# A microscope calibration protocol for single-molecule microscopy

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**Abstract:** Single-molecule microscopy allows for the investigation of the dynamics of individual molecules and the visualization of subcellular structures at high spatial resolution. For single-molecule imaging experiments, and particularly those that entail the acquisition of multicolor data, calibration of the microscope and its optical components therefore needs to be carried out at a high level of accuracy. We propose here a method for calibrating a microscope at the nanometer scale, in the sense of determining optical aberrations as revealed by point source localization errors on the order of nanometers. The method is based on the imaging of a standard sample to detect and evaluate the amount of geometric aberration introduced in the optical light path. To provide support for multicolor imaging, it also includes procedures for evaluating the geometric aberration caused by a dichroic filter and the axial chromatic aberration introduced by an objective lens.



## 1. Introduction

Single-molecule wide-field microscopy experiments have been widely used for a broad range of investigations in cell-biological studies [1]. Two of the most important types of such single-molecule experiments are single-molecule tracking and localization-based super-resolution experiments. Single-molecule tracking experiments hold the promise to reveal fundamental insights into dynamic molecular processes in live cells that remain difficult to uncover using classical microscopy approaches [2–5]. Localization-based super-resolution microscopy can yield quantitative information on the spatial distribution of a molecule of interest and the spatial characteristics of cellular structures smaller than the diffraction limit [6–9]. Both types of experiments importantly depend on the estimation of the position of single molecules with a low level of uncertainty (i.e., a small variance or standard deviation) [10].

Localization of single molecules with statistical uncertainties of tens of nanometers is routinely achievable in single-molecule microscopy [11,12], and sub-nanometer uncertainty is even possible when using fluorophores that emit large numbers of photons [13,14]. Being able to achieve sub-nanometer uncertainty is critical for our current purposes, as we seek to assess the performance of microscopes and optical components by determining differences in the positional estimates of imaged point sources that will typically be on the order of nanometers.

The complexity of advanced microscopy setups, optical imperfections, and possible misalignments of mirrors or lenses can all contribute to geometric aberrations in the image produced by a microscope. Errors due to such non-ideal conditions can lead to erroneous