaging conditions under which the calculations are performed.

Property	Value
Number of focal planes	2
Photon detection rate	10,000 photons/second
Photon count percentage	[50, 50] %
Object medium refractive index	1.515
Numerical aperture	1.45
Wavelength	0.69 μm
Exposure time	0.1 seconds
Magnification	100 X
Location of object in ROI	0.585 μm, 0.585 μm
Pixel size	13 μm × 13 μm
ROI Size	9 pixels × 9 pixels
Defocus start	-2 μm
Defocus increment	0.05 μm
Defocus end	2 μm

Table 7.1.1: Summary of simulation parameters for the MUMDesignTool

#### 7.2. Providing the inputs and configuring the MUMDesignTool

**Step 1**: Access the **Configure Setting – MUM Design Tool window** by clicking the **MUMDesignTool** option under **Tools Menu** in the main application window or through its shortcut **Ctrl+M**.

📴 Configure Settings - MUMDesignTool 🛛 😂 🗖 🗐 Σ
3D Localization Accuracy - Born-Wolf Model Pixelated with Poisson Noise
Simulation parameters
Number of focal planes = 2
Photon detection rate = 10000 photons/s
Photon count percentages = [50, 50] %
Immersion medium ref. index = 1.515
Numerical aperture = 1.45
Wavelength = 0.69 microns
Exposure time = 0.1 seconds
Magnification = 100
Location parameters
x0 = 0.325 microns Auto-center
y0 = 0.325 microns
Divelation parameters
Pixel size (height, width) = [13, 13] microns X microns
Rol size [height, width] = [5, 5] ho. or pixels X ho. or pixels
Defocus range
Defocus start = -1 microns
Defocus increment = 0.1 microns
Defocus end = 1 microns
Number of objects / data points = 21
Done

Figure 7.2.1: The MUMDesignTool configuration window

**Step 2**: In the – Simulation parameters - section of the window, enter the values for the various fields as follows: *Number of focal planes = 2, Total photon count = 10000, Photon count percentage = [50, 50], Immersion medium refractive index = 1.515, Numerical aperture = 1.45, Wavelength = 0.69, Exposure time = 0.1, Magnification = 100, x\_0 = 13 \times 4.5/100, y\_0 = 13 \times 4.5/100, Pixel size = [13, 13], and <i>ROI size = [9, 9].* 

**Note**: The center of a 9 *pixels* × 9 *pixels* pixel array where the dimensions of each pixel is  $13 \ \mu m \times 13 \ \mu m$ , is  $(4.5 \times 13) \ \mu m \times (4.5 \times 13) \ \mu m$ . To convert this location coordinates to the object space, the coordinate values are divided by the magnification. Hence the location coordinates are specified as  $13 \times 4.5/100 \ \mu m \times 13 \times 4.5/100 \ \mu m$ .

**Step 3**: In the – Defocus range - section of the window, enter the values for the various fields as follows: Defocus start = -2, Defocus increment = 0.05, Defocus end = 2.

**Step 4:** In the configure setting – MUM Design Tool window, click the Done button. The Done button text will change to Calculation in progress ... and the application will temporarily become

unresponsive while the calculations are being performed. During the calculation, a console window will be shown to inform the user about the different steps of the calculation. This console window will also display errors and warnings, if there is any, which is very useful for bug reporting purposes.

**NOTE**: The calculation could indeed take half an hour to complete depending on the hardware capabilities of the platform on which the application is being executed.

	Defocus range	
	Defocus start = -1 microns	
	Defocus increment = 0.1 microns	
	Defocus end = 1 microns	
	Number of objects / data points = 21	
	Calculation in progress	
		]
🌆 Cor	nsole	• •
MUMD	esignTool: modelobject 21/21 processed.	~
MUMD	esignTool: modelobject 20/21 processed.	
MUMD	esignTool: modelobject19/21 processed.	
MOMD	esign Looi: modelobject 18/21 processed.	-
	esignTool: modelobject17721 processed.	-
	esignTool, modelobject15/21 processed.	
	esignTool: modelobject13/21 processed.	
	esignTool: modelobject14/21 processed.	
MUMD	esignTool: modelobject12/21 processed	
MUMD	esignTool: modelobject 11/21 processed.	
MUMD	esignTool: modelobject10/21 processed.	
MUMD	esignTool: modelobject 9/21 processed.	
MUMD	esignTool: modelobject 8/21 processed.	
MUMD	ecianTool: modelphiert 7/71 processed	

Figure 7.2.2: MUMDesignTool calculation in progress

**Step 5**: When the calculation is completed, the **MUMDesignTool (Rapid Mode) window** will be brought in focus (more details about this window will be discussed in the next section).

#### 7.3. Designing the plane spacing using the Rapid Mode

Figure 7.3.1 shows the main window of the MUMDesignTool. The – Simulation summary - section of the window represents a summary of the simulation parameters. The – Focal plane offset - section of the window includes a pull-down menu for selecting the number of focal planes as well as 10 slides to change the plane spacings. Note that these settings can be performed in real-time. The right-hand side panels, which show the FIM and PLAM along the z-axis for the MUM setup, will be updated automatically by changing the plane spacing and other parameters.

The – PDR – section of the window assists us with choosing the total photon detection rate of the setup as well as the photon percentage for each focal plane. Changing the number of planes will automatically update the photon percentages to be consistent with the total photon detection rate.

35

The – Display options – section of the window provides controls for better calculation and visualization of the FIM and PLAM, and also provides tools for exporting the results. For instance, using radio buttons  $z_0$ ,  $x_0$  and  $y_0$ , user can switch the FIM and PLAM plots to visualize the behavior of the corresponding coordinate along the z-axis. The embedded checkboxes help to hide or show different curves within the figures and enhance the visibility. Moreover, a desired region in the plot can be highlighted using other controls in this panel.

The Reset button in this panel resets the MUMDesignTool by recalling the **Configure Setting – MUMDesignTool Window**. The Split windows button shows the FIM and PLAM plots in new decoupled windows. Also, the Export button exports both the results as .txt files and the plots as .pdf files. Finally, the Precise Mode button runs the precise mode calculation of the MUMDesignTool to provide accurate results for the behavior of the FIM and PLAM curves (This function will be discussed in detail later).

We next give an example for designing the focal plane spacing for a 3-plane MUM setup using the strong coupling spacing scenario (for more information see [1]). We assume that user has applied the steps in Section 7.2 (Providing the inputs and configuring the MUMDesignTool). Here are the steps:



Figure 7.3.1: MUMDesignTool Rapid Mode (main window)

**Step 1:** In the – Focal plane offset - section of the window select "3" from the Number of focal planes pull-down menu.



Figure 7.3.2: Changing the number of focal planes in real-time

**Step 2:** You will see an alert stating that "Photon count percentages have been reset". Click OK to proceed.

**Step 3:** In the – Focal plane offset - section of the window set the value of the plane spacing slider 2 to be equal to "0.6 microns". Also, set the value of the plane spacing slider 3 to be equal to "1.2 microns". This will overlap the zero of the z0-FIM of each focal plane with the local maximum of that of the adjacent focal plane (for more information see [1]). As a result, the FIM and PLAM curves will be updated.

**Step 4:** In the – Display options - section of the window, select "Sum line" from the View mode pull-down menu. This will update the FIM such that only the sum line data points will be visible.

**Step 5:** In the – Display options - section of the window, change the right-hand side field of Highlight region to "0.6" and check the "Show" checkbox. This will highlight the region between plane 1 and plane 2 for better visualization.

**Step 6:** In the – Limit ruler - section of the window, set the z0 slider to *19 nm*. At this step, you will see the z<sub>0</sub> elements of the FIM and PLAM curves.

**Step 7:** To see the  $x_0$  elements of the FIM and PLAM curves, in the – Display options – section, select the x0 radio button. The FIM and PLAM curves will be updated accordingly as can be seen in the following figure.



Figure 7.3.3: The lateral elements of FIM and PLAM (MUMDesignTool Rapid Mode)

**Step 8:** To save the calculated data points and the plots click the Export button in the – Display options – section. You will be asked to select a folder.

