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Supporting Material

Quantitative imaging of human red blood cells infected with Plasmodium falciparum

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Supporting Material

Figure S1. Confocal imaging of a cohort RBC with merozoite attached. The arrow shows a merozoite attached to the surface of the cohort RBC, ready to invade its host.

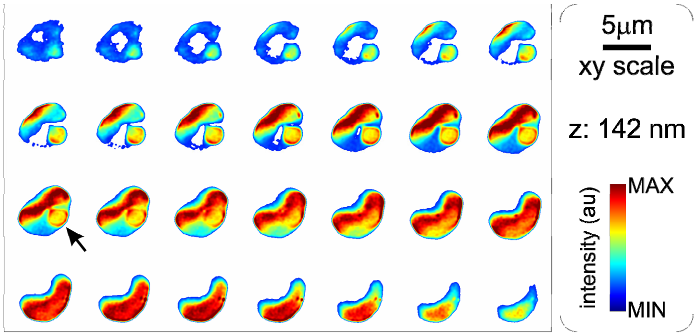


Figure S2. Examples of three dimensional rendering of normal, crenated and infected RBCs by the fit-assisted algorithm.

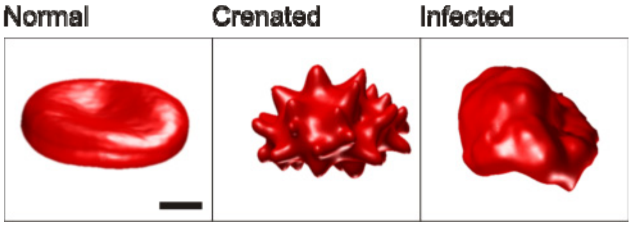
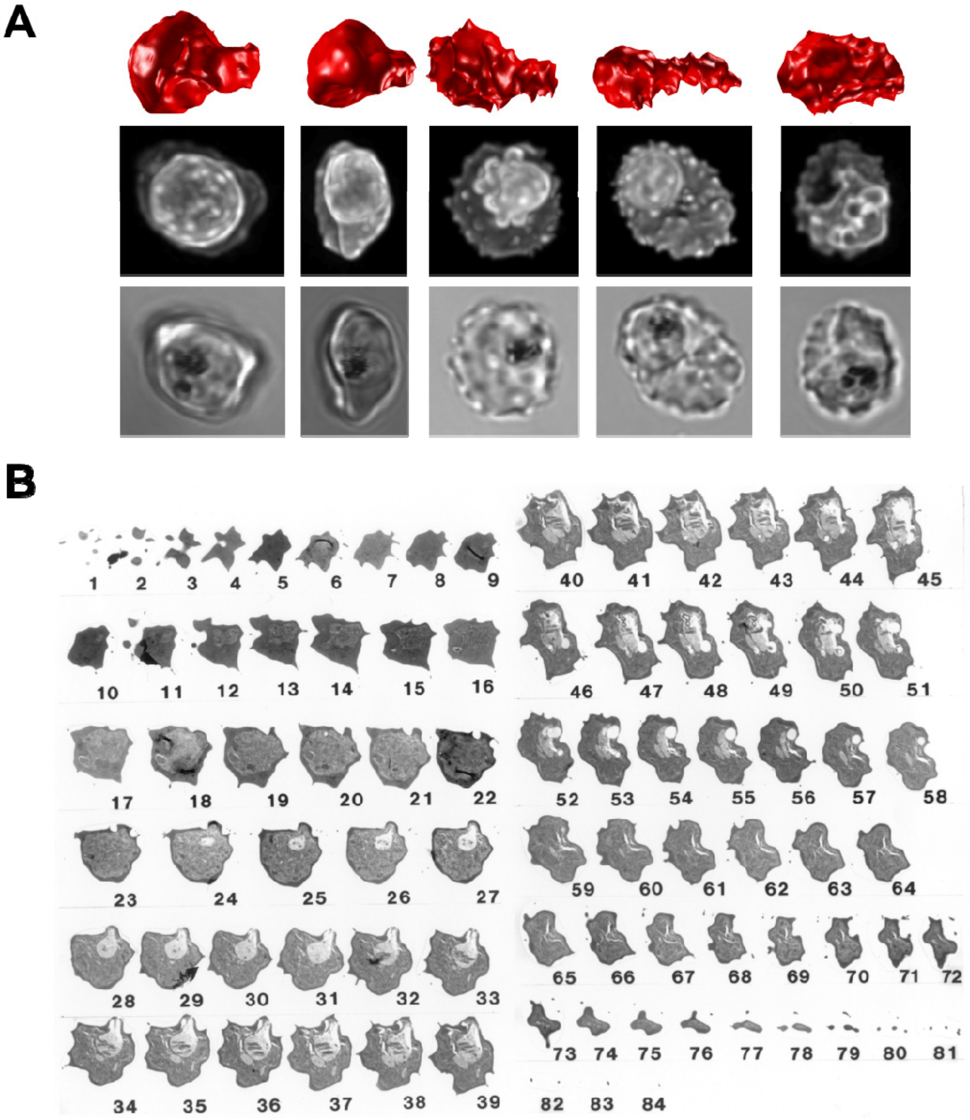


