HHS Public Access

Author manuscript

Ann Occup Hyg. Author manuscript; available in PMC 2016 January 11.

Published in final edited form as:

Ann Occup Hyg. 2015 June ; 59(5): 616–628. doi:10.1093/annhyg/mev007.

Evaluation of the Dark-Medium Objective Lens in Counting Asbestos Fibers by Phase-Contrast Microscopy

Eun Gyung Lee[1,*], **John H. Nelson**[1], **Michael L. Kashon**[2], and **Martin Harper**[1]

[1] Exposure Assessment Branch, National Institute for Occupational Safety and Health, Health Effects Laboratory Division, 1095 Willowdale Road, Morgantown, WV 26505, USA

[2] Biostatistics and Epidemiology Branch, National Institute for Occupational Safety and Health, Health Effects Laboratory Division, 1095 Willowdale Road, Morgantown, WV 26505, USA

Abstract

A Japanese round-robin study revealed that analysts who used a dark-medium (DM) objective lens reported higher fiber counts from American Industrial Hygiene Association (AIHA) Proficiency Analytical Testing (PAT) chrysotile samples than those using a standard objective lens, but the cause of this difference was not investigated at that time. The purpose of this study is to determine any major source of this difference by performing two sets of round-robin studies. For the first round-robin study, 15 AIHA PAT samples (five each of chrysotile and amosite generated by water-suspended method, and five chrysotile generated by aerosolization method) were prepared with relocatable cover slips and examined by nine laboratories. A second round-robin study was then performed with six chrysotile field sample slides by six out of nine laboratories who participated in the first round-robin study. In addition, two phase-shift test slides to check analysts' visibility and an eight-form diatom test plate to compare resolution between the two objectives were examined. For the AIHA PAT chrysotile reference slides, use of the DM objective resulted in consistently higher fiber counts (1.45 times for all data) than the standard objective (*P*-value < 0.05), regardless of the filter generation (water-suspension or aerosol) method. For the AIHA PAT amosite reference and chrysotile field sample slides, the fiber counts between the two objectives were not significantly different. No statistically significant differences were observed in the visibility of blocks of the test slides between the two objectives. Also, the DM and standard objectives showed no pattern of differences in viewing the fine lines and/or dots of each species images on the eight-form diatom test plate. Among various potential factors that might affect the analysts' performance of fiber counts, this study supports the greater contrast caused by the different phase plate absorptions as the main cause of high counts for the AIHA PAT chrysotile slides using the DM objective. The comparison of fiber count ratios (DM/standard) between the AIHA PAT chrysotile samples and chrysotile field samples indicates that there is a fraction of fibers in the PAT samples approaching the theoretical limit of visibility of the phase-contrast microscope with 3-degree phase-shift. These fibers become more clearly visible through the greater contrast from the phase plate absorption of the DM objective. However, as such fibers are

[*]Author to whom correspondence should be addressed. Tel: +1-304-285-6146; fax: +1-304-285-6041; dtq5@cdc.gov.

DISCLAIMER

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Mention of commercial product should not be construed to imply endorsement.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

not present in field samples, no difference in counts between the two objectives was observed in this study. The DM objective, therefore, could be allowed for routine fiber counting as it will maintain continuity with risk assessments based on earlier phase-contrast microscopy fiber counts from field samples. Published standard methods would need to be modified to allow a higher aperture specification for the objective.

Keywords

amosite; asbestos fiber; chrysotile; dark-medium (DM) objective lens; eight-form diatom test plate; HSE/NPL Mark II test slide; HSL/ULO Mark III test slide; NIOSH 7200 method; phase-contrast microscopy

INTRODUCTION

The current standard method to measure airborne asbestos fiber concentrations is to collect air samples by drawing air through a membrane filter on which the asbestos fibers are collected. A portion of the filter is then cleared and examined under a phase-contrast microscope, and the number of visible fibers meeting certain dimensional criteria is recorded (ISO, 1993; NIOSH, 1994; IRSST, 1995; OSHA, 1998; WHO, 1997; HSE, 2006). Resolution is defined as ‘the minimum separation of parallel lines or adjacent points in a given subject that can be made visible as separate lines or points in the image under actual condition’ (Van Duijn, 1957) and is related to the numerical aperture (NA) of the objective, i.e. the higher the NA, the smaller the separation that can be detected and the better the resolution, and condenser. Thus, two microscopes correctly setup with the same NA and condenser will have identical resolution.