9 €           10 €	Image: Omega     <	Browse For Folder	
_ Display options	PDR Max:	Select a rolder	
Switch plots:         ☑ 0         ☑ 0         ☑ 0         ☑ 0           Visibility:         ☑ Sum         Fix Y           Plane lines:         ☑ 1         ☑ 2         ☑ 3         ☑ 4         ☑ 5           ☑ 6         ☑ 7         ☑ 8         ☑ 9         ☑ 10	10000 <b>v</b> 10000 (photons/s)	■ Desktop ▷ 🧊 Libraries ▷ 🥦 Amir Tahmasbi	Î
Plane markers:  1 2 2 3 3 4 5 0 6 7 7 8 9 9 10 View mode: default		<ul> <li>Image: Computer</li> <li>Ima</li></ul>	-
Highlight region:     Image: Show       Image: Oil point of the show     Image: Oil point of the show       Reset     Export       Precise Mode     Split windows	Photon (%): [33, 34, 33]	Folder: Amir      Make New Folder OK Can	

Figure 7.3.4: Exporting the MUMDesignTool results

**Step 9:** In the **Browse For Folder window**, select a folder and click the OK button. This will export the results as text files and the plots as pdf files.

#### 7.4. Precise Mode

Once user has designed the plane spacing using the Rapid Mode, the next step is to verify whether the designed spacing is appropriate in the presence of extraneous noise sources. This verification is performed using the Precise Mode of the MUMDesignTool. We next provide an example for this verification step.

**Step 1:** Click the Precise Mode button in the – Display options – section to calculate the accurate FIM and PLAMs along the z-axis considering the extraneous noise parameters. This will load the **Noise Parameter Setting (Precise Mode) window** as shown below.

🔤 Noise Parameter Setting (Precise Mode)	Switch plots: O z0 O y0 10000 -
Noise Parameter Setting Noise type = Poisson + Gaussian + Branching	Visiolity:         Visiolity:         Visiolity:         10000           Plane lines:         I
Background level = [35 60 85] (photons/s. Readout noise std = [8 5 7] (e-/pixel) EM gain = [250 100 110] EM model = [1 1 1] 1: Geometric model 2: High gain model 3: Gaussian model	X       Examples:       Plane markers:       1 v 2 v 3 4 v 5         "Poisson" (e.g. PMT array),       fault       fault       fault         "Poisson + Gaussian" (e.g. CCD, sCMOS),       v 5 how       show       show         Color       to < 0.6 >       fault       v         Photon (%):       Precise Mode       Split windows       f33, 34, 33]

Figure 7.4.1: Entering the noise parameters

**Step 2:** In **Noise Parameter Setting (Precise Mode) window**, select the "Poisson + Gaussian + Branching" option in the Noise Type drop down menu. This will consider background, readout and branching noise for the calculations. This option is suitable when using an EMCCD detector.

**NOTE**: Holding the mouse pointer on this drop down menu will give a brief explanation (as a tool tip text) about the suitable option for different types of detectors (see Figure 7.4.1).

**Step 3:** Enter the values *Background noise* = [35 60 85], the *Readout noise std* = [8 5 7], the *EM*  $gain = [250 \ 100 \ 110]$  and the *EM*  $model = [1 \ 1 \ 1]$ . Click OK when done. The Precise Mode button text will change to Calculating ... and the application will temporarily become unresponsive while the calculations are being performed (see figure below). The console window will also be displayed.

**NOTE**: The calculation could indeed take an hour to complete depending on the hardware capabilities of the platform on which the application is being executed.

🔄 Console 📃 📼 💌	Visibility: V Sum Fix Y
MUMDesignTool: Precise Mode 2/81 processed.	Plane lines: 📝 1 📝 2 📝 3 📝 4 📝 5
MUMDesignTool: Precise Mode 1/81 processed. 📁 📼	7677879710 (photons/s)
Calculating the FIM and PLAM in Precise Mode	Plane markers: 1 1 2 2 3 7 4 7 5
MUMDesignTool: modelobject 81/81 processed.	
MUMDesignTool: modelobject 80/81 processed.	
MUMDesignTool: modelobject 79/81 processed.	View mode: default
MUMDesignTool: modelobject 78/81 processed.	1 Each Eacht an air ann
MUMDesignTool: modelobject 77/81 processed.	nigniigni region. V Snow
MUMDesignTool: modelobject 76/81 processed.	< 0 > to < 0.6 >
MUMDesignTool: modelobject 75/81 processed.	
MUMDesignTool: modelobject 74/81 processed.	Reset Export Photon (%):
MUMDesignTool: modelobject 73/81 processed.	Calculating Split windows
MUMDesignTool: modelobject 72/81 processed.	
MUMDesignTool: modelobject 71/81 processed.	
MLIMDesignTool: modelphiert 70/81 processed	



**Remark 8**: Each element in the *Background noise, Readout noise std, EM gain* and *EM model* vectors corresponds to one of the focal planes. For example, in the above case, the vectors have 3 elements associated with the 3 focal planes. For more information about the different EM models see [3].

**Step 4**: When the calculation is completed, the Calculating ... button text will change back to Precise Mode and the **MUMDesignTool (Precise Mode) window** will be brought in focus (see figure below).



Figure 7.4.3: MUMDesignTool Precise Mode

#### 8. 3D-Localization accuracy measure (Born-Wolf Model)

This section provides an example of how to use the FandPLimitTool to calculate the accuracy with which the 3D location of an object can be estimated from its image (PLAM). The image of the object is assumed to be a 3D Point Spread Function profile.

The calculation takes into account the stochastic nature of the photon detection processes and assumes the photons detected from the object to be Poisson distributed. In addition, the calculation also takes into account extraneous noise sources - in particular the background component which is modeled as Poisson noise [1], the readout noise of the detector which is modeled as independent, additive Gaussian noise [2] and the electron multiplication noise of an EMCCD detector which is modeled as a branching process [3].

It is assumed that the pixels are rectangular in shape with no dead space between any two pixels. Further, the ROI containing the image of the object is assumed to be a rectangular array of pixels. The object is assumed to be in the center of this rectangular pixel array and the location of the object is specified with respect to the object space.

Refer to [5] for details on determining the 3D localization accuracy in single molecule microscopy.

#### 8.1. Summary of imaging conditions

The following table summarizes the imaging conditions under which the calculations are performed.

Property	Value
Z <sub>0</sub>	0.5 μm
Object medium refractive index	1.515
Numerical aperture	1.3
Wavelength	0.52 μm
Photon detection rate	10,000 photons/second
Exposure time	0.13 seconds
Magnification	100 X
Location of object in ROI	0.585 μm, 0.585 μm
Pixel size	13 μm × 13 μm
ROI Size	9 pixels × 9 pixels
Background level (Poisson rate)	1000 photons/pixel/second
Gaussian noise mean	0 e <sup>-</sup>
Gaussian noise standard deviation	8 e <sup>-</sup> rms
Use EM	unchecked
EM gain	100
EM model	Geometric

Table 8.1.1: 3D Localization Accuracy - Summary of imaging conditions

#### 8.2. Providing required parameters

**Step 1**: Select the calculation option "3D Localization Accuracy - Airy: Pixelated with Poisson + Gaussian Noise" from the Calculate pull-down menu in the main application window.