Figure S3. **Variety of shapes observed in red blood cells infected with late-stage parasites**. **A**. Selected 3D rendered images (in red) are shown above the corresponding maximum-intensity projection of the deconvolved confocal 3D image stacks (second row) and transmission images (third row). Note the relatively smooth appearance of the surface bulges in the first two cells and the more spiky appearance of a substantial proportion of the membrane surface in the last three cells. The dark hemozoin pigment in the transmission images (third row) indicates the position of the food vacuole (residual body) in each cell. No difference in volume (p=0.57, t-test) was found between schizont-infected RBCs exhibiting a "smooth" or a "spiky" phenotype. The ratio of the two average volumes ("spiky" over "smooth") was 1.03±0.04 (mean±SEM). **B.** Electron-micrograph of a schizont; serial section. Sample processed for electron microscopy and serial sectioning as detailed before (30, 31). Note the relatively smooth appearance of the membrane in the regions where the parasite occupies most of the cell volume (i.e. frames 23 to 30), and the increased frequency of spikes in membrane regions distal to the parasite (i.e. frame 40 onwards).



Document S1. Effect of surface area variations on osmotic fragility

The relative tonicity at lysis depends on the fractional water volume (w) of a RBC and on its critical hemolytic volume (CHV), *i.e.*, the ratio between the maximum volume that a RBC can contain within its plasma membrane of surface area A_C and its physiological volume (V_C).

We wish to estimate the shift in osmotic fragility expected from the documented loss in membrane area in IRBCs. With the same equations, we can infer the surface area loss that would both justify our new volume measurements and the previous osmotic fragility shifts observed. In such cells w is predicted to be substantially decreased at schizont stage to about 0.5 from an original level of about 0.7 in uninfected RBCs (Saliba, Horner et al. 1998).

$$RT_L = \frac{w}{CHV - 1 + w}$$

$$CHV = \frac{A_{RBC}^{3/2}}{6\sqrt{\pi} V_{RBC}}$$

For a fractional variation of plasma membrane surface area *f*, CHV will shift at lower values accordingly to the following power law:

$$CHV' = \frac{\left[A_{RBC}(1-f)\right]^{3/2}}{6\sqrt{\pi} V_{RBC}} = CHV(1-f)^{3/2}$$

Therefore, the RT_L will be shifted to higher values:

$$RT_L' = \frac{w}{CHV(1-f)^{3/2} - 1 + w}$$

For w values of 0.7 and 0.5, *f* = 0.18 and CHV of 1.7, the resulting RT_L values for cohorts and IRBCs with late-schizont parasites is 0.5 and 0.6, respectively. On the other hand, for values of RT_L' within the 0.8-0.9 range (Lew et al., 2004) and w = 0.5, an expected reduction of surface area amounts to f = 0.24-0.27.