The ‘apparent’ resolution (i.e. the ability of the analyst to distinguish parallel lines that should be separately resolvable), however, can be dependent upon other factors as well, such as conditions of illumination, contrast between a specimen and mounting medium, quality of the human eye, and setup of the microscope. Therefore, the published standard methods for fiber counting include procedures to standardize setup (e.g. microscope alignment and graticule calibration), and the other factors are tested through application of a phase-shift test slide. Phase shift is given by

$$\phi\ (^\circ)=\frac{(n_1-n_2)\cdot d\cdot 360}{\lambda}$$

where φ is the phase shift (either positive or negative, degrees), n_1 is the refractive index (RI) of the test material, n_2 is the RI of the mounting medium, d is the thickness of transparent object (micrometer), and λ is the wavelength of the irradiating illumination (micrometer). The phase-shift test slides (references) have been created to provide a bracket of visibility around the 3-degree phase-shift agreed for standard use in fiber-counting methods. It is necessary to use these slides to calibrate performance because the small difference in RI between chrysotile and the mounting medium, coupled with relatively thin fibers, means that a proportion of the fibers could be very difficult to see. Because the risk

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

assessment is based on fiber counts by this technique, all analysts must be able to see the same proportion of fibers in a sample. (It is a lesser problem for amphibole asbestos, where the RI contrast is greater and the fibers are typically wider.) The phase-shift test slides contain seven blocks of grooves, where in each successive block the grooves become more difficult to determine by eye. Some blocks should be fully visible, some partially, and some not at all. Table 1 shows the requirements for phase-shift detection limit by various national and international standards. Although these slides do calibrate the ability of the analyst to determine blocks of parallel lines, not all analysts will see exactly the same width of fiber, as other factors, especially the degree of contrast and the ability of the eye to detect that contrast, are important. Rooker *et al.* (1982) showed that under ideal conditions chrysotile fibers with a width of 0.15 μm should be visible under a 546-nm green light with a 3-degree phase-shift in typical mounting media. The width limits published in standard methods (typically 0.2 or 0.25 μm) are consensus values based on what an average analyst might be able to see. These widths are not limits below which fibers should not be reported, even if these values are used that way in electron microscopy methods in an effort to maintain comparability with risk assessments derived from phase-contrast microscopy (PCM).

Pang and Harper (2008) performed a study to determine the quality of asbestos fiber counting using volunteer laboratories and analysts participating in the American Industrial Hygiene Association (AIHA) asbestos Proficiency Analytical Testing (PAT) program or the Asbestos Analysts Registry proficiency testing program. A total of 47 amosite and 33 chrysotile slides were prepared using the dimethylformamide/Euparal technique and relocatable cover slips. The filters were purchased from the AIHA's PAT program. Prior to circulating the slides, 'verified fibers' were determined by two experienced analysts and were considered as a 'true' value. [The 'trueness' of these values having first been evaluated in a prior study by Harper and Bartolucci (2003).] Fiber-counting errors were classified into four categories—sizing, oversight, identification, and recording—and for each category, the number of extra or missing fiber counts was recorded. The results showed that the highest error was from oversight by missing the chrysotile samples and from extra sizing [i.e. counting a shorter (<5 μm length) fiber] for the amosite samples. A subsequent study by Harper *et al.* (2009) showed performance improved during training when analysts were shown what they should be reporting on one part of the slide before asking them to count a different portion of the same slide; however, the highest errors were still found in the oversight category for chrysotile. Although all analysts followed the appropriate microscope setup and phase-shift test slide calibration, results clearly showed that performance was widely variable, with some analysts able to see almost all of the verified chrysotile fibers and some seeing none at all.

In November 2007, a Japanese group reported their results for a similar study at a meeting of the International Organization for Standardization (ISO)/Technical Committee (TC) 146 (Air Quality)/Subcommittee (SC) 2 (Workplace Atmosphere)/Working Group (WG) 5 (Inorganic Fibers). A total of 60 analysts from 30 local government laboratories in Japan participated in this test. Prior to the circulation of the reference slides, a meeting was held to ensure consistency of methodology among the analysts. An interesting result was found during this round-robin test. Six of the participants returned significant errors in the category

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

of additional chrysotile fibers. A further investigation revealed that the analysts who used a dark-medium (DM) objective lens were able to see more chrysotile fibers than those with a standard objective lens [either dark-light (DL) or dark-lower contrast (DLL)]. The background is light gray for the DL and DLL objective lenses and medium gray for the DM objective lens. Generally, the DLL is more widely used than other objective lenses. The DL objective is used for examining cells and other semitransparent living material, whereas the DM objective is recommended for examining fine fibers or particles (Olympus America Inc., 2013). The results by the Japanese group suggest that an analyst using a microscope equipped with the DM objective lens can visualize thinner chrysotile fibers than one with a standard objective.