**Step 2**: The required parameters window should be visible by default. If it is not visible, access it by clicking the Required Parameters option under the Parameters Menu.

ed Parameters 😂 🗖 I	
3D Localization Accuracy - Born-Wolf Model Pixelated with Poisson + Gaussian Noise	
Fundamental inputs	
z0 = 0.01 microns	
Object medium refractive index = 1.515	
Numerical aperture = 1.45	
Wavelength = 0.60	
Photon detection rate = 10000 photon:	
Exposure time = 0.13 second	
Additional inpute	
Additional inputs	2D Localization Accuracy - Airy: Fundamental Limit
Magnification = 100	2D Localization Accuracy - Airy: Pixelated with Poisson Noise
Location parameters	2D Localization Accuracy - Airy: Pixelated with Poisson + Gaussian Noise
x0 = 0.325 microps Auto center	2D Localization Accuracy - Gaussian Symmetric: Fundamental Limit
vo 0.025 microns <u>Adus-cente</u>	2D Localization Accuracy - Gaussian Symmetric: Pixelated with Poisson Noise
yu = 0.325 microns	2D Localization Accuracy - Gaussian Symmetric: Pixelated with Poisson + Gaussian Noise
Pixelation parameters	2D Localization Accuracy - Gaussian Asymmetric Fundamential Limit
Pixel size [height, width] = [13, 13] microns X microns	2D Localization Accuracy - Gaussian Asymmetric, Protected with Descent Accuracy
BOLsize [height width] = [5, 5] no. of pixels X no. of	2D Localization Accuracy - Gaussian Asymmetry in rotated with rotation - Gaussian Noise
teres (resident result for each second secon	3D Localization Accuracy - Born-Wolf Model: Pixelster with Prisson Noise
Extraneous noise source(s)	3D Localization Accuracy - Born-Wolf Model: Pixelated with Poisson + Gaussian Noise
	2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson Noise (Symmetric Parameterization)
background level (Poisson rate) = 1000 photons/pi	2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson + Gaussian Noise (Symmetric Parameterization)
Readout (Gaussian) noise parameters	2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson Noise (Aymmetric Parameterization)
Standard deviation = 8 e-/pixel	🛛 🔤 2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson + Gaussian Noise (Asymmetric Parameterization)
	Fi 2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson Noise (Cartesian Parameterization)
Liecti on multiplication parameters	2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson + Gaussian Noise (Cartesian Parameterization)
Use EM EM gain = 2.0082	2D Resolution Accuracy - Airy Model: Fundamental Limit (Symmetric Parameterization)
EM model = Geometric -	2D Resolution Accuracy - Airy Model: Pixelated with Poisson Noise (Symmetric Parameterization)
	3D Localization Accuracy - Born-Wolf Model: Pixelated with Poisson + Gaussian Noise
Parameters to be estimated	Perform calculations
✓ x0 ✓ z0 ✓ Background	/el
V y0 V Photon detection rate	Calculate Limits of Accuracy Calculate Model Image Simulate Data Images
Varya Param	9r

Figure 8.2.1: 3D Localization Accuracy problem scenario and its required parameters window

**Step 3**: In the - Fundamental inputs - section of the window, enter the values for the various fields as follows:  $z_0 = 0.5$ , Object medium refractive index = 1.515, Numerical aperture = 1.3, Wavelength = 0.52, Photon detection rate = 10000, and Exposure time = 0.13.

**Step 4**: In the - Additional inputs - section of the window enter the values for the various fields as follows: *Magnification* = 100,  $x_0 = 13 \times 4.5/100$ ,  $y_0 = 13 \times 4.5/100$ , *Pixel size* = [13, 13], and *ROI size* = [9, 9].

**Remark 9**: The center of a 9 pixels × 9 pixels pixel array where the dimensions of each pixel is  $13 \ \mu m \times 13$   $\mu m$ , is  $(4.5 \times 13) \ \mu m \times (4.5 \times 13) \ \mu m$ . To convert this location coordinates to the object space, the coordinate

values are divided by the magnification. Hence the location coordinates are specified as  $13 \times 4.5/100 \ \mu m \times 13 \times 4.5/100 \ \mu m$ .

**Step 5:** In the - Extraneous noise sources - section of the window, enter the values for the various fields as follows: *Background level = 1000* and *Standard deviation = 8*.

**Remark 10**: In case that the localization accuracy is required for an EMCCD detector, the *Use EM* checkbox should be checked and the desired value for the electron multiplication gain should entered in the *EM gain* field. For more information see Remark 2.

**Step 6:** In the - Parameters to be estimated - section of the window, ensure that all check-boxes for all parameters are checked.

#### 8.3. Providing advanced parameters

**NOTE:** All inputs in the **Advanced Parameters window** are optional. The default values provided have been found to work adequately for typical imaging conditions.

**Step 1**: Access the advanced parameters window by clicking the Advanced Parameters option under the Parameters Menu.

Advanced Parameters			ן				
3D L Pix — Advanced fundamental in	ocalization Accuracy - Born-Wolf M elated with Poisson + Gaussian Noi nputs Alpha* = 13.2038	odel ise te analytically					
Model profile calculation	s						
Pixel integration method :	Trapezoidal	Additional parameters					
Model integration method :	Trapezoidal	Additional parameters					
Background type :	Constant 💌	Additional parameters					
– Fisher information matrix	x calculations		F	andPLimitTool			
Single integration method :	Trapezoidal	Additional parameters	File	Export View	Parameters	Tools Help	
Double integration method :	Trapezoidal	Additional parameters			Require	ed Parameters	Ctrl+U
	Noise factor calculation parameters			elect a problem	s Advand	ed Parameters	Ctrl+N
Limits of su	mmation [lower limit, upper limit] = N/A	use default 🔻	3	D Localization Accu	iracy - born-vvo	III WOORI, PIXEIALEO Y	viin Poisson +
Limits of int	egration [lower limit, upper limit] = N/A	use_default 💌		- Perform calcul	ations ——		
Т	rapezoidal integration step size = 0.05			Calculate L	imits of Accu	ıracy C	alculate Mo
*Alpha = 2 x pi x	numerical aperture / wavelength; pi =	: 3.1416 (approx.)					

Figure 8.3.1: 3D Localization Accuracy advanced parameters window

**Step 2**: The Advanced parameters window contains an - Advanced fundamental inputs - section. The default value of the field in this section can be left unchanged.

**Step 3**: In the - Model profile calculations - section of the window, access the Pixel integration method pull-down menu and select "Trapezoidal".

- Advanced fundamental in	iputs Alpha* = 13.2038	Update analytically	🛃 Trapezo 👝 💷 💌
Model profile calculations	3		X Gridding
Pixel integration method :	Trapezoidal	Additional parameters	V Ovideline
Model integration method :	Trapezoidal	Additional parameters	13
Background type :	Constant	Additional parameters	OK Cancel

Figure 8.3.2: Pixel Integration Trapezoidal options

**Step 4**: Click the Additional parameters button next to the Pixel integration method pull-down menu. In the dialog box that appears, enter the values *XGridding* = 13 and *YGridding* = 13. Click OK when done.

**Step 5**: Access the Model integration method pull-down menu in the same section of the window and select "Trapezoidal".

Pixelated with Poisson + Gaussian Noise (Born-Wolf Model)	🛃 Trapezo 🗖 🗉 💌
Advanced fundamental inputs Alpha* = 13.2038 Update analytically	Step size 0.0005
Model profile calculations	Lower limit
Pixel integration method : Trapezoidal	Linner limit
Model integration method : Trapezoidal	1
Background type : Constant   Additional parameters	OK Cancel

Figure 8.3.3: Model Integration Trapezoidal options

**Step 6**: Click the Additional parameters button next to the Model integration method pull-down menu. In the dialog box that appears, enter the values *Step size = 0.0005, Lower limit = 0*, and *Upper limit = 1*. Click OK when done.

**Step 7**: Access the Background type pull-down menu and select "Constant". There are no additional parameters to be accessed via the Additional parameters button for the "Constant" option.

**Step 8**: In the - Fisher information matrix calculations - section of the window, access the Single integration method pull-down menu and select "Trapezoidal".

Fisher information matrix calculations	🚺 Trapezo 🗖 🔍 💌
Single integration method :       Trapezoidal       Additional parameters         Double integration method :       Trapezoidal       Additional parameters	Step size 0.0005
Limits of summation flower limit upper limit = N/A	Lower limit
Limits of summation [lower limit, upper limit] = N/A use_default	Upper limit 1
Trapezoidal integration step size = 0.05  *Alpha = 2 x pi x numerical aperture / wavelength; pi = 3.1416 (approx.)	OK Cancel

Figure 8.3.4: FIM Single Integration Trapezoidal options

**Step 9**: Click the Additional parameters button next to the Single integration method pull- down menu. In the dialog box that appears, enter the values *Step size = 0.0005, Lower limit = 0*, and *Upper limit = 1*. Click OK when done.

**Step 10**: Access the Double integration method pull-down menu in the same section of the window and select "Trapezoidal".

Fisher information matrix calculations	🛃 Trapezo 💼 🗉 💌
Double integration method :     Trapezoidal     Additional parameters	X Gridding
Noise factor calculation parameters         Limits of summation [lower limit, upper limit] = N/A       use_default ▼         Limits of integration [lower limit, upper limit] = N/A       use_default ▼         Trapezoidal integration step size = 0.05       0.05	Y Gridding 13 OK Cancel
*Alpha = 2 x pi x numerical aperture / wavelength; pi = 3.1416 (approx.)	

