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Short communication

Nanofabrication of a gold fiducial array on specimen support for electron tomography

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ABSTRACT

Here we describe the production, using lithography and micro-engineering technologies, of patterned arrays of nanofabricated gold dots on a thin Si_3N_4 electron transparent layer, supported by silicon. We illustrate that the support with a patterned structure of nanosized gold can be exploited for (cryo) electron tomography application as a specimen support with predefined alignment markers. This nanogold patterned support has several advantages. The Si_3N_4 window provides a 50 nm thin, strong and flat support with a ~0.7 mm^2 large electron-beam transparent window. The nanogold pattern has a user-defined size and density, is highly regular and stable. This facilitates accurate tracking during tilt series acquisition, provides sufficient contrast for accurate alignment during the image reconstruction step and avoids an uneven lateral distribution and movement of individual fiducials. We showed that the support is suitable for electron tomography on plastic sections.

1. Introduction

Electron tomography is a technique for three-dimensional visualization of cellular structures and macromolecular arrangements at nanometer resolution. With electron tomography, the architecture of plastic embedded and stained sections of cells, as well as the molecular structural arrangements within thin areas of vitrified cells and cryo-sections of high-pressure frozen cells and tissues, can be investigated in three dimensions. During the last decade, for a wide variety of biological systems the ultrastructure of cells, cellular organelles and their molecular components have been successfully investigated and have given great insights into cellular structure and function [1,2].

One of the parameters that define the quality of tomograms acquired with transmission electron microscopy (TEM) is the accuracy by which the individual images of an acquired tilt series can be aligned relative to each other. This alignment step can be performed in several ways. One approach is to make use of cross-correlation between pairs of individual images in the acquired tilt series. Here, the relative shifts between the images are computed making use of the inherent image contrast generated by the electron microscope specimen. This approach is often referred to as fiducial-less alignment. Another, more often used, approach makes use of fiducial markers that are deposited on the specimen support. In this approach the locations of the markers are used for alignment of the tilt series. In most cases, these fiducial markers are nanometer sized (ranging from 5 to 20 nm) spherical gold beads [3] that provide high contrast. Suspensions of (protein-stabilized) gold beads are added to and mixed with the sample or applied onto the specimen support film prior to adding the sample. An advantage of this fiducial-marker approach is that it allows retrieving not only image shifts, but that it is also a suitable approach to determine image rotations and local image distortions based on a geometrical model of the specimen tilting. Disadvantage of the fiducial marker approach is that the spatial distribution and amount of gold beads on the specimen support is hard to control. The unpredictable amount of markers and irregular distribution can result in areas on the specimen support that are suboptimal for tracking during data collection and for alignment during image reconstruction. Additionally, it is known that individual gold fiducials can move during data acquisition, both on cryo-sections which can suffer random distortions [4] and on plastic sections due to shrinkage [5,6]. This leads to an imperfect alignment of the acquired tilt series. Therefore, control over the size, concentration and positioning of fiducials on the support, as well as the firm attachment to the support would be advantageous for accurate tracking and alignment in electron tomography. Different nanofabrication techniques have been applied to engineer nano-patterns [7] and even nanogold arrays [8] on silicon surfaces, for applications in cell biology and electronics and optics [9].

We present an electron beam lithography technique to produce patterned nanogold surfaces. This enabled full control to make specific patterns of gold (30–50 nm) with a relatively wide spacing

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(0.2–5.0 μm) on a 50 nm thin electron transparent silicon nitride membrane of 417×817 μm that is supported by silicon, as required for use as fiducials in electron tomography. These silicon-based supports have the advantages that they are flat and strong, that the electron beam transparent window is not obstructed by grid bars and that the size and positioning of fiducial markers is predefined. The advantages of the patterned gold are that fiducials are evenly spread in a predefined pattern and density, and that they are firmly attached to the highly flat support, avoiding possible movement of individual fiducials. We show that the patterned gold dots can be used as fiducials to facilitate sample tracking during tomographic tilt series acquisition and for accurate alignment during tomographic reconstruction.

2. Materials and methods

2.1. The production of Si_3N_4 windows in silicon wafers

The silicon nitride windows are fabricated on 200 μm thick 4 inch (100)-silicon wafers (Fig. 1, top row). LPCVD (low-pressure chemical vapor deposition) silicon nitride with a thickness of 50 nm is deposited on the wafer. Since the deposition takes place in a furnace (Tempress Systems) the wafer is coated from both sides. In the next lithography step windows are opened in the photoresist layer (ZEP 520), defining the different chips and the transparent membrane locations. The pattern was written by using electron beam lithography with 100 keV beam energy. In order to reduce the writing time the pattern consisted of small squares and diagonals. However, mask contact lithography could be used in case of mass production (where a fixed design is used) to reduce the cost. After development the nitride layer was patterned by dry etching, using CHF_3/Ar chemistry. The nitride layer serves as a mask during the consequent wet etching step with KOH solution (30 wt%, 83 °C). The etch rate of silicon nitride in KOH is less than 1 nm/h and can be neglected. Etch rates for silicon, silicon nitride and silicon dioxide are varying with concentration and temperature of KOH [10,11]. The silicon etches anisotropically along the (111)-plane in KOH, with an angle of 54.7° from the (100)-plane. Once the wafer is etched through down to the silicon nitride layer on the opposite site, the etching is stopped. After cleaning and drying the wafer, patterns of gold markers are produced on the silicon nitride window. After that the different support samples are separated from the wafer by breaking along the predefined scribe lines.