Document S2. Algorithm

Software for fit-assisted isosurface rendering was implemented in Matlab which routines are available at the author's website (www.quantitative-microscopy.org). The version of the algorithm is described in the following section and by the flow chart shown in Fig. S3.

Parsing input: the software was developed with a Matlab function which accepts a variety of input variables. The version of the software implemented for this work currently supports the following inputs:

- *'Plot'* (Boolean, default 0) switches on/off additional data output on screen. If set to 1, the normals at each vertex of the isosurface are drawn with an arrow when used by FAIS. Intensity profile and fit of the boundary are also plotted on screen.
- *'KeepArrow'* (Boolean, default 0): when both "Plot" and "KeepArrow" are set to 1, the arrows plotted normal to the isosurface are not deleted during the scan of all vertices (see Fig. S6B).
- *'n_vertices'* or *'n_bits'* (integer, default 5) specifies the initial number of vertices to be fitted. The number of vertices is expressed as power of 2, *i.e.*, $2^{\text{n\_bits}}$.
- *'iterations'* or *'iter'* (integer, default 1) specifies the number of iterations for the FAIS algorithm (typically 2 iterations are sufficient).
- *'increment'* or *'inc'* specifies the increment in *n_bits* after each iteration; FAIS can gradually increase the resolution of the grid of vertices (<u>v-grid</u>) that is fitted.
- *'isovalue'* or *'iso'* (float, default 50.0) specifies the initial isovalue with which the first isosurface is generated. This first isosurface is the "initial guess" for FAIS.
- *'voxel_dimensions'* or *'dims'* (array of float numbers, [x y z], default [1 1 1]) specifies the metric dimension of each voxel. [50 50 150], for example, indicates that each voxel is 50nm x 50nm in the xy-plane and 150nm along the z-direction.
- *'fit_options'* or *'fopt'* (matlab optimization structure): this is a structure of optimization parameters used for data fitting; the default is generated by Matlab's function *optimset* with the following parameters: '*MaxFunEvals*', 500; '*MaxIter*', 500; '*tolfun*', 10e-8; '*tolx*', 10e-8; '*display*', 'off'; '*PrecondBandWidth*', Inf.
- *'reduce_volume'* or *'red'* (Boolean, default 0): this Boolean switch is set to 1 if a low resolution application requires the volume space to be down-sampled (see *'ropt'*).
- *'reduce_options'* or *'ropt'* (array of integer numbers, [x y z], default [3 3 2]) specifies the amount of binning used to down-sample the volume data space. Binning can differ along the three spatial dimensions (x, y, z).
- *'smooth_volume'* or *'smooth'* (Boolean, default 0) switches on/off smoothing of the volume which is carried out by convolution of the volume data with a Gaussian kernel in order to diminish noise (see *'sopt'*).
- *'smooth_options'* or *'sopt'* (array of integers, [x y z var], default [5 5 3 2]) specifies options used for the smoothing. The Gaussian kernel will span the number of pixels in the x, y and z direction indicated by the first three integers of the array. The value for the variance of the Gaussian kernel is provided by the last number of the array.
- *'reduce_patches'* or *'redpat'* (Boolean, default 1) specifies if the number of facets of the isosurface should be reduced to a maximum number (see *'popt'*).
- *'n_patches'*, *'reduce_patches_options'* or *'popt'* (integer, default 25000) specifies the maximum number of facets of the isosurface.

'photobleaching' or *'pb'* (Boolean, default 0) activates algorithms for photobleaching correction when set to one.
'fit_threshold' or *'t'* (float, default 0.5) is the local threshold level which is set to the inflex point of a sigmoid (0.5) by default.
'interactive' (Boolean, default 0) invokes graphical user interface (GUI) to input some of the parameters needed by FAIS when set to one.
'options' (array of integers, [n_bits iter inc]) provides a way to input *n_bits*, *iter* and *inc* values with an array of numbers instead of using the three different properties of the function.