Figure 8.3.5: FIM Double Integration Trapezoidal options

**Step 11**: Click on the Additional parameters button next to the Double integration method pulldown menu. In the dialog box that appears, enter the values *XGridding* = 13 and *YGridding* = 13.

**Step 12**: The - Fisher information matrix calculations - section has a subsection titled - Noise factor calculation parameters - . Enter the following values in this subsection: *Limits of summation = [1, 3000], Limits of integration = [-5000, 5000]* and *Trapezoidal integration step size = 0.05*.

**Remark 11**: The limits of integration and summation are initially set to default values which are automatically calculated based on the imaging conditions. It is possible specify these limits by changing the value of the combo box to the right of the limits of integration and summation fields.

#### 8.4. Executing the task and viewing results

**Step 1**: In the main application window, click the Calculate Limits of Accuracy button. This will present a dialog box warning that the calculation may take a long time to complete. Click Yes to proceed. The Calculate button text will change to Calculating ... and the application will temporarily become unresponsive while the calculations are being performed. During the calculation, a console window will be shown to inform the user about the different steps of the calculation.

**NOTE**: The calculation could indeed take half an hour to complete depending on the hardware capabilities of the platform on which the application is being executed.

Warning
TandPLimitTool
Select a problem scenario:
3D Localization Accuracy - Born-Wolf Model: Pixelated with Poisson + Gaussian Noise
Desfere estadator
Penorm calculations
Calculating Calculate Model Image Simulate Data Images
🕞 Console 😂 🗖 🗉 💌
Poisson Gauss Mixture 6 of 6.
Poisson Gauss Mixture 5 of 6. Poisson Gauss Mixture 4 of 6
Poisson Gauss Mixture 3 of 6.
Poisson Gauss Mixture 2 of 6. Poisson Gauss Mixture 1 of 6
Application initialized!
All rights reserved. Convright (C) 2004-2014 Ward Ober Lab
For single-molecule localization and resolution
FandPLimitTool V1.2
· · · · · · · · · · · · · · · · · · ·

Figure 8.4.1: Calculation and Final confirmation

**Step 2**: When the calculation is completed, the **Calculating** ... button text will change back to **Calculate Limits of Accuracy** and the **Results window** will be brought in focus. In addition, the console window will be hidden when the calculation is over. The console window can be accessed any time by selecting the **Console** option under the View Menu.



Figure 8.4.2: 3D localization Accuracy results window

#### 9. 2D-Resolution measure

This section provides an example of how to use the FandPLimitTool to calculate the accuracy with which the 2D location of two objects can be resolved from their image (PREM). The objects are assumed to be in focus with respect to the objective lens and the image of each object is assumed to be an Airy profile.

The calculation takes into account the stochastic nature of the photon detection processes and assumes the photons detected from the object to be Poisson distributed. In addition, the calculation also takes into account extraneous noise sources - in particular the background component which is modeled as additive Poisson noise, the readout noise of the detector which is modeled as independent, additive Gaussian noise [2] and the electron multiplication noise of an EMCCD detector which is modeled as a branching process [3].

It is assumed that the pixels are rectangular in shape with no dead space between any two pixels. Further, the ROI containing the image of the objects is assumed to be a rectangular array of pixels. The midpoint between the objects is assumed to be at the center of this rectangular pixel array and the location of the midpoint between the objects is specified with respect to the object space.

Refer to [6] for details or determining the 2D resolution accuracy in single molecule microscopy.

#### 9.1. Summary of imaging conditions

The following table summarizes the imaging conditions under which the calculations are performed.

Property	Object 1	Object 2	
Numerical aperture	1.3 1.3		
Wavelength	0.52 μm	0.52 μm	
Photon detection rate	10,000 photons/second	10,000 photons/second	
d	0.1 μm		
phi	$\pi/4$ radians		
Exposure time	0.13 seconds		
Magnification	100 X		
Location of object in ROI	0.585 μm, 0.585 μm		
Pixel size	13 μm × 13 μm		
ROI Size	9 pixels × 9 pixels		
Background level (Poisson rate)	1000 photons/pixel/second 1000 photons/pixel/second		
Gaussian noise mean	0 e		
Gaussian noise standard deviation	8 e <sup>-</sup> rms		
Use EM	unchecked		
EM gain	100		
EM model	Geometric		

Table 9.1.1: 2D Resolution Accuracy - Summary of imaging conditions

#### 9.2. Providing required parameters

**Step 1**: Select the calculation option "2D Resolution Accuracy - Airy: Pixelated with Poisson + Gaussian Noise (Symmetric Parameterization)" from the Calculate pull-down menu in the main application window.



Figure 9.2.1: 2D Resolution Accuracy calculate type and its required parameters window

**Step 2**: The required parameters window, which is visible by default, will be updated accordingly. If this window is not visible, access it by clicking the Required Parameters option under the Parameters Menu in the main application window.

**Step 3**: In the - Object selection - section of the **Required parameters window**, select "Object 1".

**Step 4**: In the - Fundamental inputs – Object 1 - section of the window, enter the values for the various fields as follows: *Numerical aperture* = 1.3, *Wavelength* = 0.52, *Photon detection rate* = 10000, d = 0.1, phi = pi/4 and *Exposure time* = 0.13.

**Step 5**: In the - Additional inputs - section of the window, enter the values for the various fields as follows: *Magnification = 100, sx = 13×4.5/100, sy = 13×4.5/100, Pixel size = [13, 13]*, and *ROI size = [9, 9]*.

**Remark 12**: The center of a 9 *pixels* × 9 *pixels* pixel array where the dimensions of each pixel is  $13 \mu m \times 13 \mu m$ , is  $(4.5 \times 13)\mu m \times (4.5 \times 13)\mu m$ . To convert this location coordinates to the object space, the coordinate values are divided by the magnification. Hence the location coordinates are specified as  $13 \times 4.5/100 \mu m \times 13 \times 4.5/100 \mu m$ .

**Step 6:** In the - Extraneous noise sources – Object 1 - section of the window, enter the values for the various fields as follows: *Background level = 1000* and *Standard deviation = 8*.

**Remark 13**: In case that the localization accuracy is required for an EMCCD detector, the *Use EM* checkbox should be checked and the desired value for the electron multiplication gain should entered in the *EM gain* field. For more information see Remark 2.

**Step 7**: In the - Object selection - section of the **Required parameters window**, select "Object 2". Then repeat steps 4 and 6 for the Object 2.

**Step 8:** In the - Parameters to be estimated - section of the window, ensure that all check-boxes for all parameters are checked.

#### 9.3. Providing advanced parameters

**NOTE:** All inputs in the **Advanced Parameters window** are optional. The default values provided have been found to work adequately for typical imaging conditions.

**Step 1**: Access the advanced parameters window by clicking the Advanced Parameters option under the Parameters Menu.

Advanced Parameters	
2D Resolution Accuracy - Airy Pixelated with Poisson + Gaussian Noise Symmetric Parameterization	
Model profile calculations	
Pixel integration method : Trapezoidal	ditional parameters
Background type : Constant	ditional parameters FandPLimitTool
Fisher information matrix calculations         Single integration method :       Trapezoidal         Double integration method :       Trapezoidal         Modulation       Addition         Addition       Addition         Imits of summation [lower limit, upper limit] =       M/A	File     Export     View     Parameters     Tools     Help       attional parameters     Required Parameters     Ctrl+U       attional parameters     Advanced Parameters     Ctrl+N       2D Resolution Accuracy - Airy wooder. Pixelated whiri Poisson + Gaus     Perform calculations       use_default     Calculate Limits of Accuracy     Calculate M

Figure 9.3.1: 2D Resolution Accuracy advanced parameters window

**Step 2**: In the - Model profile calculations - section of window, access the Pixel integration method pull-down menu and select "Trapezoidal".

Advanced Parameters			
20.1	Resolution Accuracy - Airy		🛃 Trapezo 💼 💷 👞
Pixelated	I with Poisson + Gaussian Nois nmetric Parameterization	se	X Gridding
Model profile calculations—			V. Ovideling
Pixel integration method : Trape	zoidal 🔹	Additional parameters	13
Background type : Const	ant 💌	Additional parameters	OK Cancel

Figure 9.3.2: Pixel Integration Trapezoidal options

**Step 3:** Click the Additional parameters button next to the Pixel integration method pull-down menu. In the dialog box that appears, enter values for fields as follows: *XGridding = 13, YGridding = 13.* Click OK when done.