As shown in Table 1, current national and international standards only specify the range of NA without comment on the type of objective lens (e.g. plan fluorite, plan apochromatic). The NA of the DM objective used in the Japanese round-robin test is 0.95 for a NIKON 55i microscope, greater than the recommended NA in Table 1. In addition, the DM objective has a higher absorption of the phase plate (~30% more absorption) than the DLL and thus yields higher contrast (NIKON microscope manufacturer, personal communication). It is expected that the higher NA and/or greater absorption of the phase plate of the DM objective might be the reason for seeing a greater number of fibers. Since the Japanese report at the ISO TC 146/SC 2/WG 5 meeting, however, no further information determining major factors causing the differences of fiber counts between the two objectives have been reported (H. Kosaka, personal communication).

Therefore, the purpose of this study was to determine the principle source (e.g. resolution, contrast, or combined effect of resolution and contrast) of the differences of fiber counts between two objective lens types by performing two sets of round-robins studies. Of course, detecting fibers in practice is not solely dependent upon the resolution of the microscope and/or the contrast. Other aspects, including those specific to the analyst (e.g. visual acuity, care in searching for fibers) could have a greater impact. In this study, we controlled these potential factors to focus on two factors—resolution and contrast.

METHODS

First round-robin study

Ten chrysotile and five amosite reference filters purchased from the AIHA PAT program were used in this study. Among these filters, five chrysotile samples were generated by aerosolizing chrysotile fibers in a chamber, and the remaining samples (i.e. five each of chrysotile and amosite) were generated by suspending fibers in water. A portion of each reference filter was cleared using a mixture of dimethylformamide (35% v/v), glacial acetic acid (15% v/v), and distilled water (50% v/v) and mounted with a synthetic form of Euparal (BioQuip Products, Rancho Dominguez, CA, USA). Although triacetin is a recommended mounting medium in national and international standards, Euparal was used to prevent fibers' movements, which could occur if an excessive amount of triacetin (>3.5 μl) is used. Euparal (Ogden *et al.*, 1986; Shenton-Taylor and Ogden, 1986) has been shown to be comparable to triacetin with respect to the visibility of mounted fibers (Lee *et al.*, 2011). A special cover slip imprinted with a relocatable grid was used to visit the same opening areas

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

by all analysts. Lee *et al.* (2010) provides a detailed description of sample preparation and relocatable cover slip. Once all reference sample slides were prepared, a National Institute for Occupational Safety and Health (NIOSH) analyst who did not participate in the round-robin study pre-examined each sample slide to determine the field opening areas to be examined. The allocated number of field opening areas ranged from 20 to 100 depending on fiber densities.

Nine laboratories voluntarily participated in this study. Each laboratory received 15 reference slides, two types of test slides [HSE/NPL Mark II and HSL/ULO Mark III 'green certificate' (GREEN)], instruction sheets, data logs, and a NIKON 55i microscope (Nikon Instruments Inc., Melville, NY, USA). The NIKON 55i microscope was equipped with a DLL objective (Plan Fluorite, ×40/NA 0.75), a DM objective (Plan Apochromatic, ×40/NA 0.95), and two oculars (magnification ×10 and ×12.5). The same microscope was circulated to eliminate variations between microscopes. All participants were asked to examine the same slides with the DLL objective and the DM objective lenses on different days. The NIOSH 7400 counting 'A' rules (i.e. all fibers longer than 5 μm and an aspect ratio   3:1) plus the fiber width < 3 μm were applied. Although it was expected that the AIHA reference slides might not include high number of fibers   3 μm, fiber width was limited to <3 μm as some standard methods (e.g. WHO and HSE HSG248) include this criterion.

In addition, each analyst examined the phase-contrast detection limit with the HSE/NPL Mark II test slide and the HSL/ULO Mark III GREEN test slide. Both test slides contain seven blocks of grooved lines (20 grooved lines per block) in descending order of visibility. Currently, the HSE/NPL Mark II test slide, developed by the Health and Safety Laboratory (HSL) in the UK, is no longer available and has been replaced by the HSL/ULO Mark III test slide. Based on the visibility of blocks, the new HSL/ULO Mark III test slide provides different test certificates. The 'red certificate', intended to be exactly equivalent to the HSE/NPL Mark II test slide, was not available for purchase at the time of this study. The GREEN slide is intended to have Block 5 fully visible and Block 6 partially visible. Crane and Harper (2011) showed that the GREEN slide also tests the correct degree of phase-separation required when performance meets the requirement given in the certificates. Each analyst was asked to record one of three options—clearly visible, partially visible, and invisible—for each block on each slide. A full factorial combination of two objective lens types (DM and DLL), two ocular magnifications (×10 and ×12.5), and two test slides (HSE/NPL Mark II and HSL/ULO Mark III GREEN) were tested on the same day. Each analyst was asked to perform three mandatory tests of each full factorial combination of testing parameters on different days and two optional tests.

