Co-registration Definition Review

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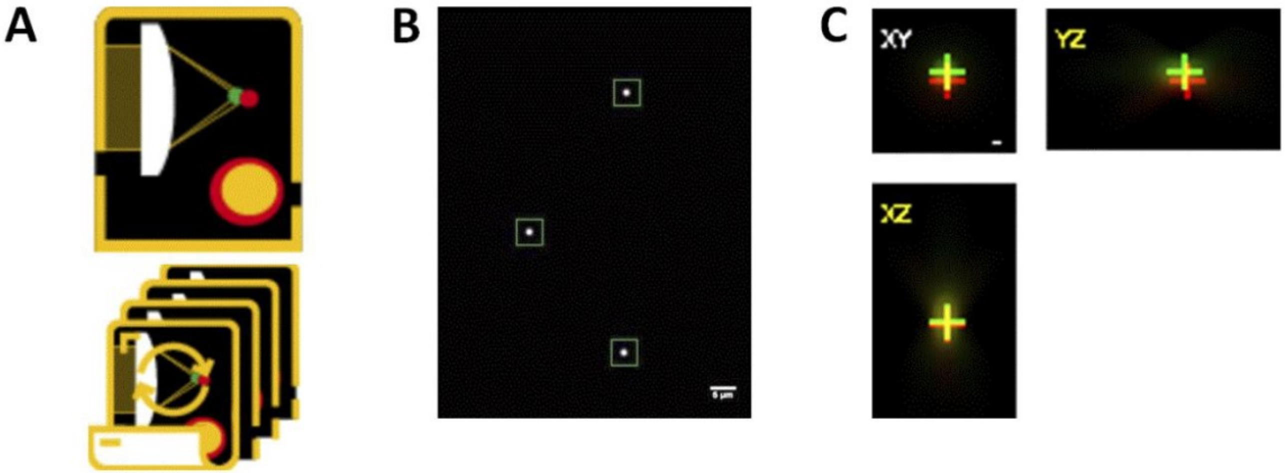
QUAREP Definition

Our metric here was the r_{exp}/r_{ref} ratio. In more detail, the acquisition consisted of 3D stacks with two or more emission channels. MetroloJ_QC plugin uses automation of the MetroloJ coregistration algorithm. Beads were identified and saturated beads or beads too close to the image border or another bead were discarded. The bead center of mass was subsequently calculated for each channel and the distances between the centers were estimated as r_{exp}. For each channel to channel distance r_{exp}, a reference distance was calculated as r_{ref}, as previously described (Cordelières and Bolte, 2008; Fig. S8 D). When $r > r_{ref}$, two points A and B are considered not colocalized. The plugin calculated the ratio r_{exp}/r_{ref} for each color couple. A ratio higher than 1 indicates, in our case, a not accepted colocalization. The shortest wavelength of the color pair was used to calculate the theoretical resolution, as it is the most stringent resolution value. When analyzing the usual four-channel images, six combinations need to be considered.

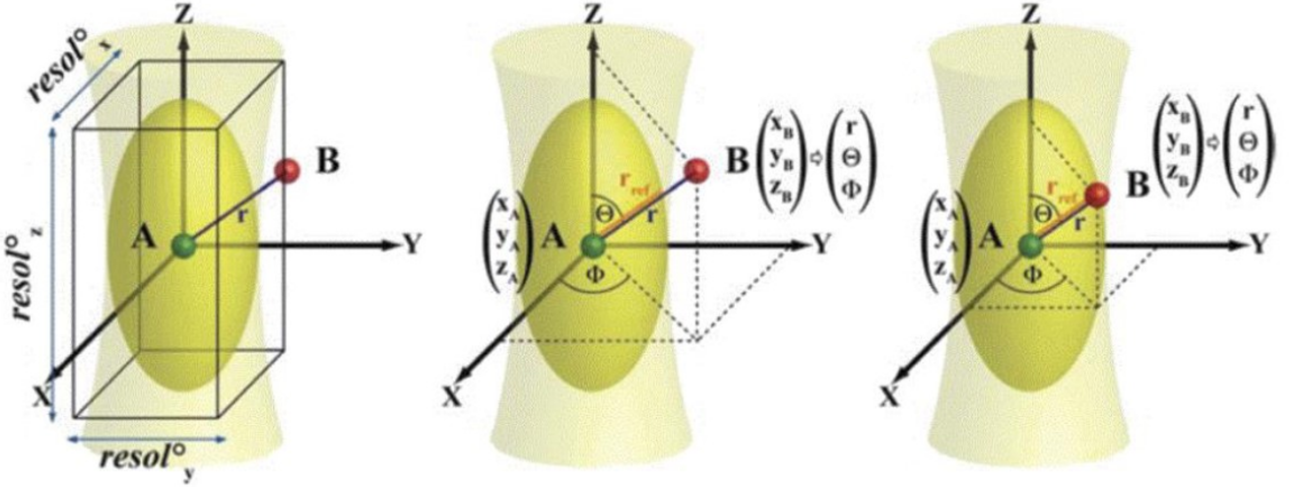
MetroloJ_QC automatically generated analyses for all possible channel combinations, measured the pixel shifts, the (calibrated/uncalibrated) intercenter distances, and compared them to their respective reference distance r_{ref}. A ratio of the measured intercenter distance to the reference distance was also calculated. Images with more than one bead can be analyzed, and a batch mode enabled the analysis of multiple datasets. Fig. S8 and the plugin manual provide a more detailed description of our coregistration workflow.



Co-registration workflow with the MetroloJ_QC. (A) Co-registration single and batch icon. **(B)** A "bead overlay" image is generated when more than one bead is in the FOV, taking into account the declared user parameters (bead size, ROI size, bead position on *z*-stack). **(C)** For each color/channel combination profile view images are composed of three maximum intensity projections, *xy*, *xz*, *yz* (side views) are generated. Crosses indicate the respective position of the green channel (first channel declared using the stack order) and the red channel. This is done for all channel combinations.

Faklaris, *et al.* Journal of Cell Biology, 2022

QUAREP Definition



Calculation of the reference distance r_{ref} Left: Centers of objects (A and B) are drawn as red and green spheres, respectively. The PSF is schematized in light yellow, while the first Airy volume appears in dark yellow. The former width, height, and depth define the resolution along the three axes. Middle: A and B are not colocalized as $r > r_{ref}$ Right: A and B are colocalized as $r \leq r_{ref}$ Illustration from Cordelières and Bolte, ImageJ User and Developer Conference Proceedings, 2008, Luxembourg.