Some of the blocks described in the flow chart of Fig. S3 are described next.

Default isovalue: initially an isosurface is computed (and rendered) with an arbitrarily selected isovalue. For the analysis of RBC three-dimensional images, this isovalue was selected manually on an image by image basis. This isovalue should be set to provide a first discrimination between object and background. According to typical outcomes, the lowest isovalue possible to achieve correct segmentation results in the best final volume rendering.

Reduce volume: when low resolution three-dimensional reconstructions are sufficient or when the signal to noise ratio of the experimental data is insufficient, it is possible to bin the image and/or to smooth the data by means of convolution with a Gaussian kernel. These options were not used for the analysis of data presented in this work. Fig. S4 shows the effects of smoothing on the robustness of volume and surface estimates.

Reduce patches: noisy experimental data result in isosurfaces with a large number of facets/patches. Although this step is not necessary for the correct computation of FAIS, computation time can be shortened by limiting the number of patches. For data analyzed in this work, an upper limit of 25000 facets was found to not affect the robustness and the estimation of the algorithms, whilst increasing execution efficiency. Fig. S4 shows also the effects of facet reduction on the robustness of volume and surface estimates.

Generate new surface: if any of the reduction algorithms is applied, the initial isosurface is recomputed.

Initialize x-grid: at each vertex location, the object-to-background boundary is fitted. However, resolution of a confocal microscope is anisotropic and different vertices exhibit different orientation and direction of the normal at the object surface. Therefore, at each location, intensity profiles have to be interpolated using a common distance coordinate. This is called **x-grid**; the x-grid is an array of float-type numbers representing the distance from the current estimated boundary along the direction of the normal to the surface. In the analysis of typical RBC images, the length of the x-grid was ~1.8µm, spanning from +0.9µm to -0.9µm with a spacing of ~80nm.

Initialize v-grid: fitting the entire ensemble of vertices for typical RBC volumes may require very long computational times. Therefore, FAIS selects only a number of vertices that will be directly fitted. These vertices (the **v-grid**) are randomly and sparsely selected. FAIS can be used iteratively: at each iteration, the FAIS-generated surface will be used as initial guess for the fit of the next iteration; the v-grid is recomputed and expanded at each iteration.

Compute normals: at each vertex location, the normal to the isosurface is computed.

Profile fit: each element of the v-grid is analyzed. For each element, an intensity profile is computed by interpolation using experimental values along the direction of the normals and mapped onto the x-grid centered on the initial position of the isosurface. Initial guesses for fitting are computed analytically and the profile is then fitted with a sigmoidal function. In this work, only sigmoid fits were used, but FAIS can be generalized with other profiles, for instance, with Gaussian profiles for the localization of membrane staining with high (sub-pixel/super-resolved) precision.

Photobleaching correction: Photobleaching may alter the apparent location of the object boundary. Photobleaching affects the apparent position of boundaries more significantly along the slowest scan direction of the microscope, *i.e.*, the z direction. This block of the algorithm provides a tool for the statistical analysis of the slopes of the fits and to infer/correct the effect of photobleaching on the boundary position. This function was not used in the analysis of RBC data because it did not yield significantly different results on initial tests.

Interpolation onto the v-grid: once the v-grid is completely analyzed, all other vertices are moved by interpolating the results of the v-grid fits; this procedure results in the elastic deformation of the initial isosurface to fit the volume data best. This step is skipped if FAIS is applied onto all the vertices.