At the end of the round-robin study, all AIHA PAT sample slides were sent to two experienced analysts who verified the true values of the AIHA PAT chrysotile and amosite samples of previous studies (Pang and Harper, 2008; Harper *et al.*, 2009) to determine 'verified fibers' in this study. Fiber densities based on the verified fibers are reported in Results.

Second round-robin study

After the first round-robin study, a second round-robin study was performed with chrysotile field samples. The location and type of workplace are unknown because these were leftover samples collected many years ago for another project. Six chrysotile sample slides were prepared in the same way as in the first round-robin study. For fiber examination, six out of nine laboratories who participated in the first round-robin study sent analysts to visit our facility to examine fibers with the same NIKON microscope used in the first round-robin study. The same counting rules were applied. The same analysts who examined the AIHA PAT samples determined the numbers of 'verified fibers' in these samples. Fiber densities based on the verified fibers are reported in Results.

Examination of an 8-form diatom test plate

After the first and second round-robin studies, we purchased an 8-form diatom test plate (Microlife Services, Somerset, England), including eight species, to compare the objectives' resolutions. Eight analysts (four from in-house and four from laboratories who participated in both round-robin studies) were asked to examine each species with the DM and DLL objective lens and to indicate if he/she was able to view the minute lines and/or dots of each species images. At the end of examination, each analyst was also asked which objective is his/her preference regardless of resolution.

Transmission electron microscopy analysis

After making the AIHA PAT reference sample slides for the first round-robin study, the leftover AIHA PAT chrysotile filters were sent to the NIOSH contract laboratory for the transmission electron microscopy (TEM) analyses to determine if fiber widths prepared using different-generation methods were similar. The TEM specimen grids from the filters were prepared according to the NIOSH 7402 method (i.e. direct-transfer method). Seven chrysotile reference filters (four filters generated by aerosolization method and three filters generated by water-suspension method) were used. The contract lab was asked to record the dimensions of fibers that met the NIOSH 7400 counting 'A' rules plus the fiber width 0.15 and <3 μm.

Statistical data analysis

The analysis of fiber counts was performed using SAS/STAT software, Version 9.3 of the SAS system for Windows (SAS Institute, Cary, NC, USA). We used PROC MIXED to run a two-way factorial analysis of variance (objective lens type by fiber type) with laboratory treated as a random variable. All data were transformed using the square root function to meet the assumptions of the analysis (Poisson distribution rather than normal distribution). Outliers were established using the Mahalanobis distance metric. In this study, only test results with all data were presented because statistical conclusions with and without outliers were the same. The data of the phase-shift test slides were analyzed using contingency tables and Fisher exact test. Separate analyses were performed to compare objectives, oculars, and test slides. All differences were considered signifi-cant at $P < 0.05$ with a 95% level of confidence.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

RESULTS

Comparison of fiber counts between the DM and DLL objective lenses

Figure 1 shows the results of fiber counts including all data by each laboratory. Note that fiber examination by Lab F was incomplete for the AIHA PAT chrysotile reference slides (i.e. Lab F only counted three each chrysotile_water and chrysotile_aero reference slides). In general, Lab G showed considerably lower fiber counts for the PAT chrysotile fibers, compared with the other laboratories, regardless of the type of objective lens and filter generation method. The majority of data was above the diagonal (1:1) line for the PAT chrysotile_water and _aero samples, indicating higher fiber counts with the DM. On the other hand, the fiber counts using the DM objective were not noticeably different compared with those using the DLL objective for the AIHA PAT amosite _water and chrysotile field samples (i.e. close to the 1:1 line).