QUAREP Definition

E

	Channel 0	Channel 1	Channel 2	Channel 3
Channel 0		0.603	0.751	1.195
Channel 1	0.603		0.65	1.036
Channel 2	0.751	0.65		0.539
Channel 3	1.195	1.036	0.539	
Resolutions (µm)	0.168 0.168 0.629	0.191 0.191 0.718	0.222 0.222 0.835	0.24 0.24 0.903
Bead centres'coord.(µm)	24.5 24.0 16.0	23.5 23.5 16.0	24.5 21.5 15.5	24.5 20.5 15.5

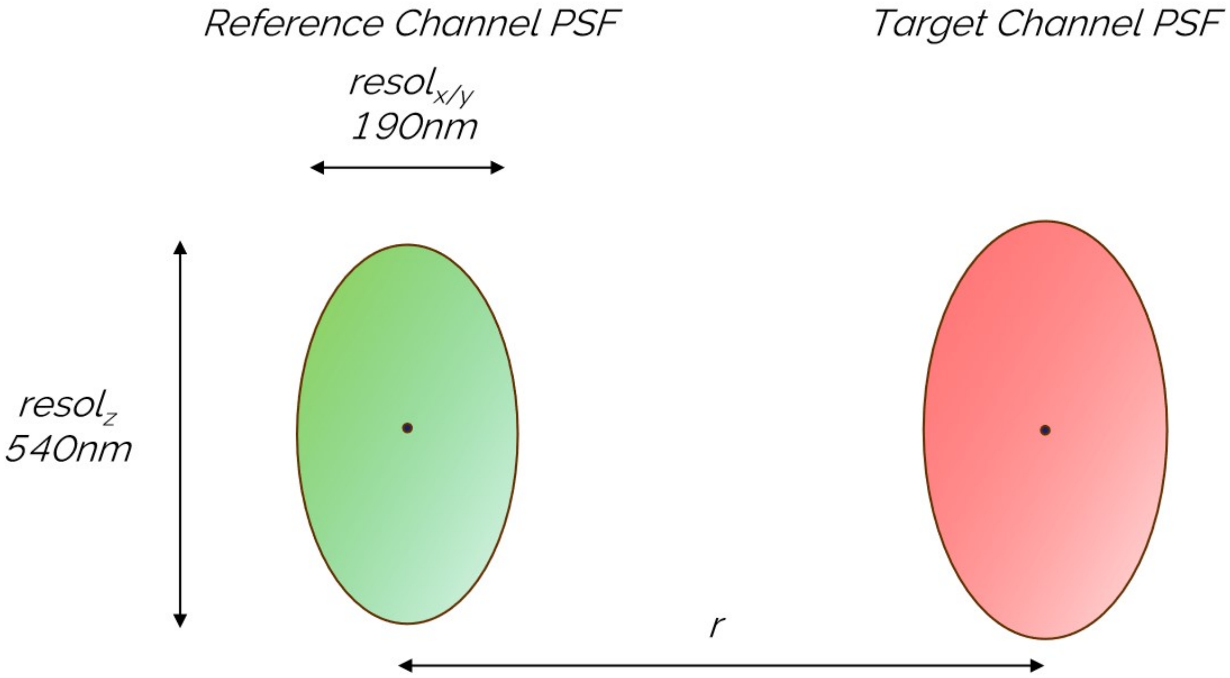
F

	Channel 0	Channel 1	Channel 2	Channel 3
Channel 0		0.39 +/- 0.149 (n=7.0)	1.02 +/- 0.3 (n=7.0, 42.0% failed)	1.073 +/- 0.277 (n=7.0, 42.0% failed)
Channel 1	0.39 +/- 0.149 (n=7.0)		0.592 +/- 0.03 (n=5.0)	0.783 +/- 0.17 (n=7.0, 14.0% failed)
Channel 2	1.02 +/- 0.3 (n=7.0, 42.0% failed)	0.592 +/- 0.03 (n=5.0)		0.39 +/- 0.137 (n=7.0)
Channel 3	1.073 +/- 0.277 (n=7.0, 42.0% failed)	0.783 +/- 0.17 (n=7.0, 14.0% failed)	0.39 +/- 0.137 (n=7.0)	

