**Brain image simulation protocol**

**File required:** ‘imageSimulation’ folder

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

1. Prepare **negative control images** where no fluorescent puncta were labelled. These images are considered as background images.

2. Select one or several cropped images containing **large aggregates** in the library and apply a **sigmoid function** to the selected cropped image where x in the sigmoid function is determined by **the distance to the large aggregate in the cropped image**.

3. Simulate **small puncta** with 2D gaussian distribution on a **blank image** (same image size as the negative control image), the number of which should be determined from **real images**. The sigma of puncta should be determined from **real images** as well.

4. **Add** cropped **large aggregates** onto the **background images** at a random location. Then **add** the **simulated puncta** onto **the background image** to form a simulated image with **both small and large features**.

5. Record position, intensity and background information per small puncta expect for those overlapping with the large aggregates in the simulated image and save the results.

6. **Repeat** step2 to 5 again for other background images.