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ORIGINAL PAPER

Evaluation of Sensitivity of Fluorescence-Based Asbestos Detection by Correlative Microscopy

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Abstract Fluorescence microscopy (FM) has recently been applied to the detection of airborne asbestos fibers that can cause asbestosis, mesothelioma and lung cancer. In our previous studies, we discovered that the *E. coli* protein DksA specifically binds to the most commonly used type of asbestos, chrysotile. We also demonstrated that fluorescent-labeled DksA enabled far more specific and sensitive detection of airborne asbestos fibers than conventional phase contrast microscopy (PCM). However, the actual diameter of the thinnest asbestos fibers visualized under the FM platform was unclear, as their dimensions were below the resolution of optical microscopy. Here, we used correlative microscopy (scanning electron microscopy [SEM] in combination with FM) to measure the actual diameters of asbestos fibers visualized under the FM platform with fluorescent-labeled DksA as a probe. Our analysis revealed that FM offers sufficient sensitivity to detect chrysotile fibrils as thin as 30–35 nm. We therefore conclude that as an analytical method, FM has the potential to detect all countable asbestos fibers in air samples, thus approaching the sensitivity of SEM. By visualizing thin asbestos fibers at approximately tenfold lower magnifications, FM enables markedly more rapid counting of fibers than SEM. Thus, fluorescence microscopy represents an advanced analytical tool for asbestos detection and monitoring.

Keywords Asbestos detection · Fluorescence · Asbestos-binding protein · Chrysotile · Correlative microscopy · DksA

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Introduction

Asbestos is a generic term for several types of naturally occurring fibrous silicate minerals that have been widely used in construction materials because of their chemical and thermal stability [1, 2]. While most developed countries have banned the use of asbestos, residual contamination remains a widespread problem, with airborne asbestos fibers contributing to the increasing incidence of lung cancer and mesothelioma [3, 4]. Continued monitoring of airborne asbestos levels is thus essential to limit or avoid adverse health effects.

For determination of airborne concentrations of asbestos fibers, air is typically filtered through a membrane filter, which is then cleared with acetone vapor, and the fibers are counted using an optical phase contrast microscopy (PCM) [5]. Although simple and cheap, PCM has two major limitations: it is unable to detect asbestos fibers that are thinner than approximately 0.25 μm, which are abundant in chrysotile asbestos, and it cannot distinguish asbestos fibers from natural or man-made fibers of similar dimensions [6]. However, some epidemiological studies have suggested that both lung cancer and asbestosis are most strongly associ-

ated with exposure to thin fibers (<0.25 μm) [7], while mesothelioma is most closely associated with exposure to fibers thinner than approximately 0.1 μm [8]. PCM analysis may seriously underestimate the number of such fibers present in air samples, and may also lead to overestimations of asbestos exposure due to its inability to distinguish asbestos from non-asbestos fibers. Due to these limitations, the National Institute for Occupational Safety and Health has made the development of improved analytical methods for asbestos fibers a strategic research goal [6].

The simplest way to achieve the necessary detection sensitivity for thin asbestos fibers is by using scanning electron microscopy (SEM). SEM could be also used to determine the elemental composition of fibers by energy dispersive x-ray analysis (EDX), making it possible to distinguish asbestos from non-asbestos fibers. However, the primary goal of asbestos monitoring is not simply to detect the presence of fibers, but rather to estimate total fiber loads, which requires the counting of a sufficient number of fibers across a large area of the filter membrane. As the sensitivity of SEM is achieved through the use of much higher magnifications than those used for PCM, examination of a large filter area using SEM is a tedious and time-consuming task. Therefore, electron microscopy is generally used as a supplementary method to confirm asbestos presence and fiber identity, and determine the percentage of asbestos fibers in total PCM fiber counts [6].