Table 2 shows a summary of fiber count ratios between the DM and DLL objectives along with the statistical test results. The median ratios of fiber counts (DM/DLL) were greater for the AIHA PAT chrysotile_water and _aero samples than for the AIHA PAT amosite _water and chrysotile field samples. For both types of AIHA PAT chrysotile samples, all labs except for Lab D showed median ratios of DM/DLL > 1.0. An exception is the results from Lab D, where the median ratio is less than but close to 1.0 (0.96 for the chrysotile_water samples and 0.95 for the chrysotile_aero samples). The AIHA PAT amosite _water and chrysotile field samples showed less variation in fiber count ratios (DM/DLL) across the participating labs than the AIHA PAT chrysotile_water and _aero samples. The relative standard deviation (RSD) of individual samples across the labs ranged from 0.16 to 0.59 for the AIHA PAT chrysotile_water and _aero samples, whereas the RSDs were <0.3 for all AIHA PAT amosite_water and chrysotile field samples. For the AIHA PAT chrysotile samples, regardless of filter generation methods, statistically significant differences in fiber counts between two objectives were observed (all P-values < 0.05), and the estimates using the DM objective were always higher than those using the DLL objective (Table 2). For the amosite_water and chrysotile field samples, the fiber counts were not significantly different (P-values > 0.05) between the two objectives.

Table 3 shows the median values of the fiber counts divided by ‘verified fibers’, which indicate that, overall, fiber counts of the PAT chrysotile_water and _aero samples were less than those of the verified fiber counts regardless of the objective type, whereas fiber counts of the PAT amosite_water and chrysotile field samples were similar. Also, higher variation was observed from the PAT chrysotile_water and _aero samples than the other type of fibers. Figure 2 shows that the range of fiber densities of the chrysotile field samples was markedly narrower (i.e. <250 fibers mm^{-2}) than those of the AIHA PAT samples.

Test slides examination

Table 4 shows the results of visibility using the Fisher exact test. Because Blocks 1 and 2 were clearly visible and Block 7 was invisible to all laboratories, only Blocks 3, 4, 5, and 6 were considered for the analysis. Also, the results of Lab I were excluded due to a misunderstanding of the guidance provided. Comparisons of lens types (DM versus DLL)

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

and optical magnification (×10 versus ×12.5) did not show statistical differences of block visibility for all test conditions (all P-values > 0.05). On the other hand, significant differences were observed from the comparison of two test slides (Mark II versus Mark III Green) except for the 3-day results under the condition of ×12.5/DM objective, which is as expected because the certificates of block visibility are different. There are no statistical differences between days (3 day versus all).

TEM results

The number of fibers examined by TEM was 827 for the AIHA PAT chrysotile_water samples and 635 for the AIHA PAT chrysotile_aero samples. The fiber width distribution for the PAT chrysotile fibers in the range of 0.15–3.0 μm was similar for samples generated by aerosolization and water-suspension. For the AIHA PAT chrysotile_water samples, the average fiber length was 10.7 μm and width was 0.181 μm. For the AIHA PAT chrysotile_aero samples, the average fiber length was 8.91 μm and width was 0.182 μm.

8-form diatom test plate examination

As shown in Table 5, the analyst's responses for viewing the 8-form diatom test plate were not consistent (i.e. no patterns). Analysts 1, 2, and 4 responded that both objectives were equivalent for determining lines and/or dots for all species (i.e. equal resolution). Analysts 5 and 6 reported that the DM objective had a better resolution than the DLL for some species images (3, 4, 7, and 8), whereas Analysts 7 and 8 reported the opposite. Regardless of objectives' resolution, five out of eight analysts preferred the DM objective and two preferred the DLL objective (one response missing).

DISCUSSION

The round-robin studies to evaluate the DM objective lens against a standard objective lens (DLL objective in this study) demonstrated statistically significant differences for the AIHA PAT chrysotile sample slides regardless of filter generation method (P-values < 0.05), with the DM objective allowing greater numbers of fibers to be counted. On the other hand, no statistical differences were found between objectives for fiber counts from the AIHA PAT amosite_water and chrysotile field samples (P-values > 0.05). The results of fiber count comparison between two objectives for the AIHA PAT chrysotile and amosite samples were consistent with the findings from the Japanese study. For the AIHA PAT amosite_water samples, the DM objective did not improve performance (median ratio of fiber counts DM/DLL = 1.02). Kenney *et al.* (1987) reported that they were able to determine all amosite fibers >5 μm in length and >0.125 μm in width with the PCM using a standard objective when compared with TEM analysis of the same samples. The results imply that amosite fiber widths and the contrast between the fiber (RI 1.69–1.70) and the Euparal mounting medium (RI 1.48) is already sufficient to determine all amosite fibers that might be present. Also, the analysts reported similar numbers of chrysotile fibers in the field samples regardless of the objective type (median ratio of fiber counts DM/DLL = 1.03), whereas considerably less fiber counts using the DLL were reported compared with those using the DM for the AIHA PAT chrysotile fibers. This finding indicates that the PAT chrysotile fibers, regardless of filter generation method, were on average thinner than the chrysotile