**Step 4**: Access the Background type pull-down menu and select "Constant". There are no additional parameters to be accessed via the Additional parameters button for the "Constant" option.

**Step 5**: In the - Fisher information matrix calculations - section of the window, access the Single integration method and select "Trapezoidal".

Fisher information matrix	x calculations		🛃 Trapezo 🗖 🗉 🗾
Single integration method :	Trapezoidal	Additional parameters	Step size
Double integration method :	Trapezoidal	Additional parameters	0.05
Limits of su Limits of int	Noise factor calculation parameters mmation [lower limit, upper limit] = N/A egration [lower limit, upper limit] = N/A	use_default ▼ use_default ▼	OK Cancel
I			

Figure 9.3.3: FIM Single Integration Trapezoidal options

**Step 6**: Click on the Additional parameters button next to the Single integration method pulldown menu. In the dialog box that appears, enter *Step size* = 0.05.

**Step 7**: Access the Double integration method pull-down menu in the - Fisher information matrix calculations - section of the window and select "Trapezoidal".

**Step 8**: Click on the Additional parameters button next to the Double integration method pulldown menu. In the dialog box that appears, enter the values *XGridding* = 13 and *YGridding* = 13.

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Fisher information matri	x calculations		
Single integration method :	Trapezoidal	<ul> <li>Additional parameters</li> </ul>	
Double integration method :	Trapezoidal	<ul> <li>Additional parameters</li> </ul>	X Gridding
	Noise factor calculation paramet	ers	Y Gridding
Limits of su	mmation [lower limit, upper limit] = [	V/A use_default ▼	13
Limits of in	egration [lower limit, upper limit] = [	use_default ▼	OK Cancel

Figure 9.3.4: FIM Double Integration Trapezoidal options

**Step 9**: The - Fisher information matrix calculations - section has a subsection titled - Noise factor calculation parameters - . Enter values *Limits of summation = [1, 3000]* and *Limits of integration = [-5000, 5000]* in this subsection.

**Remark 14**: The limits of integration and summation are initially set to default values which are automatically calculated based on the imaging conditions. It is possible specify these limits by changing the value of the combo box to the right of the limits of integration and summation fields.

#### 9.4. Executing the task and viewing results

**Step 1**: In the main application window, click the Calculate Limits of Accuracy button. This will present a dialog box warning that the calculation may take a long time to complete. Click Yes to proceed. The Calculate button text will change to Calculating ... and the application will temporarily become unresponsive while the calculations are being performed.



Figure 9.4.1: Calculation and Final confirmation

**NOTE**: During the calculation, a console window will be shown to inform the user about the different steps of the calculation. The calculation could indeed take half an hour to complete depending on the hardware capabilities of the platform on which the application is being executed.

**Step 2**: When the calculation is completed, the **Calculating** ... button text will change back to **Calculate Limits of Accuracy** and the **Results window** will be brought in focus. In addition, the console window will be hidden when the calculation is over. The console window can be accessed any time by selecting the **Console** option under the View Menu.

🔤 Results	🗢 🗖 💌
2D Res Pixelated wit Symme	olution Accuracy - Airy h Poisson + Gaussian Noise etric Parameterization
Results : Limit o	of the accuracy of
sx	= 5.9669 nanometers
sy	= 5.9669 nanometers
d	= 11.7311 nanometers
phi	i = 0.023825 radians

Figure 9.4.2: Results window (2D Resolution)

### 10. 3D-Resolution measure (Born-Wolf Model)

This section provides an example of how to use the FandPLimitTool to calculate the accuracy with which the 3D location of two objects can be resolved from their image (PREM). The image of each object is assumed to be 3D Point Spread Function.

The calculation takes into account the stochastic nature of the photon detection processes and assumes the photons detected from the object to be Poisson distributed. In addition, the calculation also takes into account extraneous noise sources - in particular the background component which is modeled as additive Poisson noise, the readout noise of the detector which is modeled as independent, additive Gaussian noise [2] and the electron multiplication noise of an EMCCD detector which is modeled as a branching process [3].

It is assumed that the pixels are rectangular in shape with no dead space between any two pixels. Further, the ROIs containing the images of the object are assumed to be rectangular arrays of pixels. The object is assumed to be in the center of this rectangular pixel array and the location of the object is specified with respect to the object space.

Refer to [6] for details on 3D resolution accuracy in single molecule microscopy.

#### **10.1. Summary of imaging conditions**

The following table summarizes the imaging conditions under which the calculations are performed.

Property	Object 1 Object 2		
Numerical aperture	1.3	1.3	
Wavelength	0.52 μm	0.52 μm	
Photon detection rate	10,000 photons/second	10,000 photons/second	
Object medium refractive index	1.515		
d	0.1 μm		
phi	π/4 radians		
omega	π/3 radians		
Exposure time	0.13 seconds		
Magnification	100 X		
Location of object in ROI (sx, sy)	0.585 μm, 0.585 μm		
Axial location of object (sz)	0 μm		
Pixel size	13 μm × 13 μm		
ROI Size	9 pixels × 9 pixels		
Background level (Poisson rate)	1000 photons/pixel/second	1000 photons/pixel/second	
Gaussian noise mean	0 e <sup>-</sup>		
Gaussian noise standard deviation	8 e <sup>-</sup> rms		
Use EM	unchecked		
EM gain	100		
EM model	Geometric		

Table 10.1.1: 3D Resolution Accuracy - Summary of imaging conditions

#### **10.2. Providing required parameters**

**Step 1**: Select the calculation option "3D Resolution Accuracy – Point Spread Function (Born-Wolf Model): Pixelated with Poisson + Gaussian Noise" from the Calculate pull-down menu in the main application window.

**Step 2**: The required parameters window, which is visible by default, will be updated accordingly. If this window is not visible, access it by clicking the Required Parameters option under the Parameters Menu in the main application window.

**Step 3**: In the - Object selection - section of the **Required parameters window**, select "Object 1".

🖥 Required Parameters 🛛 🖘 🗖 🖻 Σ	
3D Resolution Accuracy - Born-Wolf Model Pixelated with Poisson + Gaussian Noise Symmetric Parameterization	
- Object selection-	
Object 1 Object 2	
- Fundamental inputs - Object 1	
Numerical aperture = 1.3	
Wavelength = 0.52 microns	
Photon detection rate = 10000 photons/s	
Immersion medium ref. index** = 1.515	
d** = 0.5 microps	
nbitt = 0.7854 radiene	
omera# = 1.5708 radiana	
Evnosure timett = 0.13 seconds	
Exposure time = 0.13 seconds	
Additional inputs	1
Magnification** = 100	2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson Noise (Symmetric Parameterization)
Location parameters	2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson + Gaussian Noise (Symmetric Parameterization) 2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson Noise (Aymmetric Parameterization)
sx** = 0.325 microns Auto-center	2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson Holes (Aymmetric Parameterization)
sy** = 0.325 microns	2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson Noise (Cartesian Parameterization)
sz** = 0 microns	2D Resolution Accuracy - Gaussian Symmetric Model: Pixelated with Poisson + Gaussian Noise (Cartesian Parameterization)
Pixelation parameters	2D Resolution Accuracy - Airy Model: Fundamental Limit (Symmetric Parameterization)
Pixel size [height, width]** = [13, 13] microns X microns	2D Resolution Accuracy - Airy Model: Pixelated with Poisson Noise (Symmetric Parameterization)
ROI size [height, width]** = [5, 5] no. of pixels X no. of pixels	2D Resolution Accuracy - Airy Model: Hixelated with Poisson + Gaussian Noise (symmetric Parameterization) 2D Resolution Accuracy - Airy Model: Fundamental Limit (Asymmetric Parameterization)
	2D Resolution Accuracy - Airy Model: Pixelated with Poisson Noise (Asymmetric Parameterization)
Extraneous noise source(s) - Object 1	2D Resolution Accuracy - Airy Model: Pixelated with Poisson + Gaussian Noise (Aymmetric Parameterization)
Background level (Poisson rate) = 1000 photons/pixel/s	2D Resolution Accuracy - Airy Model: Fundamental Limit (Cartesian Parameterization)
Readout (Gaussian) noise parameters	2D Resolution Accuracy - Airy Model: Pixelated with Poisson Noise (Cartesian Parameterization)
Standard deviation** = 8 e-/pixel	2D Resolution Accuracy - Airy Model: Pixelated with Poisson + Gaussian Noise (Cartesian Parameterization)
Electron multiplication parameters	3D Resolution Accuracy - Born-Wolf Model: Pixelated with Phisson Noise (Symmetric Parameterization)
	3D Resolution Accuracy - Born-Wolf Model: Pixelated with Poisson + Gaussian Noise (Symmetric Parameterization)
	3D Localization Accuracy - Multi-focal: Pixelated with Possion Noise
	3D Localization Accuracy - Multi-focal: Pixelated with Possion + Gaussian Noise
Parameters to be estimated	3D Resolution Accuracy - Born-Wolf Model: Pixelated with Poisson + Gaussian Noise (Symmetric Parameterization)
▼ sx ▼ sy ▼ sz ▼ d ▼ phi ▼ omega ■ Photon detection rate 1	Perform calculations
Background level	Calculate Limits of Accuracy Calculate Model Image Simulate Data Images
** Values apply to both objects. Vary a Parameter	]