Fluorescence microscopy (FM) is one of the most important analytical tools in modern life sciences. The sensitivity and sophistication of FM is continually improving, with single-molecule fluorescence imaging now being widely used in a number of specialized applications [9]. Therefore, an FM-based asbestos detection method could be expected to offer higher sensitivity than PCM analysis. However, most fluorescent dyes are not capable of specifically binding to inorganic materials. In our previous studies, we discovered that the *E. coli* protein DksA specifically binds to the most commonly used type of asbestos, chrysotile [10], and demonstrated that fluorescent-labeled DksA enables far more specific and sensitive detection of airborne asbestos fibers than conventional PCM [11]. In addition, parallel counting of fibers by FM and PCM indicated that the former is able to detect several times as many chrysotile fibers as the latter in an identical sample [11]. However, it was not possible to estimate the actual gain in sensitivity using this indirect approach. To directly measure the diameter of the thinnest fibers visible under FM, it is necessary to observe the same area on the membrane filter using both FM and SEM. The best tool for this task is correlative microscopy, which allows a region of interest identified by FM to be examined using the markedly higher resolution offered by the SEM platform, by means of a specially designed sample holder, as well as a motorized stage and automated calibration [12]. FM capabilities can thus be supplemented by high-resolution morphological examination and/or elemental analysis of fibers by EDX.

In this study, we used correlative microscopy to determine whether FM can achieve sufficient sensitivity to detect all countable asbestos fibers irrespective of their diameter. Chrysotile asbestos is known to produce the thinnest airborne fibers, reportedly in the range of 20–40 nm [13], and was therefore selected to evaluate the sensitivity of FM.

Materials and Methods

Materials Chrysotile (JAWE 111), amosite (JAWE 231), and crocidolite (JAWE 331) asbestos were obtained from the Japan Association for Working Environment Measurement (Tokyo, Japan). Glass wool, rock wool, micro glass fibers, refractory fibers, potassium titanium whiskers, silicon carbide whiskers, titanium oxide whiskers, and wollastonite standard reference samples were provided by the Japan Fibrous Material Research Association (Kanagawa, Japan). Membrane filters were purchased from Millipore Co. (Bedford, MA). Cy3 dye (Ex. 550 nm; Em. 570 nm) was purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). All other reagents were purchased from Wako Chemicals (Osaka, Japan) or Sigma (St. Louis, MO) and were of the highest available quality.

Preparation of Fluorescent Probes DksA was conjugated with Cy3 fluorescent dye according to the manufacturer's instructions to a final dye (D)/protein (P) ratio of 0.98. Excitation and emission peaks of DksA-Cy3 were 555 nm and 565 nm, respectively. Fluorescence properties of the conjugated Cy3 dye were measured using a spectrofluorometer (FP-6500, Jasco, Tokyo, Japan).

