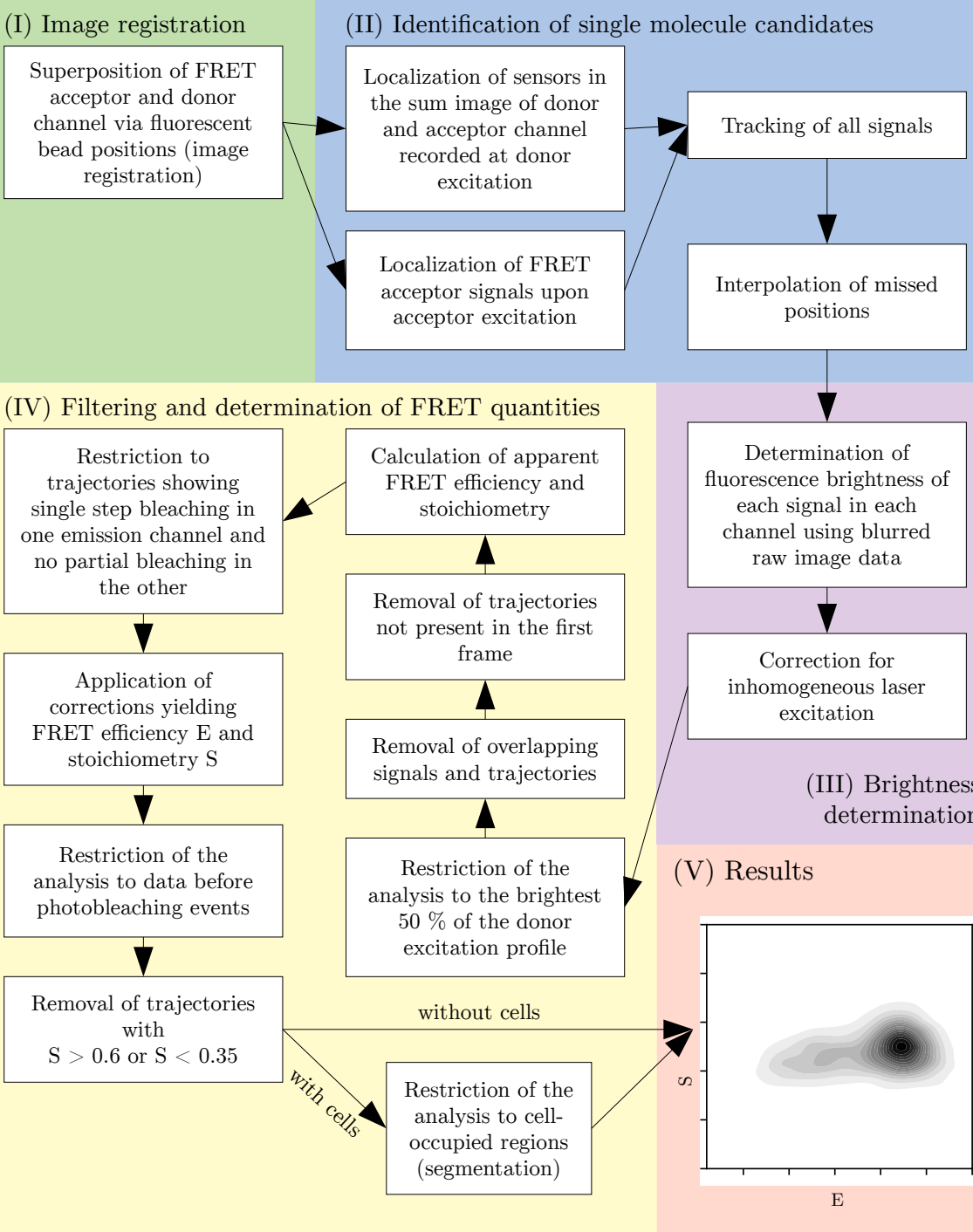
(I) Image registration
Superposition of FRET acceptor and donor channel via fluorescent bead positions (image registration)
(II) Identification of single molecule candidates
Localization of sensors in the sum image of donor and acceptor channel recorded at donor excitation
Localization of FRET acceptor signals upon acceptor excitation
Tracking of all signals
Interpolation of missed positions
(III) Brightness determination
Determination of fluorescence brightness of each signal in each channel using blurred raw image data
Correction for inhomogeneous laser excitation
(IV) Filtering and determination of FRET quantities
Restriction to trajectories showing single step bleaching in one emission channel and no partial bleaching in the other
Application of corrections yielding FRET efficiency E and stoichiometry S
Restriction of the analysis to data before photobleaching events
Removal of trajectories with S > 0.6 or S < 0.35
Calculation of apparent FRET efficiency and stoichiometry
Removal of trajectories not present in the first frame
Removal of overlapping signals and trajectories
Restriction of the analysis to the brightest 50 % of the donor excitation profile
without cells
with cells
Restriction of the analysis to cell-occupied regions (segmentation)
(V) Results
S
E