**Spot detection on structured background protocol**

**File required:** ‘main\_RADSpot’ folder

**Toolbox required:** Statistics and Machine Learning Toolbox, image processing toolbox

1. Put sub-diffraction beads images in the folder ‘**area\_threshold’** and run **determineAreaThres.m** to find the maximum area in pixel for a diffraction-limited object.

2. Put negative control images in the folder **‘negative\_control’** and run **determineRad.m** to determined the steepness and integrated gradient used in the study.

3. Put images in **‘image’** folder and run **detection.m** for diffraction-limited puncta detection