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

fibers collected from the field. The process of breaking up thick chrysotile fibers in laboratory preparation releases more individual fibrils and bundles containing fewer fibrils than are produced by the processes that release airborne chrysotile in field situations and hence fibers in the field are generally wider than those observed in laboratory preparations. This finding is also consistent with the many reports that the use of TEM does not count a very much greater number of fibers in field samples than does PCM. For example, Lynch *et al.* (1970): 'The counts of longer fibers (>5 μm length) on electron micrographs did not appear to be greater than those obtained by optical microscopy', Dement and Wallingford (1990): '... estimated the electron microscope fiber concentration to be 1.07 times the phase-contrast concentration for fibers > 5 μm in length' and Marconi *et al.* (1984): 'The median of the ratios between TEM and LM (PCM) counts has been found to be ... 1.2 for total fibres (length > 5 μm)'. Although Pang *et al.* (1984) did report a much greater number of chrysotile fibers counted under TEM versus PCM, ultrasonication was used to disrupt the thick bundles and produced many fibrils thinner than 0.1 μm. Note that our finding is limited to only six chrysotile sample slides from a single, unknown, field site with fiber density <230 fibers mm^{-2} (three slides < 100 fibers mm^{-2} and three slides between 100 and 230 fibers mm^{-2}). Although Cherrie *et al.* (1986) recommended a fiber density ranging from 100 to 1000 fibers mm^{-2} to minimize bias in fiber counts, the comparison of the ratio of fiber counts (DM/DLL) between the samples <100 and >100 fibers mm^{-2} was not visually different in this study (Fig. 2).

Analysts using the DLL objective can easily see the AIHA PAT amosite and chrysotile field fibers but can have severe problems in seeing all the AIHA PAT chrysotile fibers, which is a result consistent with previous studies (Pang and Harper, 2008; Harper *et al.*, 2009). Several factors can lead to the difference in fiber-counting performance between the DM and DLL objective lenses. Note that although the study design was limited to two factors (resolution and contrast) by controlling other potential factors, we have included other potential factors in this discussion.

1. *Human factors*. The quality of the human eye (i.e. visual acuteness of the eye) and the interpretation of the fiber-counting rules of the analysts do affect performance and could be the primary reason for causing variation between analysts. In order to minimize between-analyst variation, we asked the same analysts to examine sample slides with both objectives on different days for both round-robin studies. Thus, we conclude that this variation was controlled for, although there might be still slight differences of fiber counts using two objectives depending on the tiredness of the analysts' eyes.

2. *Microscope setup*. The setup of the microscope—including condition of optics, type and intensity of illumination, and microscope alignment (adjusting the field diaphragm and centering the phase ring)—was controlled in this study by circulating the same microscope along with the detailed guidance for the microscope setup and counting procedures. Thus, an effect of the microscope maintenance status should be minimal in fiber counts using different objectives.

3. *Resolution of objective lenses*. The DLL objective has a plan fluorite objective with 0.75 NA, whereas the DM objective has a plan apochromatic objective with 0.95

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

NA. One characteristic of the apochromatic objective is that it is more highly corrected for chromatic aberrations (i.e. dispersion) and thus has higher NA than the fluorite objective (McCrone *et al.*, 1984). The national and international standards recommend an NA ranging from 0.65 to 0.75, whereas the NA of the DM objective was 0.95. If higher fiber counts using the DM lens were from the higher NA than the recommended NAs, the increased fiber counts by a factor of 45% for the DM objective cannot be compatible with earlier exposure data on which risk assessment was based. In this study, the comparison of analyst's responses to the eight-form diatom test plate does not allow us to conclude that increased NA accounts for the significant enhancement in fiber counts. This finding also suggests that the NA range recommended in the current national/international standards could be extended from 0.75 to 0.95 NA without changing counting performance.

4. *Contrast sensitivity caused by phase-shift change*. Rooker *et al.* (1982) investigated the visibility of fibers under the PCM by applying different RI liquids to the glass wool, microquartz, and chrysotile fibers. They reported clear changes of visibility of fibers as the contrast between fibers and mounting medium (i.e. phase-shift change) increased. They, however, found that a substantial change in contrast (the RI difference between the fiber and mounting medium from 0.1 to 0.056) had no considerable change of chrysotile fiber counts. In this study, the sample slides, mounted with a synthetic form of Euparal, were examined, that is, no change of phase shift ($n_1–n_2$) was considered. Additionally, the comparison of visibility of the phase-shift test slide blocks between the DM and DLL objectives showed no statistically significant differences. The result also supports that the contrast between the fibers and the mounting medium was irrelevant to the higher fiber counts using the DM objective. Note that the results of the phase-shift slides include effects of other factors including microscope maintenance and the quality of the human eye. Thus, this factor has been controlled.