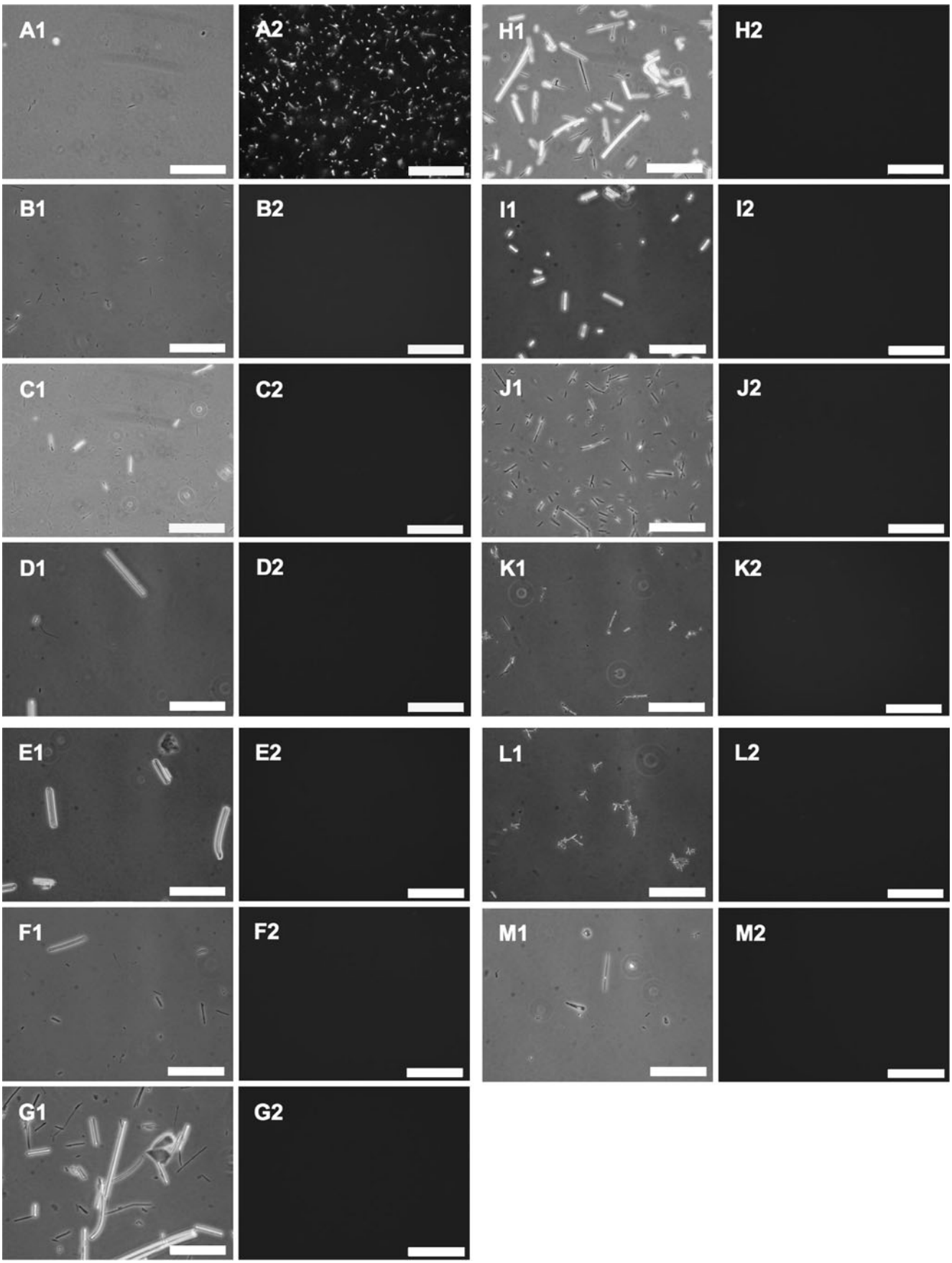
Determination of Binding Specificity of DksA-Cy3 Chrysotile, five kinds of amphibole asbestos, glass wool, rock wool, micro glass fibers, three kinds of refractory fibers, potassium titanium whiskers, silicon carbide whiskers, titanium oxide whiskers and wollastonite were dispersed by sonication in deionized water at a concentration of 1 mg/mL. Each sample was appropriately diluted with deionized water and filtered under reduced pressure through a nitrocellulose filter (pore size 0.8 μm; Millipore Co., Billerica, MA). The use of 0.8-μm pore size nitrocellulose filters for the PCM asbestos test is specified in the Asbestos Monitoring Manual published by the Japanese Ministry of the Environment [14]. In the United States, the NIOSH 7400 method also recommends