Estimates: volume and surface area of the fitted object are computed at each iterative step and returned to the calling function of the FAIS routine once the iterations are completed. These estimates were obtained adapting code distributed by Dr John Burkardt under the GNU LGPL license

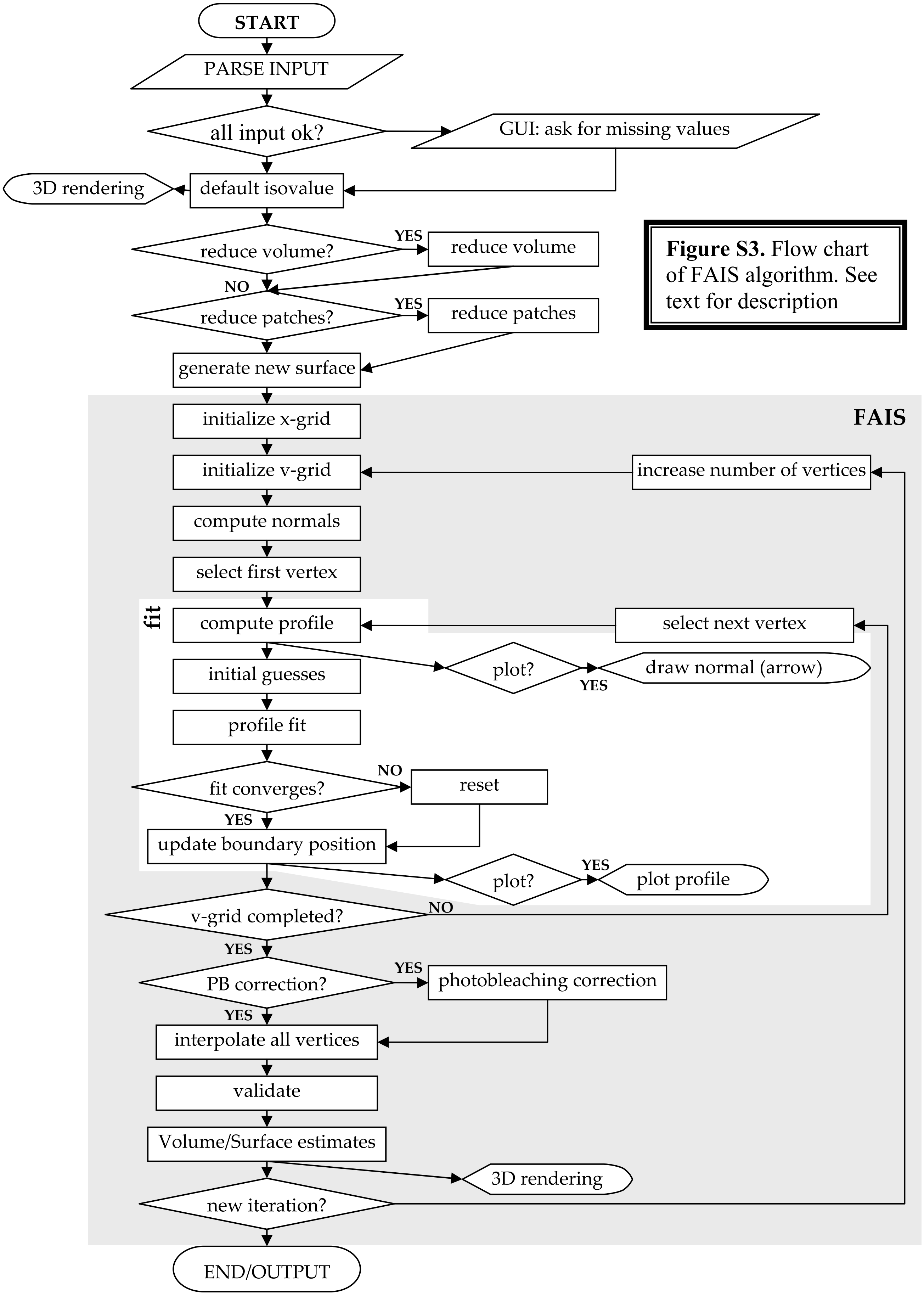


Figure S3. Flow chart of FAIS algorithm. See text for description

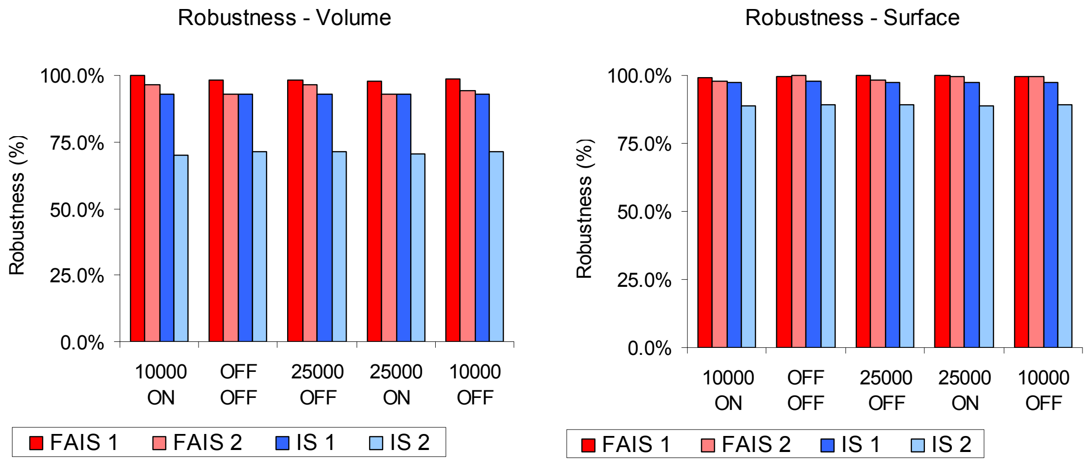


Figure S4. Robustness for volume and surface estimations plotted as a function of parameters used in the "reduce volume" block of FAIS algorithm (see Fig. S3 and text). The robustness estimator that is shown (*y-axis*) is the relative change of volume or surface estimator for a variation of the initial isovalue (between 0-255 for 8 bit images) from 70 to 90 (FAIS 1 and IS 1) or from 50 to 130 (FAIS 2 and IS 2). Different conditions for maximum number of rendered patches (first row) and smoothing (Gaussian kernel [3 3 2] with variance equal to 2, second row) are shown in the category axis. The sample was a representative IRBC.

Document S3. Testing the algorithms

The flow chart of the routines used to generate Fig. 3A-C is shown in Fig. S5. One dimensional profiles are generated and convolved with a Gaussian point spread function. Then, background and Poissonian noise are added to simulate experimental intensity profiles. The absolute position of the boundaries and the length of the simulated segment are estimated with three different techniques: 1D-FAIS data fitting, detection by the analysis of gradients with profiles smoothed