5. *Contrast sensitivity caused by different phase plate absorptions*. The DM objective has a higher absorption of the phase plate (~30% more absorption) than the DLL and thus generates a darker background, which yields higher contrast. Historically, the importance of phase plate absorption has been recognized by several authors who evaluated the effects of phase plate absorption on image generation (Bennett *et al.* 1946; Brice and Keck, 1947; Oettle, 1950; Barer 1952; Françon, 1961; Ross, 1967; Goldstein, 1982; Yamamoto and Taira, 1983). For example, Oettle (1950) examined the images of unstained human blood fixed in methyl alcohol and mounted in glycerine at various phase changes and amplitude changes. Oettle reported that under a quarter-wave phase change, both positive and negative phase contrast showed better contrast of the image (by qualitative visual results) as the percent of absorption increased. Ross (1967) also reported the same conclusion from the study of image contrasts at phase change of ordinary 90 positive phase plates with absorptions of 0%, 25%, and 75%. The findings of this study can be supported by findings reported by earlier studies. Thus, it is this higher contrast, especially with a bright Becke line, that makes the DM objective useful in detecting fine fibers.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

In addition, no statistically significant difference was observed between test slides examined under magnifications of ×400 (×10 ocular) and ×500 (×12.5 ocular), confirming that in this range either ocular can be used for the PCM as recommended by national and international standards. The comparison of HSE/NPL Mark II and HSL/ULO Mark III GREEN test slides, as expected, showed significant difference due to different levels of visibility in accordance with their certificates.

CONCLUSIONS

The findings of this study support earlier studies that the contrast caused by different phase plate absorptions between the DM and DLL objectives is the main factor affecting the fiber count as there is no difference in phase-shift. Not all analysts could see all verified fibers in AIHA PAT chrysotile samples (however produced) with the DLL objective, but visibility was improved and fiber counts were higher with the DM objective, which has a higher percent of phase plate absorption. It might then be hypothesized that higher counts would also result from use of the DM objective on field samples, but this was not observed. AIHA PAT chrysotile samples and any reference slides made from them include a fraction of fibers approaching the limit of visibility of PCM that were not also found in our field samples. It is likely that these are thinner fibers, which would be observable under the electron microscope if they were present. Several historical investigations of the difference between TEM and PCM fiber counts, however, suggest that very thin fibers are not common in field samples. Thus, there is no expectation that use of the DM objective for routine fiber counting will produce results incompatible with risk assessments. The DM objective, therefore, could be allowed for routine fiber counting as it will maintain continuity with risk assessments based on earlier PCM fiber counts from field samples. However, published standard methods would need to be modified to allow a higher aperture specification for the objective.

ACKNOWLEDGEMENTS

The authors are sincerely thankful for all the participating laboratories that contributed their time and efforts and to Dan Crane (OSHA) and Peter M. Cooke (Microscopy Instruction, Consultation, & Analysis (MICA), Chicago, IL) for reviewing this manuscript prior to journal submission. The authors are also grateful to the anonymous Journal reviewers whose keen insights allowed us to improve the manuscript considerably.

FUNDING

This project was funded internally by the National Institute for Occupational Safety and Health (Project 927ZKEB, Evaluation of the Dark-Medium (DM) objective in counting asbestos fibers).

REFERENCES

Barer R. A vector theory of phase contrast and interference contrast. I. Positive phase contrast. J R Microsc Soc. 1952; 72:10–30. [PubMed: 13000815]

Bennett AH, Jupnik H. Phase microscopy. Trans Am Microsc Soc. 1946; 65:99–131. [PubMed: 20989161]

Brice AT, Keck PH. The most favorable absorption of the phase plate in phase contrast microscopy. J Opt Soc Am. 1947; 37:647–51. [PubMed: 20258247]

Cherrie J, Jones AD, Johnston AM. The influence of fiber density on the assessment of fiber concentration using the membrane filter method. Am Ind Hyg Assoc J. 1986; 47:465–74.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Crane, D.; Harper, M. The phase shift test slide interpretation. AIHA Laboratory Accreditation Program, LLC; Falls Church, VA: 2011. Available at http://www.aihaaccreditedlabs.org/programs/Documents/Phase%20Shift%20Interpretation%20OSHA%20NIOSH.pdf. [26 September 2014]

Dement J, Wallingford K. Comparison of phase contrast and electron microscopic methods for evaluation of occupational asbestos exposures. App Occup Environ Hyg. 1990; 5:242–7.