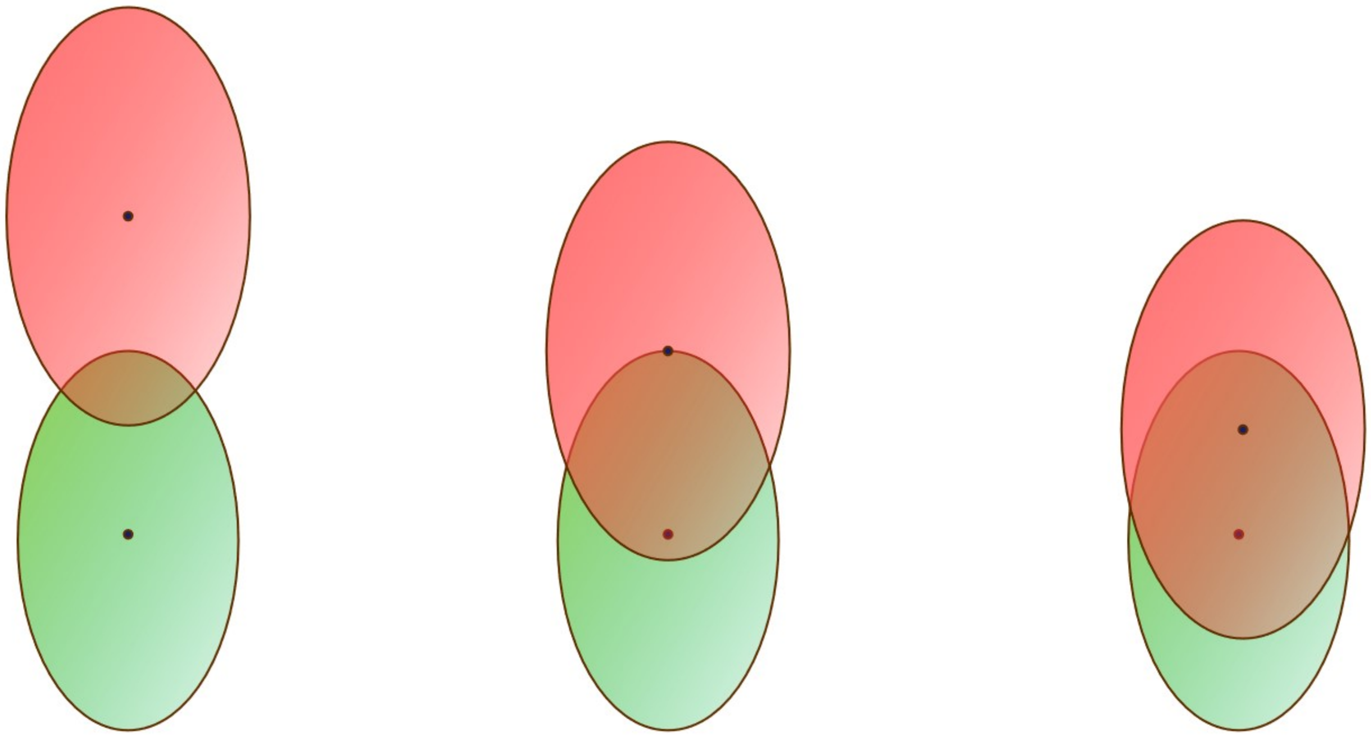
(E) A ratio table is generated for each bead indicating the measured co-registration ratios of all channel combinations, the theoretical resolution for each channel, and the bead position coordinates. **(F)** If ratio values are within specs they are highlighted in green; if not, they are highlighted in red, when more than one bead is in the FOV, a ratio table gives the mean ratio values with their SD values. The number of beads taken into account for each channel is also given.

QUAREP Definition – Summarized

- Two channels are co-localised if the centre of target channel lies within an ellipsoid defined by the PSF of the reference channel (defined as the channel with shorter emission wavelength).



Special cases: no XY mis-registration

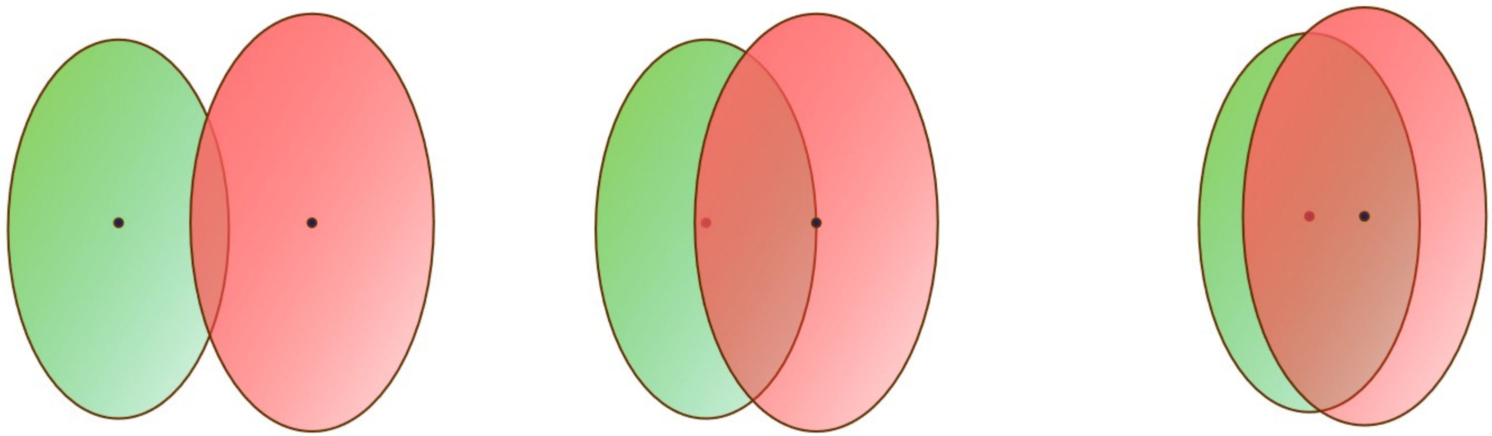


Not co-registered
$r > 1$

Co-registered
$r = 1$

Co-registered
$r < 1$

Special cases: no Z mis-registration



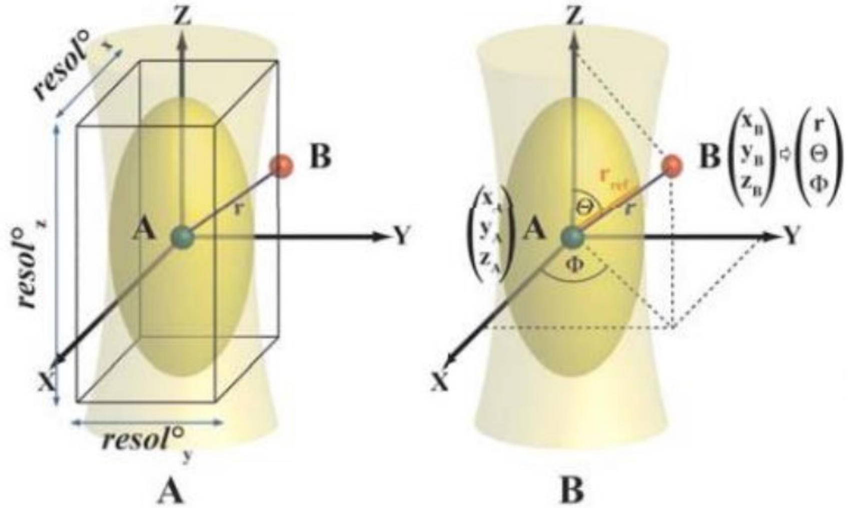
Not co-registered $r > 1$

Co-registered $r = 1$

Co-registered $r < 1$

Origin of the reference surface definition

…the reference distance is quite easy to determine in 2D as it corresponds to the *xy* resolution: while considering the center of the structure on image A, a structure of image B will be co-localized if it is present within a circle traced around center A of a radius equal to the *xy* resolution. Due to the disparate resolutions over the three dimensions, this distance is not so easy to calculate in 3D. However, the answer might come from the observation of the factor limiting the resolution: the PSF (Point Spread Function) and more precisely the first Airy disc which might be approximated in 3D as having an ovoid shape. Therefore in 3D, the reference distance is calculated by considering a reference point and fitting a 3D ellipse around it for which the two characteristic radii correspond to *x*/*y* and *z* resolutions. In this matter changing from Cartesian coordinates to **Polar coordinates** make it more easy to calculate the reference distance. In JACoP the two characteristic angles, the azimuth Φ and the zenith Θ (see expression 6) are first calculated, based on the coordinates of the two centers to analyze. Knowing this orientation, as well as the *x*, *y* and *z* resolutions ($resol_x$, $resol_y$ and $resol_z$ respectively), the distance from the reference center to the border of the ovoid shape r_{ref} is calculated (see expression 7). The inter-center distance *r* is then compared to this reference distance to assess if co-localization occurs or not.



