NOVEL APPROACH FOR 3D LIVE CELL FLUORESCENCE MICROSCOPY BASED ON MICROFABRICATED MIRRORS
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ABSTRACT
We have developed a novel 3D fluorescence microscopy technique, which is based on micromachining technology with stereovision analysis. Our method relies on the fabrication of V-shaped micromirrors, which are placed above fluorescent samples in order to simultaneously provide different point of views of the same specimen. We demonstrate that our technique is readily adapted to standard bright field microscopes, and that it can be applied for in vitro single particle tracking and for in vivo single gene 3D positioning.

KEYWORDS: Silicon micromachining, live cell imaging, fluorescence microscopy

INTRODUCTION
Fluorescence microscopy plays a central role in modern biology. Indeed, using the continuously growing library of fluorescent probes, it becomes possible to visualize virtually any protein in living cells, and gain understanding on the molecular interactions that govern the processes of life. Confocal microscopy has become an essential tool in this field of research because it enables real time 3D visualization. However, this technique suffers from some intrinsic limitations. First, 3D reconstruction is achieved by acquiring several confocal sections of the biological sample, meaning that specimens are exposed to high doses of illumination, which may induce photodamages. Second, 3D spatial resolution can only be improved at the expense of a poor temporal sampling because the acquisition of large numbers of confocal sections takes time. Due to these problems, several approaches have recently been proposed to perform fast and undamaging 3D microscopy [1,2], we propose a method readily adapted to standard wide field microscopes.

MATERIAL AND METHODS
Facetted mirrors are fabricated by wet etching of (100) or (110) silicon wafers (Fig. 1a-b). Silicon V-grooves are subsequently covered with gold, platinum or aluminum to increase their reflectivity. Fluorescent objects placed in between the facetted mirrors can be observed together with their two reflected images (Fig. 1c). Because the mirrors are tilted, the simultaneous visualization of the three images provides different points of view of the same scene. This situation is reminiscent of human 3D vision: each eye captures one projected view of a scene with a slightly
tilted angle in comparison to the other one, and the visual cortex reconstructs the surrounding 3D environment. This process is called stereovision, and it solely depends on the mirrors tilt angle, i.e. 54° or 35° for (100) or (110) wafers, respectively. Notably, because two views are sufficient for 3D reconstruction, each image gives access to three independent measurements, which can be compared in order to assess the consistency of our 3D tracking.

RESULTS AND DISCUSSION

First, we recorded the diffusive motion of 1 µm fluorescent particles placed beneath 35° and 54° mirrors (Fig. 2a), and we reconstructed 3D trajectories based on the relative position of the bead vs. its reflections (Fig. 2b). We then compared the tracking accuracy along the z-direction. Z positioning was more accurate for mirrors tilted by 54°: with a 20X objective and considering the three z-measurements ob-

Figure 1. Experimental approach. (a) Micromachining involves deposition of a nitride layer (blue), photoresist masking (red, 1), followed by reactive ion etching (2), anisotropic wet etching (3), and metal evaporation (gray layer in 4). (b) SEM image of silicon V-grooves (scale bar=400 µm). (c) 3D reconstruction is based on stereovision: two objects are observed from different point of views, and positions in z are projected along the y axis on the reflected images.

Figure 2. Tracking fluorescent particles. (a) Image of a bead placed beneath the mirrors with its left side and right side reflections (scale bar=5 µm). Tracks are obtained using one ImageJ plug-in [3]. (b) 3D trajectories of the bead and its reflections. Planes correspond to the mirrors. (c) Histograms showing the consistency of the z-measurement for 35° and 54° mirrors (green and blue datasets, respectively).
obtained at each time point, we evaluated that the quadratic error in z was 133 nm (n=757) and 668 nm (n=529) for 54° and 35° micromirrors, respectively (Fig. 2c). We also tested different objectives with the 54° mirrors, and observed that measurements were more consistent for 60X objectives, for the quadratic error in the z measurement decreased to 88 nm (n=1189).

Finally, we applied our method to live cell imaging, and we focused on chromatin in vivo dynamics. We used yeasts as a model system in which one or several genes were fluorescently tagged with green fluorescent protein [4]. We successfully followed genes 3D movements in real time (Fig. 3a), and we were able to reconstruct their trajectories in a realistic cellular geometry (Fig. 3b). We also optimized micromirrors geometry for our live cell assay. Whereas yeasts nuclei appeared to overlap with their reflections using 35° mirrors, well-separated points of views were obtained with 54° mirrors, and fluorescent genes could be accurately positioned in the nucleus. We also evaluated the consistency of the z-measurement, and we observed that 55° mirrors provided measurements twice more accurate.

CONCLUSIONS

We propose a new method for 3D live cell imaging that combines state-of-the-art microfabrication and fluorescence microscopy. We prove that this method is adapted for in vitro and in vivo applications, and we optimize the manufacturing process to obtain reliable 3D information. We now start analyzing genes motions quantitatively based on mean square displacement analyses, and we intend to obtain fundamental insights into chromatin organization with this real time microscopy approach.

REFERENCES