using such filters for personal sampling [5]. The filters were air-dried for 24 h and cut in four wedges of equal size. To reduce non-specific adsorption of the protein probe on the surface of the nitrocellulose filter, one wedge of each filter was prewetted with 40 μL of assay buffer (0.1 M sodium carbonate buffer [pH 9.5], 1% Tween80, and 1% polyethyleneimine). After prewetting, 100 μL of assay buffer containing 100 nM DksA-Cy3 was applied to each filter wedge, followed by washing with 20 μL of washing buffer (0.1 M sodium carbonate buffer [pH 9.5] and 1% Tween80). During the prewetting, binding and washing steps, the wedges were placed on a paper sheet (Whatman 3MM, Kent, UK) to remove excess liquid via capillary action. The samples were then observed under a fluorescence microscope (Olympus BX60, Tokyo, Japan) equipped with phase contrast optics. Fluorescence micrographs were obtained using an Olympus U-MNG filter (Ex: 530–550 nm, DM: 570 nm, Em: >590 nm) and a 40× objective lens (Olympus UplanFl N 40×/0.75 NA Ph2). For phase contrast observation, one filter wedge of each sample was cleared with acetone vapor according to the NIOSH 7400 method [5]. Phase contrast micrographs were obtained using the same 40x objective lens used for FM. Images were captured using a cooled charge-coupled device camera (Olympus DP70).

Modifications of the FM Asbestos Assay Method To measure the diameter of the thinnest chrysotile fibers visible with FM and to enable direct comparison of FM and SEM sensitivity for the same filter sample, it was necessary to considerably modify our regular FM asbestos assay method. The most important modification was the change of the filter material from nitrocellulose to polycarbonate. The nitrocellulose filters could be rendered transparent for PCM examination, and are therefore routinely used for collecting airborne asbestos fibers. However, they have an uneven, fibrous texture, making it nearly impossible to find thin chrysotile fibers by SEM. In contrast, polycarbonate filters have a flat, smooth, glass-like surface, and are therefore used for SEM analysis of asbestos in Japan [14] and worldwide (ISO 14966 method, [15]). The filter pore size was also changed from 0.8 to 0.4 μm due to greater potential for thin fibers to pass through the straight pores of track-etched polycarbonate filters as compared to the tortuous-path pores of nitrocellulose filters. As polycarbonate filters have much lower protein adsorption than that of nitrocellulose filters, we could omit filter prewetting in the modified staining procedure for the correlative fluorescence and electron microscopy. On the other hand, the surface of polycarbonate filters is relatively hydrophobic, necessitating the use of negative pressure during both staining and washing steps.

Another modification to the assay method involved altering the composition of the buffer used for probe binding and washing. Our regular assay buffer contains Tween80 surfactant and polyethyleneimine polymer, which reduce nonspecific adsorption of the protein probe on the surface of the nitrocellulose filter. The drying of the buffer was found to leave various residues on the polycarbonate filter surface, which interfered with SEM analysis. To remove this source of interference, we selected deionized water for the final filter washing step. However, polyethyleneimine in the assay buffer could not be washed off the filter surface, likely due to its strong binding to the negatively charged filter material. Since polyethyleneimine is a high-molecular weight polymer, it could potentially obscure thin chrysotile fibers during SEM imaging, and was therefore excluded from the modified assay buffer. Due to the inherently low protein adsorption on the surface of polycarbonate filters, this modification did not affect fiber visibility under a fluorescence microscope. We also confirmed that all modifications of our regular assay method did not influence the degree of chrysotile staining.