$$\Phi = \arccos \frac{x_B - x_A}{\sqrt{(x_B - x_A)^2 + (y_B - y_A)^2}} \quad \text{and} \quad \Theta = \arccos \frac{z_B - z_A}{\sqrt{(x_B - x_A)^2 + (y_B - y_A)^2 + (z_B - z_A)^2}} \tag{6}$$

$$r_{ref} = \sqrt{(resol_x^\circ \times \sin\Theta \times \cos\Phi)^2 + (resol_y^\circ \times \sin\Theta \times \sin\Phi)^2 + (resol_z^\circ \times \cos\Theta)^2} \tag{7}$$

Cordelières and Bolte, ImageJ User and Developer Conference Proceedings, 2008, Luxembourg.

Origin of the reference surface definition

$$r_{ref} = \sqrt{(resol_x^\circ \times \sin\Theta \times \cos\Phi)^2 + (resol_y^\circ \times \sin\Theta \times \sin\Phi)^2 + (resol_z^\circ \times \cos\Theta)^2} \quad (1)$$

$$\Phi = \arccos \frac{x_B - x_A}{\sqrt{(x_B - x_A)^2 + (y_B - y_A)^2}} \quad (2)$$

$$\Theta = \arccos \frac{z_B - z_A}{\sqrt{(x_B - x_A)^2 + (y_B - y_A)^2 + (z_B - z_A)^2}} \quad (3)$$

$$\text{Lateral resolution : } res_{x,y}^{o} = \frac{0.51 \times \lambda_{em}}{NA} = r_{x,y} \quad (4)$$

$$\text{Axial resolution : } res_{z}^{o} = \frac{\lambda_{em}}{n - \sqrt{n^2 - NA^2}} = r_z \quad (5)$$

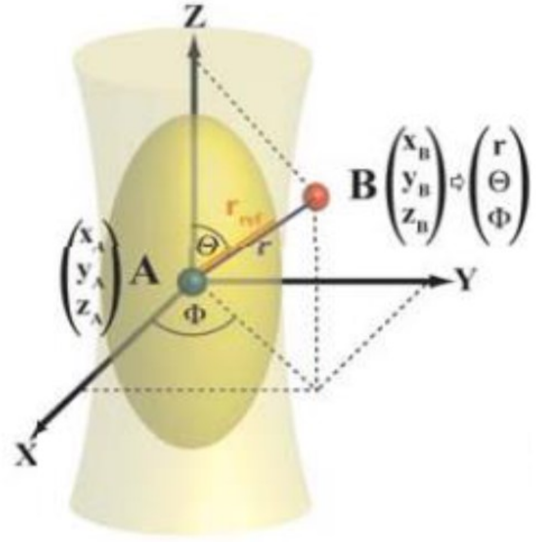
(Amos *et al.* Comprehensive Biophysics, 2012)

- In the following slides, we will show that the equation for r_{ref} is incorrectly defined.
- The choice of how one defines the PSF width (FWHM, first zero, etc) is ultimately not important for what follows.

Spherical Coordinate Angles

$$\Phi = \arccos \frac{x_B - x_A}{\sqrt{(x_B - x_A)^2 + (y_B - y_A)^2}} \quad (2)$$

$$\Theta = \arccos \frac{z_B - z_A}{\sqrt{(x_B - x_A)^2 + (y_B - y_A)^2 + (z_B - z_A)^2}} \quad (3)$$



- Cordelières and Bolte have defined the azimuthal angle and polar angle using spherical coordinates.
- For simplicity, place the center of the reference channel point at the origin ($x_A = y_A = z_A = 0$). The angle equations can now be rewritten as:

$$\Phi = \arccos \frac{x}{\sqrt{x^2+y^2}} \quad (2')$$

$$\Theta = \arccos \frac{z}{\sqrt{x^2+y^2+z^2}} \quad (3')$$

- In this form, the reference ovoid surface equation given by Cordelières and Bolte, generalized for any ovoid with axes lengths r_x, r_y and r_z becomes:

$$r = \sqrt{r_x^2 \cos^2\Phi \sin^2\Theta + r_y^2 \sin^2\Phi \sin^2\Theta + r_z^2 \cos^2\Theta} \quad (1')$$

Equation of ovoid/ellipsoid

- The equation of an ovoid/ellipsoid in cartesian coordinates is:

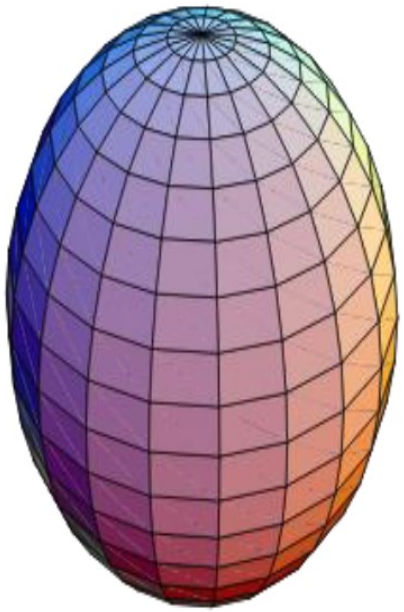
$$\frac{x^2}{r_x^2} + \frac{y^2}{r_y^2} + \frac{z^2}{r_z^2} = 1 \qquad (6)$$