with Gaussian kernels, detection by the analysis of profile enhanced by diffusional (adaptive) filtering. Data fitting can identify a boundary positioned between neighbouring pixels; therefore, data fitting returns estimations that are more precise that the pixel unit. For comparison, 1D-FAIS estimations are also rounded to the nearest pixel. The four estimators are computed for a variety of signal-to-noise ratios and repeated several times to obtain statistics. Diffusional filtering was performed with the Matlab toolbox "Nonlinear Diffusion Toolbox" developed by Federico D'Almeida and distributed under BSD licence.

The flow chart of the routines used to engineer and validate the Matlab code for FAIS is shown in Fig. S6A. This routine allowed to automatically apply FAIS on synthetic three-dimensional objects: ellipsoids, hollow ellipsoids or indented (hollow) ellipsoids. Fig. S6B show a typical image of an indented hollow ellipsoid which served as reference for convex objects (*e.g.*, red blood cells) with structured surface. Signal-to-noise ratios and initial isovalues could be adjusted iteratively for the estimation of the effects of these parameters on the efficiency of the algorithm. Each test was repeated a number of time (typically 20) in order to provide meaningful statistics. Fig. S6B shows also black arrows to demonstrate the surface normals that are computed; also, the inset of Fig. S6B shows a magnified area of the reconstructed surface (*red*) after a low resolution iteration of FAIS applied to an initial isosurface (*grey transparent surface*).

