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3D Fluorescence Localization in Frozen Cells for Targeted Lamella Milling for Electron Cryo-Tomography

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In-situ imaging of proteins in cells allows to characterize their native structure, conformation, and interactions with their environment. This can be achieved through electron cryo-tomography but requires the milling of a 100-200 nm thick lamella with a focused ion beam [1]. To include the fluorescent feature of interest in the lamella correlative targeting methods are utilized [2,3]. These methods can however be laborious and prone to contamination of the sample, hindering the acquisition of high-quality electron tomograms. Integration of a light microscope into a cryo-FIB-SEM allows for a simplified workflow to target fluorescent features [4-6]. Regular wide-field fluorescence microscopy, however, does not bring sufficient resolving power to allow localization and targeting of sparse fluorescence features in the 100-200 nm thick lamella. We are therefore implementing 3D localization using astigmatic imaging in an integrated cryo-fluorescence-FIB-SEM [6,7]. The cryogenic conditions pose two unknown constraints on the localization accuracy: (1) a limited numerical aperture (NA) of the objective lens, and (2) cryogenic immobilization of the fluorescence transition dipole moment of the molecule.

Using vectorial point spread function (PSF) software [8], we simulated fluorescence microscopy reflecting the conditions of the experimental integrated cryo-fluorescence-FIB-SEM in our lab [6]. This includes a limited NA of 0.85 and induced astigmatism. We evaluated different biological targeting scenarios where the features are composed either of multiple fluorescent molecules (*e.g.* fluorescently labeled vesicles) or a single fluorescent molecule (*e.g.* fluorescently labeled proteins). To localize the fluorescent features, we used both classical astigmatic localization algorithms [9], as well as direct vectorial PSF fitting [8].

We find that for features of multiple fluorescent molecules, standard 3D astigmatic localization techniques suffice to reach localization accuracies smaller than the needed lamella thickness at realistic photon counts. The fixed dipole orientation of the molecule in features composed of a single fluorescent molecule leads to large errors in the localization using standard techniques [10,11]. Vectorial PSF fitting of both the position *and* orientation of the dipole in astigmatic imaging overcomes this problem and leads to accuracies well below the lamella thickness required for electron cryo-tomography.

Our results demonstrate that 3D localization of fluorescent features, from single molecules to fluorescently labeled vesicles, can be achieved at accuracies below the thickness of lamellae required for electron cryo-tomography. Using the aforementioned integrated cryo-fluorescence-FIB-SEM [6], we put our findings to use in the targeted lamella preparation from vitreous cells with fluorescently labeled features. We will discuss further implementation of and prospects for a simplified workflow for the preparation of lamellae with minimized contamination. In the future, the localization methods as presented here, in combination with the integrated cryo-fluorescence-FIB-SEM [6], will allow for correlative super-resolution localization microscopy and electron cryo-tomography [12].

References:

[1] A Rigort and JM Plitzko, Archives of Biochemistry and Biophysics **581** (2015), p. 122. doi:10.1016/j.abb.2015.02.009
[2] J Arnold et al., Biophysical Journal **110** (2016), p. 860. doi:10.1016/j.bpj.2015.10.053
[3] S Li et al., Journal of Structural Biology **201** (2018), p. 63. doi:10.1016/j.jsb.2017.11.002
[4] S Gorelick et al., Elife **8** (2019), p. e45919. doi:10.7554/eLife.45919.001
[5] M Smeets et al., Microscopy Today **29** (2021), p. 20. doi:10.1017/S1551929521001280
[6] DB Boltje et al., Microscopy & Microanalysis **27** (2021), p. 3228. doi:10.1017/S1431927621011120
[7] B Huang et al., Science **319** (2008), p. 810. doi:10.1126/science.1153529
[8] CN Hulleman et al., Nature Communications **12** (2021), p. 5934. doi:10.1038/s41467-021-26228-5
[9] M Ovesny et al., Bioinformatics **30** (2014), p. 2389. doi:10.1093/bioinformatics/btu202
[10] J Enderlein et al., Optics Express **14** (2006), p. 8111. doi:10.1364/OE.14.008111
[11] S Stallinga and B Rieger, Optics Express **18** (2010), p. 18. doi:10.1364/OE.18.024461
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