- In spherical coordinates, this is (see same link above):

$$\frac{r^2 \cos^2\Phi \sin^2\Theta}{r_x^2} + \frac{r^2 \sin^2\Phi \sin^2\Theta}{r_y^2} + \frac{r^2 \cos^2\Theta}{r_z^2} = 1 \qquad (7)$$

- Solving for *r*, this becomes:

$$r = \frac{r_x r_y r_z}{\sqrt{r_y^2 r_z^2 \cos^2\Phi \sin^2\Theta + r_x^2 r_z^2 \sin^2\Phi \sin^2\Theta + r_x^2 r_y^2 \cos^2\Theta}} \qquad (8)$$

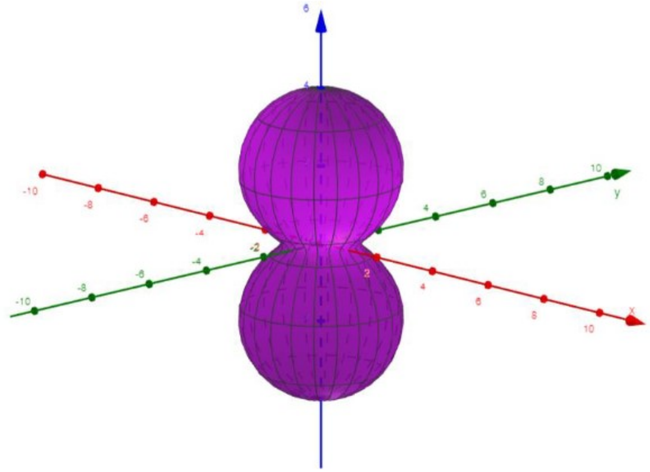


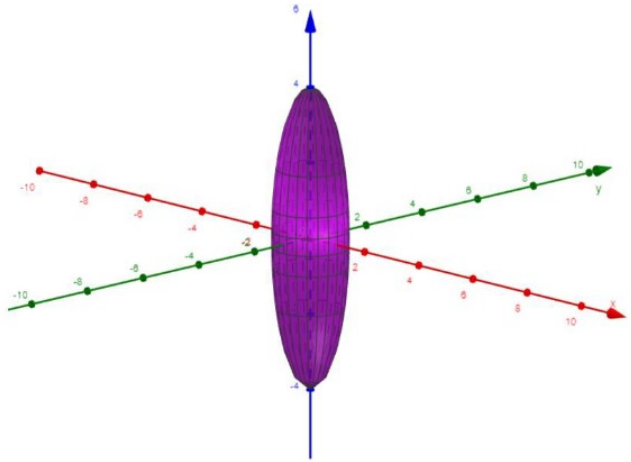
Differences between these equations

- Equation 1' does not match the Expected equation 8 – WHY?

$$r = \sqrt{r_x^2 \cos^2\Phi \sin^2\Theta + r_y^2 \sin^2\Phi \sin^2\Theta + r_z^2 \cos^2\Theta} \qquad (1')$$

$$r = \frac{r_x r_y r_z}{\sqrt{r_y^2 r_z^2 \cos^2\Phi \sin^2\Theta + r_x^2 r_z^2 \sin^2\Phi \sin^2\Theta + r_x^2 r_y^2 \cos^2\Theta}} \qquad (8)$$





- Cordelières and Bolte have used the **parametric form** of an ovoid surface; the angles Φ and Θ in Equation 1' **do not** correspond to physical azimuthal and polar angles in spherical coordinates that they defined in Equations 2 and 3!
- In the following slide, we will prove this discrepancy using the 2D equivalent of an ovoid (an ellipse and its parametric form).

Polar and Parametric Polar Equations of a 2D Ellipse

- Consider an ellipse oriented vertically in the *xy* plane with major and minor axes r_y and r_x respectively (see diagram).

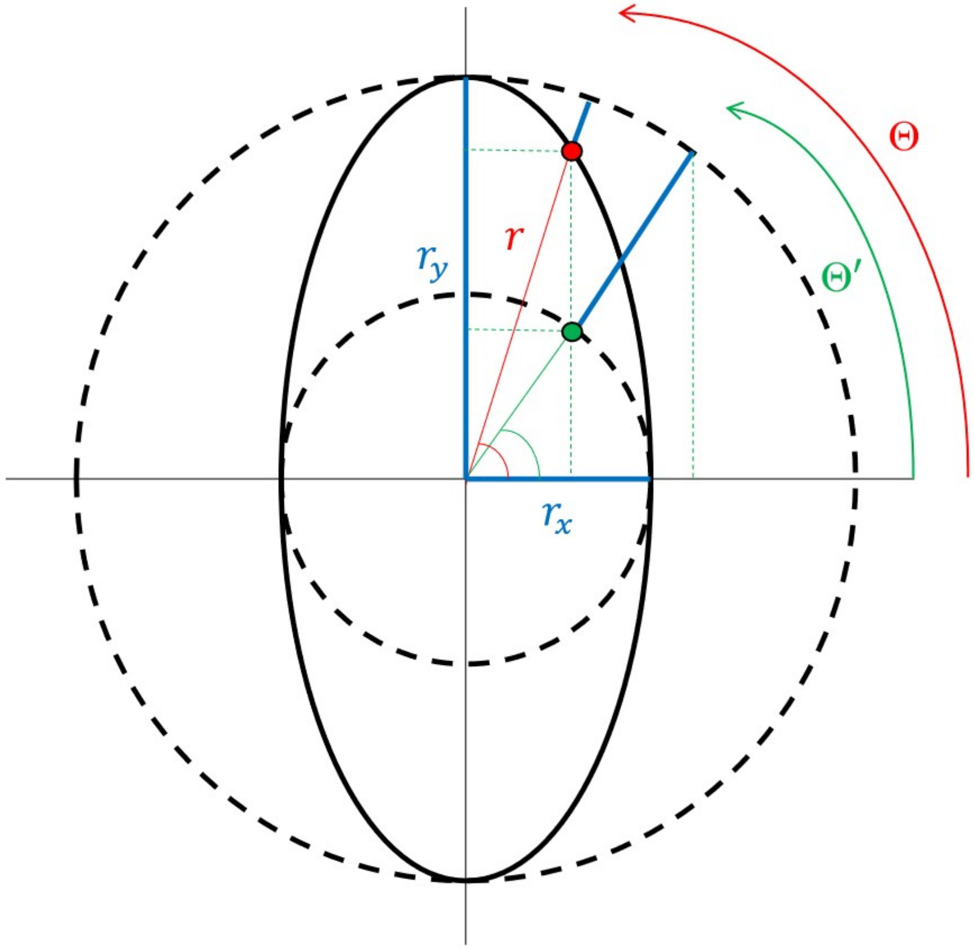
$$\tan\Theta' = \frac{\sin\Theta'}{\cos\Theta'} = \frac{r_x \sin\Theta}{r_y \cos\Theta} = \frac{r_x}{r_y}\tan\Theta \qquad (9)$$