2.2. The production of gold marker patterns on Si_3N_4 windows

The gold marker patterns are produced using a combination of electron beam lithography (EBPG) and metal evaporation (Fig. 1A). First, a 350 nm thick layer of polydi(m)ethyl glutarimide (PMGI, 7% in anisol) and 60 nm polymethyl-methacrylate (PMMA, 950 K, 2% in anisol) was deposited by spin-coating on a 50 nm thick Si_3N_4 membrane (Fig. 1B). To facilitate the removal of both layers by a lift-off process the PMGI layer is etched away under the PMMA layer. Both layers are subsequently prebaked – PMGI at 200 °C for 15 min and the PMMA layer had to harden by baking it at 175 °C for at least 60 min to prevent it later from collapsing after development of the PMGI layer. Then a pattern of dots of two different sizes with diameters of ~30 nm for TEM imaging and ~250 nm for light microscopy and low magnification TEM imaging are written by exposure of the Si_3N_4/PMGI/PMMA to a 100 keV electron beam. The electron beam exposure dose for the small dots was in the range of 40,000 μC/cm^2. The pattern consisted of 10 nm diameter dots with a 500 nm pitch. After electron beam etching and dry etching the exposure the irradiated PMMA is removed by solubilization in 25% methylisobutylketone (MIBK) in isopropylalcohol (IPA). In order to get a good pattern resolution a developer temperature of 5 °C and a development time of 150 s were used. In the next step the PMGI was etched under the PMMA layer using MF-321 developer (Sigma-Aldrich). The etching time was found to be very critical. Too short times resulted in a not completely developed PMGI layer and too long times resulted in the collapse

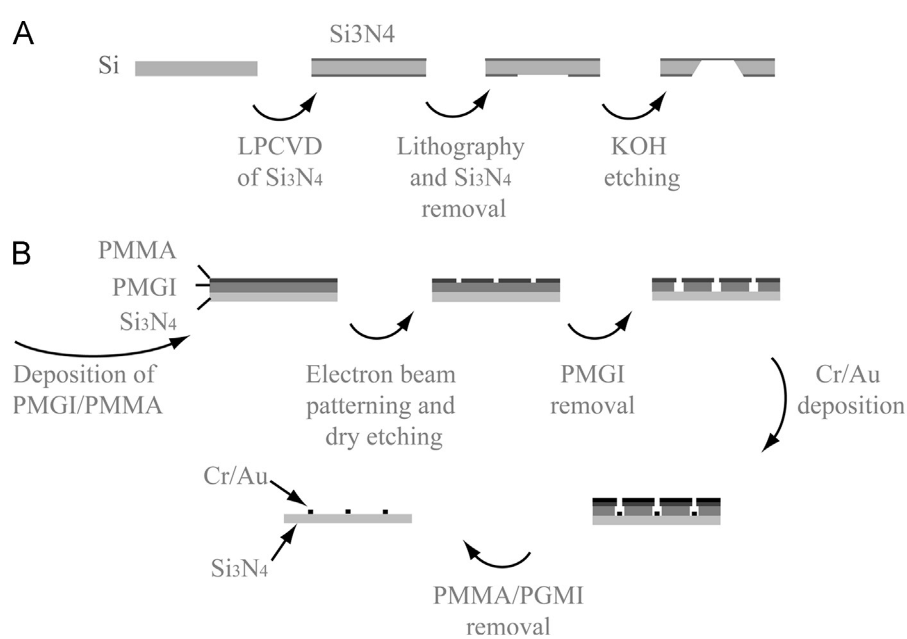

Fig. 1. Schematic fabrication of the production of gold marker patterns on silicon nitride windows on silicon wafers. (A) On a (100)-silicon wafer a 50 nm thick layer of Si_3N_4 is deposited on both sides of the wafer by low pressure chemical vapor deposition (LPCVD). A Si_3N_4 window is created by electron beam lithography and dry etching. The silicon is removed by KOH wet etching. (B) On the 50 nm silicon nitride window a 350 nm layer of PMGI and a 60 nm layer of PMMA are deposited by spin coating. A desired pattern of 10 nm spots is written by an electron beam. The exposed areas of the top PMMA layer are removed. The PMGI underlying the open areas in the PMMA layer is chemically washed away. A 5 nm layer of chrome and a 15 nm gold layer are evaporated (onto the Si_3N_4 window). The PMGI/PMMA with the Cr/Au is removed and the pattern of gold dots on the Si_3N_4 window remains.