Correlative Microscopy Analysis of Chrysotile Chrysotile was dispersed in isopropanol by sonication at a concentration of 1 mg/mL. Serial dilutions with isopropanol were then prepared, and 100 μL of the diluted dispersion was mixed with 900 μL of deionized water (final chrysotile concentration of 1 μg/mL) and filtered under reduced pressure through a polycarbonate filter (pore size 0.4 μm; Millipore Co.). The filter was air-dried for 24 h and then cut into four wedges of equal size. Staining was conducted by applying 100 μL of assay buffer (0.1 M sodium carbonate buffer [pH 9.5] and 1% Tween80) containing 100 nM DksA-Cy3 and subsequently washing the filter wedge with 20 μL of deionized water. Both staining and washing were performed under negative pressure. The sample was attached to the correlative microscopy stage (Carl Zeiss, Gottingen, Germany) with an adhesive carbon tape and observed under a fluorescence microscope (AxioImager M2, Carl Zeiss) equipped with a 43 HE DsRed filter (Ex: 537.5–562.5 nm, DM: 570 nm, Em: 570–640 nm, Carl Zeiss) and 50x objective lens (EC Epiplan-Apochromat 50×/0.95 HD DIC, Carl Zeiss). Images were captured using a cooled charge-coupled device camera (AxioCam MR3, Carl Zeiss) with a 281-ms exposure. Following the FM observation, the stage was directly transferred to a Field Emission SEM microscope (Ultra Plus, Carl Zeiss) operated at 5 kV for high-magnification analysis. EDX analysis was conducted using an INCAPentaFET-x3 Si(Li) detector (Oxford Instruments, Abingdon, Oxfordshire, UK).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◀ **Fig. 1** Phase contrast (1) and fluorescence (2) micrographs of different fiber types. **a** chrysotile; **b** amosite; **c** crocidolite; **d** glass wool; **e** rock wool; **f** micro glass fibers; **g** and **h** amorphous refractory fibers; **i** crystalline refractory fibers; **j** potassium titanium whiskers; **k** silicon carbide whiskers; **l** titanium oxide whiskers; and **m** wollastonite. Bar, 50 μm

Results and Discussion

The probe selected for evaluating the sensitivity of fluorescence-based asbestos detection was Cy3-labeled DksA protein (DksA-Cy3), which was previously found to bind to chrysotile with the dissociation constant (Kd) of approximately 3.5 nM by Scatchard analysis [10]. To ensure that the sensitivity of asbestos detection was not achieved at the expense of selectivity, we tested the DksA-Cy3 probe for non-specific binding to other types of asbestos, as well as standard reference samples of non-asbestos fibrous minerals provided by the Japan Fibrous Material Research Association [16]. While the reference samples certainly do not cover the full variety of natural and man-made fibrous materials, they could provide a useful indication of the probe selectivity. We found that DksA-Cy3 bound to chrysotile, but neither amphibole asbestos nor the other examined mineral fibers (Fig. 1). Fiber diameter did not affect the selectivity of the DksA-Cy3 probe. As proteins are typically 1–3 orders of magnitude smaller than the diameter of airborne fibers, binding selectivity probably depends on some properties of the fiber surface, rather than "recognition" of fiber size or shape.

A polycarbonate filter with a high chrysotile fiber load was used to evaluate fiber visibility under correlative FM and SEM microscopic examination (Fig. 2). The filter was first examined under FM (Fig. 2a) and a filter region containing thin fibers (indicated by the white rectangle in Fig. 2a) was then identified and further analyzed under SEM at both low and high magnification (Fig. 2c and e). To facilitate comparison of the fiber visibility between the SEM images and the original FM image, we digitally magnified the corresponding filter region of the FM image (Fig. 2b and d). A clear difference in the visibility of thin fibers was observed at lower magnifications, with the FM platform offering lower resolution, but superior fiber visibility. In our analyses, SEM at magnifications below× 4,000 did not provide reliable identification of the thinnest chrysotile fibers present on the filter samples (Fig. 2e). This finding means that approximately 100 SEM micrographs are required to cover a single FM or PCM field of view (we generally use×400 magnification for FM observation) during sample analysis. This limits SEM's usefulness for routine airborne fiber counting, which involves examination of a large number of graticule fields.

The apparent diameter of the thinnest fibers (single fibrils) of chrysotile in our sample, as estimated by SEM under×28,800 magnification, was approximately 30–35 nm (Fig. 3). These fibers were also visible on the original FM micrograph, indicating that FM can provide sufficient sensitivity to detect single chrysotile fibrils and produce asbestos counts that are equivalent to those of SEM. Our results demonstrate that conventional fluorescence micros-