Figure 10.2.1: 3D Resolution Accuracy calculate type and its required parameters window

**Step 4**: In the - Fundamental inputs – Object 1 - section of the window, enter the values for the various fields as follows: *Numerical aperture* = 1.3, *Wavelength* = 0.52, *Photon detection rate* = 10000, *Object medium refractive index* = 1.515, d = 0.1, *phi* = *pi/4*, *omega* = *pi/3* and *Exposure time* = 0.13.

**Step 5**: In the - Additional inputs - section of the window, enter the values for the various fields as follows: *Magnification = 100, sx = 13×4.5/100, sy = 13×4.5/100, sz = 0, Pixel size = [13, 13],* and *ROI size = [9, 9].* 

**Remark 15**: The center of a 9 *pixels* × 9 *pixels* pixel array where the dimensions of each pixel is  $13 \ \mu m \times 13 \ \mu m$ , is  $(4.5 \times 13) \ \mu m \times (4.5 \times 13) \ \mu m$ . To convert this location coordinates to the object space, the coordinate values are divided by the magnification. Hence the location coordinates are specified as  $13 \times 4.5/100 \ \mu m \times 13 \times 4.5/100 \ \mu m$ .

**Step 6:** In the - Extraneous noise sources – Object 1 - section of the window, enter the values for the various fields as follows: *Background level = 1000* and *Standard deviation = 8*.

**Step 7**: In the - Plane selection - section of the **Required parameters window**, select "Object 2". Then repeat steps 4 and 6 for the Object 2.

#### 10.3. Providing advanced parameters

**NOTE:** All inputs in the **Advanced Parameters window** are optional. The default values provided have been found to work adequately for typical imaging conditions.

3D Resolution Accuracy - Born-Wolf Model Pixelarded with Poisson + Gaussian Noise Symmetric Parameterization         Object selection       Object 1         Advanced fundamental inputs (Object 1)         Advanced fundamental inputs (Object 1)         Alpha* = 15.708       Update analytically         Model profile calculations         Pixel integration method:       Trapezoidal         Additional parameters         Background type :       Constant         Additional parameters         Double integration method:       Trapezoidal         Additional parameters         Double integration method:       Trapezoidal         Limits of summetion (lower limit, upper limit) =       Additional parameters         Limits of summetion (lower limit, upper limit) =       N/A         Limits of integration (lower limit, upper limit) =       N/A         Limits of integration (lower limit, upper limit) =       N/A         Limits of integration (lower limit, upper limit) =       N/A         VAlpha = 2 x pi x numerical aperture / wavelengty;       pi = 3.1416 (approx.)	Advanced Parameters	
Object selection         Object 1       Object 2         Advanced fundamental inputs (Object 1)         Alpha* = 15.708       Update analytically         Model profile calculations         Pixel integration method :       Trapezoidal         Additional parameters         Background type :       Constant         Additional parameters         Background type :       Constant         Fisher information method :       Trapezoidal         Trapezoidal       Additional parameters         Double integration method :       Trapezoidal         Limits of summation [lower limit, upper limit] =       MAA         Limits of integration flower limit, upper limit] =       MAA         Limits of integration flower limit, upper limit] =       MAA         Limits of integration step size =       0.05         *Alpha = 2 x pi x numerical aperture / wavelength;       pi = 3.1416 (approx.)	3D Resolution Accuracy - Born-Wolf Model Pixelated with Poisson + Gaussian Noise Symmetric Parameterization	
Advanced fundamental inputs (Object 1) Alpha* = 15.708 Update analytically Model profile calculations Pixel integration method : Trapezoidal Additional parameters Background type : Constant Additional parameters Background type : Constant Additional parameters Fisher information matrix calculations Single integration method : Trapezoidal Additional parameters Double integration method : Trapezoidal Additional parameters Double integration method : Trapezoidal Additional parameters Limits of summation [lower limit, upper limit] = N/A Limits of integration [lower limit, upper limit] = N/A Limits of integration flower limit, upper limit] = N/A Limits of integration step size = 0.05 *Alpha = 2 x pi x numerical aperture / wavelength; pi = 3.1416 (approx.)	Object selection Object 1  Object 2	
Model profile calculations         Pixel integration method :         Trapezoidal         Additional parameters         Background type :         Constant         Additional parameters         Background type :         Constant         Additional parameters         Background type :         Constant         Additional parameters         Single integration method :         Trapezoidal         Additional parameters         Double integration method :         Trapezoidal         Additional parameters         Limits of summation [lower limit] =         N/A         Use default         Limits of integration step size =         0.05         *Alpha = 2 x pi x numerical aperture / wavelength;         pi = 3.1416 (approx.)	Advanced fundamental inputs (Object 1) Alpha* = 15.708 Update analytically	
Single integration method :       Trapezoidal <ul> <li>Additional parameters</li> <li>Double integration method :</li> <li>Trapezoidal</li> <li>Additional parameters</li> <li>Additional parameters</li></ul>	Model profile calculations Pixel integration method : Trapezoidal  Additional parameters Model integration method : Trapezoidal Additional parameters Background type : Constant Additional parameters Fisher information matrix calculations	
Double integration method :       Trapezoidal <ul> <li>Additional parameters</li> <li>Noise factor calculation parameters</li> <li>Limits of summation [lower limit, upper limit] =</li> <li>N/A</li> <li>use_default </li> <li>Trapezoidal integration (lower limit, upper limit) =</li> <li>N/A</li> <li>use_default </li> <li>Trapezoidal integration step size =</li> <li>0.05</li> </ul> Select a problem s     Required Parameters Ctrl +           Besolution Accuracy - Born - voon wooder, Pixerated with Poisson         Perform calculations           *Alpha = 2 × pi × numerical aperture / wavelength;         pi = 3.1416 (approx.)         Calculate Limits of Accuracy         Calculate	Single integration method : Trapezoidal   Additional parameters	File Export View Parameters Tools Help
***Values apply to both objects	Double integration method :       Trapezoidal <ul> <li>Additional parameters</li> <li></li></ul>	Perform calculations     Calculate Limits of Accuracy     Calculate Mathematics

#### Figure 10.3.1: 3D Resolution Accuracy advanced parameters window

**Step 1**: Access the advanced parameters window by clicking the Advanced Parameters option under the Parameters Menu.

**Step 2**: Skip the - Object selection - and - Advanced fundamental inputs (Object 1) - sections of the window. Their fields do not need to be changed as they are automatically calculated from the values entered in the Required Inputs window.

**Step 3**: In the - Model profile calculations - section of window, access the Pixel integration method pull-down menu and select "Trapezoidal".

- Advanced fundamental in	nputs (Object 1)———		
	Alpha* = 15.708	Update analytically	
			X Gridding
- Model profile calculation	S		13
Pixel integration method :	Trapezoidal	Additional parameters	Y Gridding
Model integration method :	Trapezoidal	Additional parameters	13
Background type :	Constant	Additional parameters	OK Cancel

Figure 10.3.2: Pixel Integration Trapezoidal options

**Step 4:** Click the Additional parameters button next to the Pixel integration method pull-down menu. In the dialog box that appears, enter values for fields as follows: *XGridding = 13, YGridding = 13.* Click OK when done.