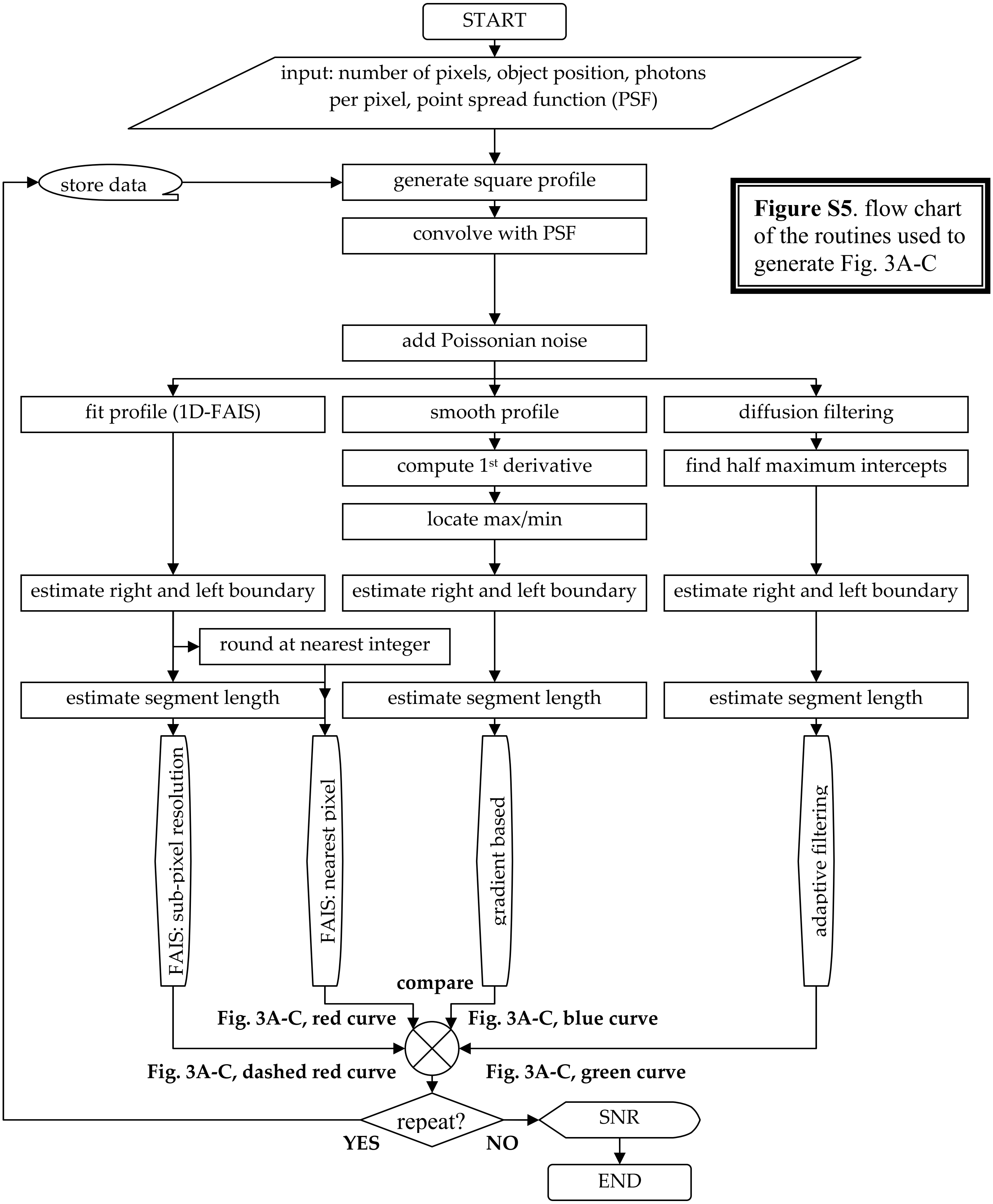


Figure S5. flow chart of the routines used to generate Fig. 3A-C

Figure S6. A) flow chart of the routines used to generate Fig. 3D; B) typical synthetic samples.