- Using polar coordinates (Θ), the ellipse is described by:

$$\frac{x^2}{r_x^2} + \frac{y^2}{r_y^2} = 1 \qquad (10)$$

$$r = \frac{r_x r_y}{\sqrt{r_y^2 \cos^2\Theta + r_x^2 \sin^2\Theta}} \qquad (11)$$

- Note the similarity of Equation 11 to Equation 8.



Polar and Parametric Polar Equations of a 2D Ellipse

- In contrast, the parametric polar equation of the 2D ellipse is:

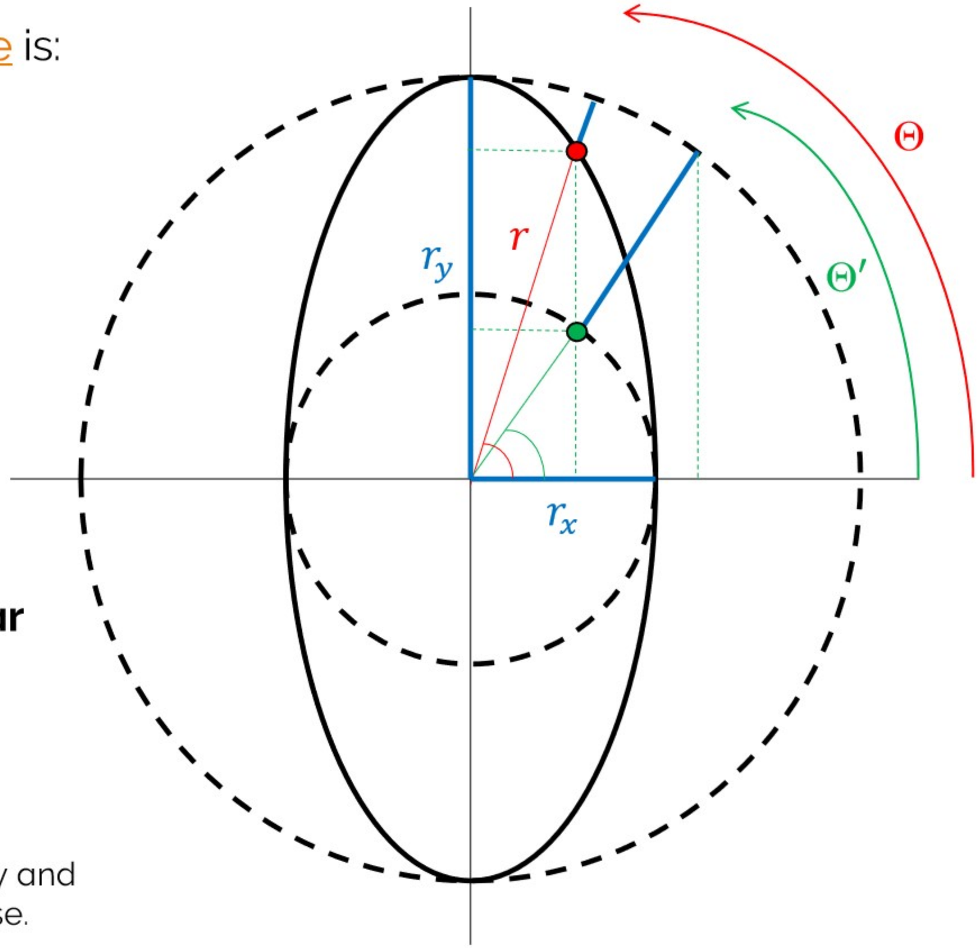
$$r = \sqrt{r_x^2 \cos^2 \Theta' + r_y^2 \sin^2 \Theta'} \quad (12)$$

- Equation 12 is the 2D version of the equation used by Cordelières and Bolte (Equation 1'):

$$r = \sqrt{r_x^2 \cos^2 \Phi \sin^2 \Theta + r_y^2 \sin^2 \Phi \sin^2 \Theta + r_z^2 \cos^2 \Theta} \quad (1')$$

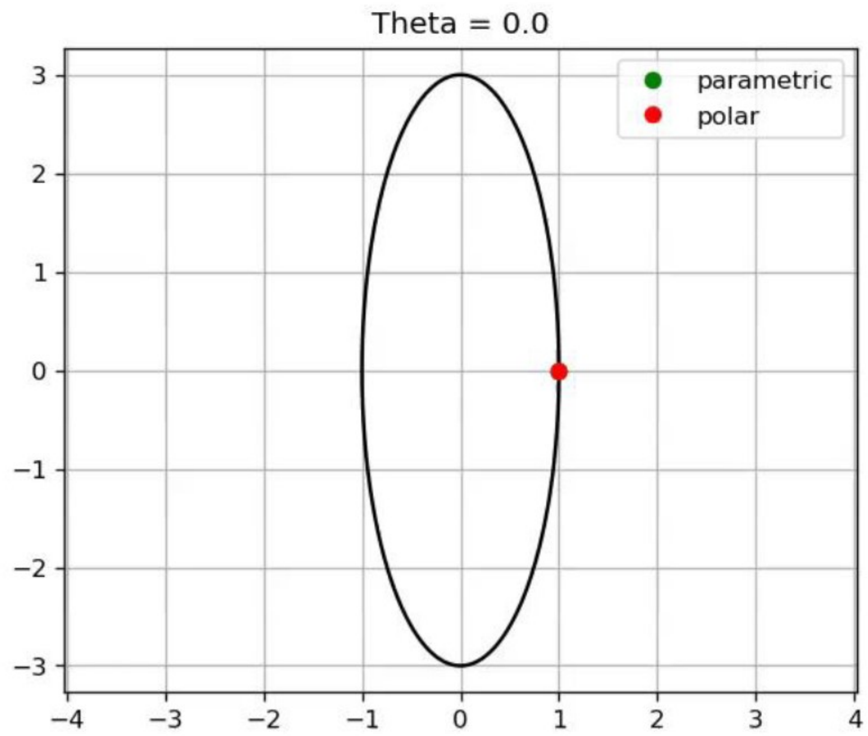
- But Cordelières and Bolte have **incorrectly used the parametric form of the ovoid using the azimuth and polar angles of spherical coordinates**, not the parameterized versions of these angles (Equation 9)!
- $\Theta \neq \Theta'$