**Step 5**: Access the Model integration method pull-down menu in the same section of the window and select "Trapezoidal".

- Object selection	Object 1 ()	Object 2	🚺 Trapezo 💼 🔳 💽
Advanced fundamental ir	nputs (Object 1) Alpha* = 15.708	Update analytically	Step Size
Model profile calculation:	s		Lower Limit
Pixel integration method :	Trapezoidal	<ul> <li>Additional parameters</li> </ul>	Upper Limit
Model integration method :	Trapezoidal	Additional parameters	1
Background type :	Constant	Additional parameters	OK Cancel

Figure 10.3.3: Model Integration Trapezoidal options

**Step 6**: Click the Additional parameters button next to the Model integration method pull-down menu. In the dialog box that appears, enter the values *Step size = 0.0005, Lower limit = 0*, and *Upper limit = 1*. Click OK when done.

**Step 7**: Access the Background type pull-down menu and select "Constant". There are no additional parameters to be accessed via the Additional parameters button for the "Constant" option.

**Step 8**: In the - Fisher information matrix calculations - section of the window, access the Single integration method pull-down menu and select "Trapezoidal".

Fisher information matrix calculations		
Single integration method : Trapezoidal	arameters 🛛 🚺 Trapezo 👝 🗉 💌	
Double integration method : Trapezoidal	arameters Step size	
Noise factor calculation parameters	0.0005	
Limits of summation [lower limit, upper limit] = N/A use	default  Lower limit	
Limits of integration [lower limit, upper limit] = N/A use	_default 👻	
Trapezoidal integration step size = 0.05	Upper limit	
*Alpha = 2 x pi x numerical aperture / wavelength; pi = 3.1416 (approx.)		
***Values apply to both objects.		

Figure 10.3.4: FIM Single Integration Trapezoidal options

**Step 9**: Click the Additional parameters button next to the Single integration method pull- down menu. In the dialog box that appears, enter the values *Step size = 0.0005, Lower limit = 0,* and *Upper limit = 1*. Click OK when done.

**Step 10**: Access the Double integration method pull-down menu in the same section of the window and select "Trapezoidal".

Fisher information matr	ix calculations———			
Single integration method :	Trapezoidal	▼ Addit	ional parameters	M Trapezo
Double integration method :	Trapezoidal	- Addit	ional parameters	X Gridding
	Noise factor calculation param	neters		
Limits of s	ummation [lower limit, upper limit] =	= N/A	use_default 💌	Y Gridding
Limits of in	ntegration [lower limit, upper limit] =	= N/A	use_default 💌	
	Trapezoidal integration step size =	0.05	]	OK Cancel
*Alpha = 2 × pi :	x numerical aperture / wavelength	n; pi = 3.1416 (	approx.)	
***∀alues apply to both objects				

Figure 10.3.5: FIM Double Integration Trapezoidal options

**Step 11**: Click on the Additional parameters button next to the Double integration method pulldown menu. In the dialog box that appears, enter the values *XGridding* = 13 and *YGridding* = 13.

**Step 12**: The - Fisher information matrix calculations - section has a subsection titled - Noise factor calculation parameters - . Enter the following values in this subsection: *Limits of summation = [1, 3000], Limits of integration = [-5000, 5000]* and *Trapezoidal integration step size = 0.05*.

**Remark 16**: The limits of integration and summation are initially set to default values which are automatically calculated based on the imaging conditions. It is possible specify these limits by changing the value of the combo box to the right of the limits of integration and summation fields.

#### 10.4. Executing the task and viewing results

**Step 1**: In the main application window, click the Calculate Limits of Accuracy button. This will present a dialog box warning that the calculation may take a long time to complete. Click Yes to proceed. The Calculate button text will change to Calculating ... and the application will temporarily become unresponsive while the calculations are being performed.

**NOTE**: The calculation could indeed take half an hour to complete depending on the hardware capabilities of the platform on which the application is being executed.

🔄 FandPLimitTool 🚺 Warr	ing 🗖 🗖 🔤 🔜
File Export View Paran Select a problem scenari	WARNING: This calculation may take a long time to complete. Are you sure you want to continue?
3D Resolution Accuracy - Bor	Yes No
Calculating	Calculate Model Image Simulate Data Images

Figure 10.4.1: Calculation and Final confirmation (3D Resolution)

**Step 2**: When the calculation is completed, the Calculating ... button text will change back to Calculate Limits of Accuracy and the **Results window** will be brought in focus.

E Results					
3D Resolution Accuracy - Born-Wolf Model Pixelated with Poisson + Gaussian Noise Symmetric Parameterization					
Results : Limit of the a	accuracy	of			
sx =	4.3882	nanometers			
sy =	4.3882	nanometers			
sz =	33121041	nanometers			
d =	41.7225	nanometers			
phi =	0.017509	radians			
omega =	12984231	radians			
Photon detection rate 1 =	NaN	photons/s			
Photon detection rate 2 =	NaN	photons/s			
Background level =	NaN	photons/pixel/s			
1					

Figure 10.4.2: Results window (3D Resolution)

#### **11. 3D-Localization accuracy measure - Multifocal Plane**

This section provides an example of how to use the FandPLimitTool to calculate the accuracy with which the 3D location of an object can be estimated from the images acquired by a 2-plane MUM setup (PLAM). The images of the object are assumed to be 3D Point Spread Function profiles.

The calculation takes into account the stochastic nature of the photon detection processes and assumes the photons detected from the object to be Poisson distributed. In addition, the calculation also takes into account extraneous noise sources - in particular the background component which is modeled as additive Poisson noise, the readout noise of the detector which is modeled as independent, additive Gaussian noise and the electron multiplication noise which in modeled as a branching process.

It is assumed that the pixels are rectangular in shape with no dead space between any two pixels. Further, the ROIs containing the images of the object are assumed to be rectangular arrays of pixels. The object is assumed to be in the center of this rectangular pixel array and the location of the object is specified with respect to the object space. Moreover, it is assumed that the photon detection process at each detector is independent of that of other detectors. Refer to [1] and [7] for details on determining the 3D localization accuracy in multifocal plane microscopy (MUM).

#### 11.1. Summary of imaging conditions

The following table summarizes the imaging conditions under which the calculations are performed.

Property	Plane 1	Plane 2		
Z <sub>0</sub>	0.5 μm			
Alpha	15.708			
Object medium refractive index	1.515			
Numerical aperture	1.3			
Wavelength	0.52 μm			
Photon detection rate	10,000 photons/second			
Exposure time	0.13 seconds			
Magnification	100 X			
Photon detection rate ratio	[0.5, 0.5]			
Focal plane distance	[0, 0.5] μm			
Location of object in ROI	0.585 μm, 0.585 μm			
Pixel size	13 μm × 13 μm			
ROI Size	9 pixels × 9 pixels			
Background level (Poisson rate)	25 photons/pixel/second 25 photons/pixel/second			
Gaussian noise standard deviation	8 e <sup>°</sup> rms			
Use EM	unchecked			
EM gain	100			
EM model	Geometric			

Table 11.1.1: 3D Localization Accuracy (MUM) - Summary of imaging conditions

#### **11.2. Providing required parameters**

**Step 1**: Select the calculation option "3D Localization Accuracy - Multifocal: Pixelated with Poisson + Gaussian Noise" from the Calculate pull-down menu in the main application window.

**Step 2**: The required parameters window, which is visible by default, will be updated accordingly. If this window is not visible, access it by clicking the Required Parameters option under the Parameters Menu in the main application window.

Step 3: In the - Plane selection - section of the Required parameters window, select "Plane 1".

**Step 4**: In the - Fundamental inputs – Plane 1 - section of the window, enter the values for the various fields as follows:  $z_0 = 0.5$ , Alpha = 15.708, Object medium refractive index = 1.515, Numerical aperture = 1.3, Wavelength = 0.52, Photon detection rate = 10000, and Exposure time = 0.13.



Figure 11.2.1: 3D Localization Accuracy (MUM) calculate type and its required parameters window

**Step 5**: In the - Additional inputs - section of the window, enter the values for the various fields as follows: *Magnification* = 100, *Photon detection rate ratio* = [0.5, 0.5], *Focal plane distance* = [0, 0.5],  $x_0 = 13 \times 4.5/100$ ,  $y_0 = 13 \times 4.5/100$ , *Pixel size* = [13, 13], and *ROI size* = [9, 9].