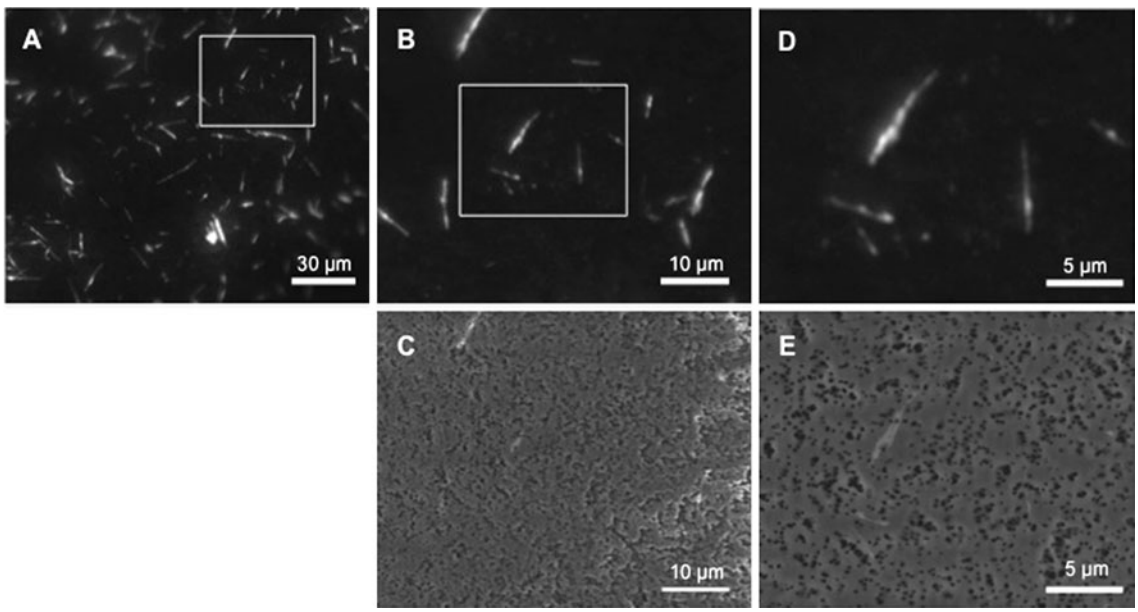

Fig. 2 Correlative microscopic analysis of a chrysotile asbestos sample. **a** Original FM micrograph at×500 magnification. **b** White rectangular area in (**a**), rescaled to correspond to the SEM micrograph (**c**, ×1,800). **d** White rectangular area in (**b**), rescaled to correspond to the SEM micrograph (**e**, ×4,000). Image sizes and resolution were adjusted in Adobe Photoshop

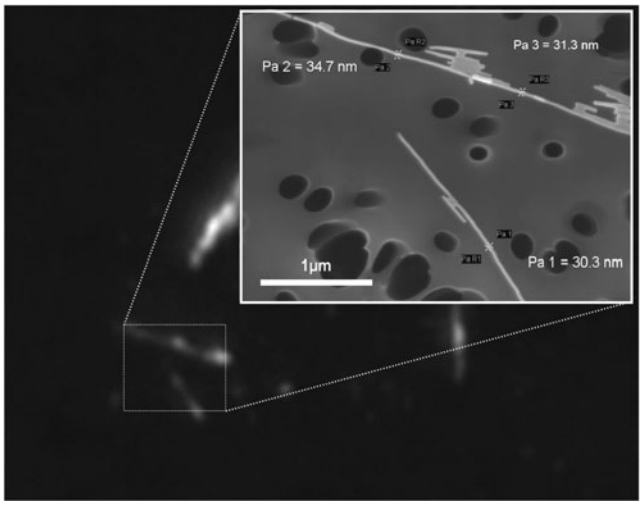

Fig. 3 Estimation of the diameter of the chrysotile fibrils using high-magnification (×28,800) SEM. The estimated diameter of the fibrils (30–35 nm) appeared quite uniform in the SEM micrograph (inset) that is overlaid on the enlarged area of the original FM micrograph

copy can offer both the SEM-level sensitivity and the convenience of a wide field of view. In addition, because several protein probes can be used to stain different asbestos fiber types, our method is not limited to chrysotile. We previously identified a bacterial protein (GatZ) that selectively binds to the remaining five types of asbestos which belong to the amphibole mineral group [11]. Thus, a combination of fluorescently labeled DksA and GatZ can be used to detect all asbestos types, and to distinguish between chrysotile (serpentine mineral group) and more toxic amphibole asbestos [11].

