**Supplementary software readme**

The software contains Matlab code using ANN for single molecule localization. It has been test on **Matlab (**64-bit, R2010a**)** under **Windows 64-bit**. The software requires a freely available imaging processing toolbox for Matlab, **DIPimage 2.3** (<http://www.diplib.org/>).

**Please use the software as following:**

1. Run **ANN\_generationData.m** in Matlab. This script demonstrates the generation of training data for ANN. Three types of images (free dipole, fixed dipole and restricted dipole) can be generated as training data, respectively.
2. Run **ANN\_training.m** in Matlab. This script demonstrates the training of ANN. **First, you should load one type of training data generated as above.** After noise corruption, it creates four neural networks for position, photons, phi and theta (delta) training (for free dipole, only two networks are created). Finally, it simulates with training data and displays the precision of trained networks.
3. Run **ANN\_demo.m** in Maltab. This script demonstrates the use of ANN for simulation of real images. **You should finish training first!** Three images of single molecule are provided. Data 1 (free.tif) is an image of biotin-conjugated mEos2, so it represents free dipole and is simulated with free ANN. Data 2 (fixed.tif) is an image of fixed mEos2 immobilized in a PMMA layer, hence it represents fixed dipole and is simulated with fixed ANN. Data 3 (restricted.tif) is an images of mEos2 from fixed cell PALM experiment, so it represents restricted dipole and is simulated with restricted ANN.
4. **ANN\_test.m:** This script demonstrates the basic steps of ANN for single molecule localization. Load test.tif as example.

**Other:**

1. **ANN\_simulation.m:** This function can be called for simulation of single molecule images.
2. **ANN\_getPSF.m:** This is the core code for generating the theoretical PSF of dipoles. It’s based on following papers:
3. Mortensen, K. I., Churchman, L. S., Spudich, J. A. & Flyvbjerg, H. Nat. Methods 7, 377-381 (2010).
4. Stallinga, S. & Rieger, B. Opt. Express 18, 24461-24476 (2010).
5. Patra, D., Gregor, I., Enderlein, J. & Sauer, M. Applied Physics Letters 87,(2005).
6. Irving, M. Biophys. J. 70, 1830-1835 (1996).
7. **ANN\_gui:** We also provided a program with graphical user interface, which makes the use of ANN more convenience.