**Remark 17**: The center of a 9 *pixels* × 9 *pixels* pixel array where the dimensions of each pixel is  $13 \mu m \times 13 \mu m$ , is  $(4.5 \times 13)\mu m \times (4.5 \times 13)\mu m$ . To convert this location coordinates to the object space, the coordinate values are divided by the magnification. Hence the location coordinates are specified as  $13 \times 4.5/100 \mu m \times 13 \times 4.5/100 \mu m$ .

**Step 6:** In the - Extraneous noise sources – Plane 1 - section of the window, enter the values for the various fields as follows: *Background level = 25* and *Standard deviation = 8*.

**Step 7**: In the - Plane selection - section of the **Required parameters window**, select "Plane 2". Then repeat step 6 for Plane 2.

**Remark 18**: In case that the localization accuracy is required for an EMCCD detector, the *Use EM* checkbox should be checked and the desired value for the electron multiplication gain should entered in the *EM gain* field. For more information see Remark 2.

**Step 8:** In the - Parameters to be estimated - section of the window, ensure that all check-boxes for all parameters are checked.

#### 11.3. Providing advanced parameters

**NOTE:** All inputs in the **Advanced Parameters window** are optional. The default values provided have been found to work adequately for typical imaging conditions.

**Step 1**: Access the advanced parameters window by clicking the Advanced Parameters option under the Parameters Menu.

🔄 Advanced Parameters							
3i Pix — Advanced fundamental in	D Localization Accuracy - Multi-focal elated with Poisson + Gaussian Nois nputs Alpha* = 15.708	se					
- Model profile calculation	s						
Pixel integration method :	Trapezoidal 💌	Additional parameters					
Model integration method :	Trapezoidal	Additional parameters					
Background type :	Constant 💌	Additional parameters					
Fisher information matrix	calculations	Additional parameters	File	andPLimitTool Export View	Parameters	Tools Help	
Double integration method :  Limits of su Limits of int T	Trapezoidal Trapezoidal Noise factor calculation parameters mmation [lower limit, upper limit] = N/A egration [lower limit, upper limit] = N/A rapezoidal integration step size = 0.05	Additional parameters	Se 3D	elect a problem s Docalization Accu Perform calcula Calculate Li	Requir Advan acy - mani-roc ations	ed Parameters ced Parameters al: Pixeialeu will Po al: accy C:	Ctrl+U Ctrl+N ssion + Gauss alculate Mo
*Alpha = 2 × pi ×	numerical aperture / wavelength; pi = :	3.1416 (approx.)					

Figure 11.3.1: 3D Localization Accuracy (MUM) advanced parameters window

**Step 2**: The Advanced parameters window contains an - Advanced fundamental inputs - section. The default value of the field in this section can be left unchanged.

**Step 3**: In the - Model profile calculations - section of window, access the Pixel integration method pull-down menu and select "Trapezoidal".

Advanced fundamental in	nputs		<b>Tranezo D</b>
	Alpha* = 15.708	Update analytically	X Gridding
- Model profile calculation	s		3
Pixel integration method :	Trapezoidal	Additional parameters	Y Gridding
Model integration method :	Trapezoidal	Additional parameters	
Background type :	User Defined	Additional parameters	OK Cancel

Figure 11.3.2: Pixel Integration Trapezoidal options

**Step 4:** Click the Additional parameters button next to the Pixel integration method pull-down menu. In the dialog box that appears, enter values for fields as follows: *XGridding = 13, YGridding = 13.* Click OK when done.

**Step 5**: Access the Model integration method pull-down menu in the same section of the window and select "Trapezoidal".

3 Pix	🛃 Trapezo 💼 💷 💌					
– Advanced fundamental i	Step Size					
	Alpha* = 15.708 Update analytically					
— Model profile calculation	s		0			
Pixel integration method :	Trapezoidal	Additional parameters	Upper Limit			
Model integration method :	Trapezoidal	<ul> <li>Additional parameters</li> </ul>				
Background type :	User Defined	Additional parameters	OK Cancel			

Figure 11.3.3: Model Integration Trapezoidal options

**Step 6**: Click the Additional parameters button next to the Model integration method pull-down menu. In the dialog box that appears, enter the values *Step size = 0.0005, Lower limit = 0*, and *Upper limit = 1*. Click OK when done.

**Step 7**: Access the Background type pull-down menu and select "Constant". There are no additional parameters to be accessed via the Additional parameters button for the "Constant" option.

**Step 8**: In the - Fisher information matrix calculations - section of the window, access the Single integration method pull-down menu and select "Trapezoidal".

[	Fisher information matrix calculations						
	Single integration method :	Trapezoidal	•	Additi	onal parameter	s	
	Double integration method :	Trapezoidal	•	Additi	onal parameter	s	Step size 0.0005
		Noise factor calculation par	ameters			_	Lower limit
	Limits of summation [lower limit, upper limit] = N/A use_default 💌					•	
	Limits of integration [lower limit, upper limit] = N/A use_default						
	Trapezoidal integration step size = 0.05						1
'	*Alpha = 2 × pi ×	numerical aperture / wavelen	gth; pi=	3.1416 (a	approx.)		OK Cancel

Figure 11.3.4: FIM Single Integration Trapezoidal options

**Step 9**: Click the Additional parameters button next to the Single integration method pull- down menu. In the dialog box that appears, enter the values *Step size = 0.0005, Lower limit = 0,* and *Upper limit = 1*. Click OK when done.

**Step 10**: Access the Double integration method pull-down menu in the same section of the window and select "Trapezoidal".

[	— Fisher information matri	x calculations			
	Single integration method :	Trapezoidal	•	Additional parameters	
	Double integration method :	Trapezoidal	•	Additional parameters	🚺 Trapezo 🗖 🗉 💌
		Noise factor calculation paramete	ers		X Gridding
	Limits of su	ummation [lower limit, upper limit] = [\	/A	use_default 💌	13
	Limits of in	tegration [lower limit, upper limit] = 🕅	/A	use_default 💌	Y Gridding
	1	Frapezoidal integration step size = 0	.05		13
1	*Alpha = 2 × pi ×	numerical aperture / wavelength;	pi =	- 3.1416 (approx.)	OK Cancel

Figure 11.3.5: FIM Double Integration Trapezoidal options

**Step 11**: Click on the Additional parameters button next to the Double integration method pulldown menu. In the dialog box that appears, enter the values *XGridding* = 13 and *YGridding* = 13.

**Step 12**: The - Fisher information matrix calculations - section has a subsection titled - Noise factor calculation parameters - . Enter the following values in this subsection: *Limits of summation = [1, 3000], Limits of integration = [-5000, 5000]* and *Trapezoidal integration step size = 0.05*.

**Remark 20**: The limits of integration and summation are initially set to default values which are automatically calculated based on the imaging conditions. It is possible specify these limits by changing the value of the combo box to the right of the limits of integration and summation fields.

#### 11.4. Executing the task and viewing results

**Step 1**: In the main application window, click the Calculate Limits of Accuracy button. This will present a dialog box warning that the calculation may take a long time to complete. Click Yes to proceed. The Calculate button text will change to Calculating ... and the application will temporarily become unresponsive while the calculations are being performed. During the calculation, a console window will be shown to inform the user about the different steps of the calculation.

**NOTE**: The calculation could indeed take half an hour to complete depending on the hardware capabilities of the platform on which the application is being executed.



Figure 11.4.1: Calculation and Final confirmation (3D localization MUM)

**Step 2**: When the calculation is completed, the Calculating ... button text will change back to Calculate Limits of Accuracy and the **Results window** will be brought in focus. In addition, the console window will be hidden when the calculation is over.

E Results	
3D Localiza Pixelated wi	ation Accuracy - Multi-focal th Poisson + Gaussian Noise
Results : Limit (	of the accuracy of
	x0 = 2.3687 nanometers
	y0 = 2.3687 nanometers
	z0 = 12.7615 nanometers
Photon detection	rate = 0.29457 photons/s
Background	level = 57.7687 photons/pixel/s
1	

Figure 11.4.2: Results window (3D localization MUM)

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