DksA-Cy3 binding tests using three types of asbestos and ten kinds of typical non-asbestos fibrous materials (Fig. 1) indicated very high selectivity of the probe for chrysotile asbestos. The materials tested included nearly all of the asbestos substitutes commonly used in the construction industry, as well as several industrially important fiber types. DksA did not bind to any of the examined fibers except chrysotile, implying near-zero false positive rate for actual air samples. The reliable identification of asbestos is a considerable improvement over the PCM method, which is incapable of fiber identification. The level of selectivity observed in our method would be more than sufficient for most applications that currently rely on PCM, such as the initial testing for the presence of asbestos or the ongoing monitoring of asbestos contamination from known sources. For such applications, FM is capable of much faster chrysotile detection and counting than SEM, while offering comparable sensitivity at the fraction of SEM cost. However, a few limitations of asbestos detection with FM prevent this approach from fully replacing electron microscopy-based methods. For example, our method does not provide sufficient specificity to distinguish between the different types of amphibole asbestos [11]. In addition, it is not practically feasible to test all of the existing and newly developed fibrous materials for non-specific binding of the DksA-Cy3 probe to completely exclude the possibility of false positives.

In cases where definitive fiber identification is required, it would be desirable to combine the capabilities of FM and electron microscopy platforms to enable sensitive and convenient identification and counting of asbestos fibers. For this purpose, correlative microscopy has a clear advantage due to the possibility of elemental analysis using X-ray spectroscopy (EDX). Following the initial examination of our sample under FM, the fibers were further analyzed using EDX under SEM (Fig. 4). The observed X-ray peaks indicated the presence of Mg, Si, and O, and are consistent with the composition of chrysotile fibers. Staining of the fibers with the fluorescent probe did not interfere with EDX analysis. Correlative microscopy can thus combine rapid fiber counting by FM with definite fiber identification by SEM, and represents an advanced analytical tool for asbestos detection and monitoring.

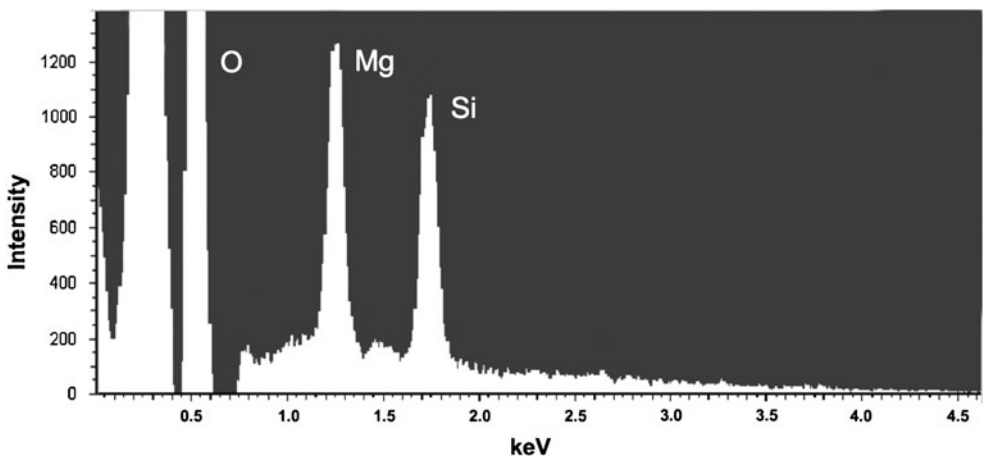

Fig. 4 EDX analysis of the sample fibers. Peaks of Mg, Si, and O are shown

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