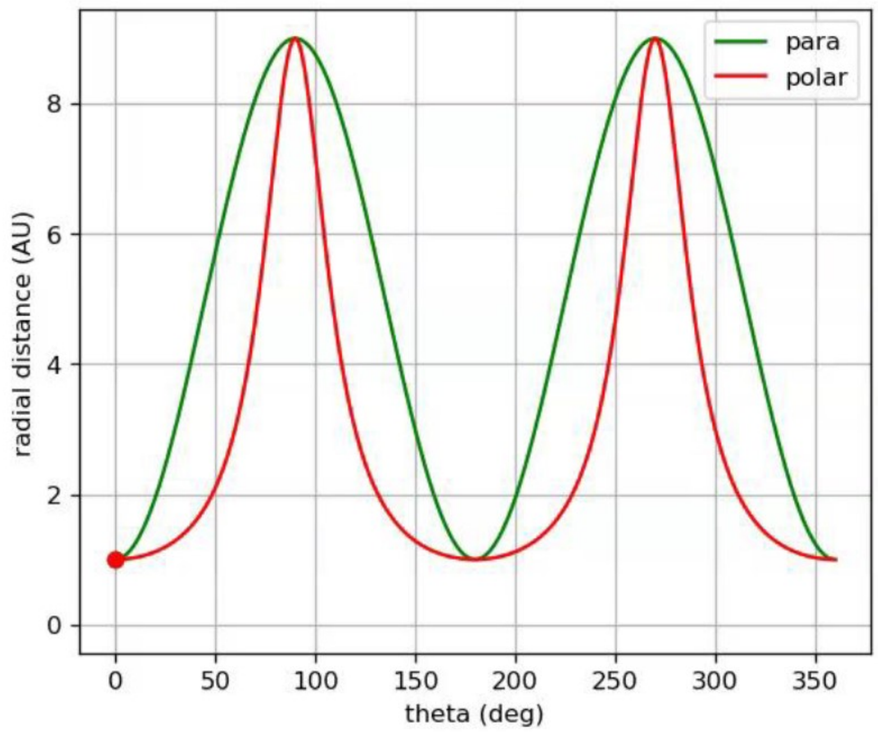
Fun fact: Most often Θ' is written as t to denote a time coordinate which evenly and proportionately oscillates the lengths of the major and minor axes of the ellipse.



Implications

- These angles, $\color{green}{\Theta'} \neq \color{red}{\Theta}$, sweep over ellipse at different speeds.





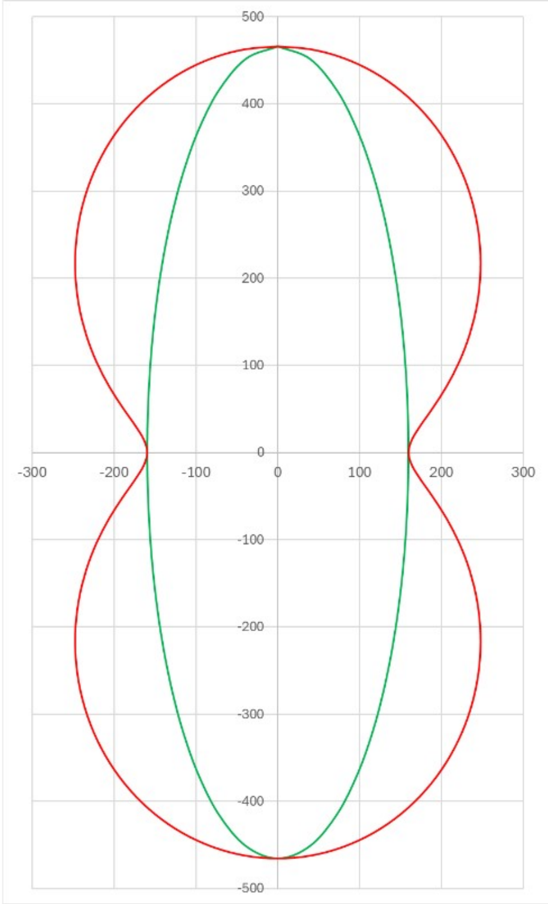
Implications

- If polar angle Θ (as defined by Equation 3') is used with Equation 1' instead of the parameterized angle Θ' (Equation 9), then r_{ref} will be inflated (except when on x- or y-axis), and therefore co-registration r_{exp} / r_{ref} ratios will be incorrectly calculated lower than expected!
- As before, consider the 2D version of this situation:

$$r = \frac{r_x r_y}{\sqrt{r_y^2 \cos^2\Theta + r_x^2 \sin^2\Theta}} \qquad (11)$$

$$r = \sqrt{r_x^2 \cos^2\Theta + r_y^2 \sin^2\Theta} \qquad \text{(12, modified using polar angle according to Equation 3')}$$

- Notice that when the modified version of 12 is plotted from 0 to 2π, the resultant curve **is not an ellipse**! (It is in fact a “cassini oval” or “peanut” shape. In 3D, this is called a “cassinoid”).
- This is the incorrect reference surface!

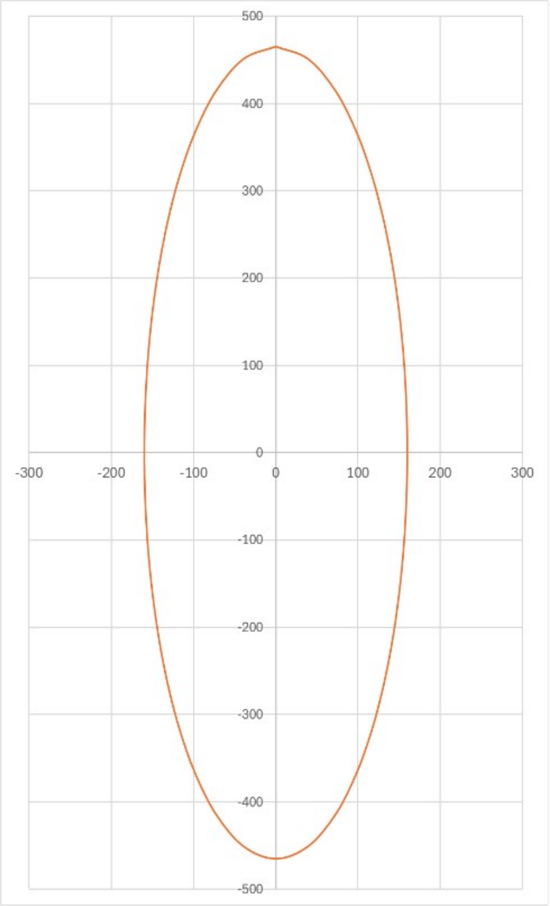


Implications

- However, Equation 12 *will* yield a proper ellipse in 2D ***if the parameterized polar angle Θ' according to Equation 9*** is used instead:

$$\tan\Theta' = \frac{\sin\Theta'}{\cos\Theta'} = \frac{r_x \sin\Theta}{r_y \cos\Theta} = \frac{r_x}{r_y}\tan\Theta \qquad (9)$$

$$r = \sqrt{r_x^2\cos^2\Theta' + r_y^2\sin^2\Theta'} = \sqrt{r_x^2\cos^2\left(\arctan\left(\frac{r_x}{r_y}\tan\Theta\right)\right) + r_y^2\sin^2\left(\arctan\left(\frac{r_x}{r_y}\tan\Theta\right)\right)} \qquad (12)$$



The correct 3D reference surface

- We contend that Equation 1' as utilized by Cordelières and Bolte must use the parameterized polar and azimuthal angles in the same manner as has been shown for the 2D ellipse in order the yield the correct 3D reference surface:

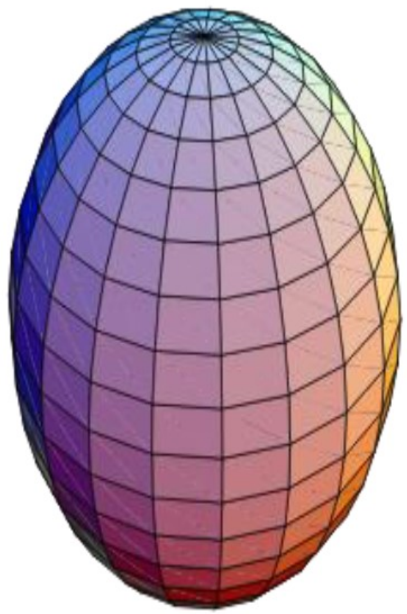
$$r = \sqrt{r_x^2 \cos^2\Phi' \sin^2\Theta' + r_y^2 \sin^2\Phi' \sin^2\Theta' + r_z^2 \cos^2\Theta'} \quad \text{(1' using parameterized angles)}$$

- Alternatively, the 3D reference surface can be calculated using Equation 8 and the standard azimuthal and polar angles of spherical coordinates, as was originally defined by Cordelières and Bolte:

$$r = \frac{r_x r_y r_z}{\sqrt{r_y^2 r_z^2 \cos^2\Phi \sin^2\Theta + r_x^2 r_z^2 \sin^2\Phi \sin^2\Theta + r_x^2 r_y^2 \cos^2\Theta}} \quad (8)$$

$$\Phi = \arccos\frac{x}{\sqrt{x^2+y^2}} \quad (2')$$

$$\Theta = \arccos\frac{z}{\sqrt{x^2+y^2+z^2}} \quad (3')$$



Examination of the MetroloJ_QC Co-registration Code

- The MetroloJ_QC code can be found on GitHub here.
- The relevant lines of code are between line 463 and 479:

```
463   private double calcRefDist(double[] coordA, double[] coordB, microscope micro, int channel) {
464     double x = (coordB[0] - coordA[0]) * micro.cal.pixelWidth;
465     double y = (coordB[1] - coordA[1]) * micro.cal.pixelHeight;
466     double z = (coordB[2] - coordA[2]) * micro.cal.pixelDepth;
467     double distXY = Math.sqrt(x * x + y * y);
468     double distXYZ = Math.sqrt(distXY * distXY + z * z);
469     double theta = 0.0D;
470     if (distXYZ != 0.0D)
471       theta = Math.acos(z / distXYZ);   (Equation 3)
472     double phi = 1.5707963267948966D;
473     if (distXY != 0.0D)
474       phi = Math.acos(x / distXY);   (Equation 2)
475     double xRef = ((double[])micro.resolutions.get(channel))[0] * Math.sin(theta) * Math.cos(phi);
476     double yRef = ((double[])micro.resolutions.get(channel))[1] * Math.sin(theta) * Math.sin(phi);
477     double zRef = ((double[])micro.resolutions.get(channel))[2] * Math.cos(theta);
478     return Math.sqrt(xRef * xRef + yRef * yRef + zRef * zRef);   (Equation 1)
479   }
```

This is the same mistake of Cordelières and Bolte; The azimuthal and polar angles of spherical coordinates are being used with the parameterized equation of an ovoid/ellipsoid.

- **The code can be corrected by replacing line 478 with Equation 8 and keeping the angles (Equations 2 and 3) the same.**

Suggested Code Correction

```
    double xRef = ((double[]micro.resolutions.get(channel))[0]*Math.sin(theta)*Math.cos(phi);
    double yRef = ((double[]micro.resolutions.get(channel))[1]*Math.sin(theta)*Math.sin(phi);
    double zRef = ((double[]micro.resolutions.get(channel))[2]*Math.cos(theta);
    double xRes = ((double[]micro.resolutions.get(channel))[0];
    double yRes = ((double[]micro.resolutions.get(channel))[1];
    double zRes = ((double[]micro.resolutions.get(channel))[2];
    return xRes * yRes * zRes / Math.sqrt(xRef * xRef + yRef * + zRef * zRef);
}
```