Françon, M. Progress in microscopy. Pergamon Press Ltd; Oxford, UK: 1961.

Goldstein DJ. A simple quantitative analysis of phase contrast microscopy, not restricted to objects of very low retardation. J Microsc. 1982; 128:33–47. [PubMed: 6754949]

Harper M, Bartolucci A. Preparation and examination of proposed consensus reference standards for fiber-counting. AIHA J. 2003; 64:283–7.

Harper M, Slaven JE, Pang TWS. Continued participation in an asbestos fiber-counting proficiency test with relocatable grid slides. J Environ Monit. 2009; 11:434–48. [PubMed: 19212603]

HSE. Asbestos: the analysts' guide for sampling, analysis and clearance procedures (HSG 248). Health and Safety Executive; Sheffield, UK: 2006.

IRSST. Numération des fibres 243-1. Institut de recherché Robert-Sauvé en santé et en Sécurité du travail; Québec, Canada: 1995. IRSST

ISO. Air quality-determination of the number concentration of airborne inorganic fibres by phase contrast optical microscopy—membrane filter method. International Organization for Standardisation; Geneva, Switzerland: 1993. ISO 8672. ISO

Kenney LC, Rood AP, Blight BJN. A direct measurement of the visibility of amosite asbestos fibres by phase contrast optical microscope. Ann Occup Hyg. 1987; 33:261–4.

Lee EG, Nelson J, Hintz PJ, et al. Field performance of the CATHIA-T sampler and two cyclones against the standard Cowled sampler for thoracic fiber concentrations. Ann Occup Hyg. 2010; 54:545–56. [PubMed: 20457784]

Lee EG, Pang TWS, Nelson J, et al. Comparison of mounting methods for the evaluation of fibers by phase contrast microscopy. Ann Occup Hyg. 2011; 55:644–57. [PubMed: 21642614]

Lynch JR, Ayer HE, Johnson DL. The interrelationships of selected asbestos exposure indices. Am Ind Hyg Assoc J. 1970; 31:598–604. [PubMed: 5488306]

Marconi A, Menichini E, Paoletti L. A comparison of light microscopy and transmission electron microscopy results in the evaluation of the occupational exposure to airborne chrysotile fibres. Ann Occup Hyg. 1984; 28:321–31. [PubMed: 6095717]

McCrone, WC.; McCrone, LB.; Delly, JG. Polarized light microscopy. McCrone Research Institute; Chicago, IL: 1984.

NIOSH. NIOSH manual of analytical methods. 5th edn.. National Institute for Occupational Safety and Health; Cincinnati, OH: 1994. NIOSH method 7400—Asbestos and other fibers by PCM..

Oettle AG. Experiments with a variable amplitude and phase microscope. J R Microsc Soc. 1950; 70:232–54. [PubMed: 24538700]

Ogden TL, Thomson DJM, Ellwood PA. Euparal and its use in measurement of asbestos. Microscope. 1986; 34:173–9.

Olympus America Inc. [4 April 2013] Olympus microscopy resource center. 2013. Available at http://www.olympusmicro.com/primer/anatomy/specialobjectives.html.

OSHA. OSHA manual of sampling and analytical methods. Occupational Safety and Health Administration, OSHA Salt Lake Technical Center; Sandy, UT: 1998. Method ID-160. Asbestos in air..

Pang TWS, Dicker WL, Nazar MA. An evaluation of the precision and accuracy of the direct transfer method for the analysis of asbestos fibers with comparison to the NIOSH method. Am Ind Hyg Assoc J. 1984; 45:329–35. [PubMed: 6377863]

Pang TWS, Harper M. The quality of fiber counts using improved slides with relocatable fields. J Environ Monit. 2008; 10:89–95. [PubMed: 18175021]

Rooker SJ, Vaughan NP, LeGuen JM. On the visibility of fibers by phase contrast microscopy. Am Ind Hyg Assoc J. 1982; 43:505–15.

Ross, KFA. Phase contrast and interference microscopy for the cell biologist. St. Martin's Press; New York, NY: 1967.

Author Manuscript

Shenton-Taylor T, Ogden TL. Permanence of membrane filter clearing and mounting methods for asbestos measurement. Microscope. 1986; 34:161–72.

Van Duijn C. Visibility and resolution of microscopical detail. The Microscope. 1957; 11:196–209.

WHO. Determination of airborne fibre concentrations. A recommended method, by phase-contrast optical microscopy (membrane filter method). World Health Organization; Geneva, Switzerland: 1997.

Yamamoto K, Taira A. Some improvements in the phase contrast microscope. J Microsc. 1983; 129:49–62.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

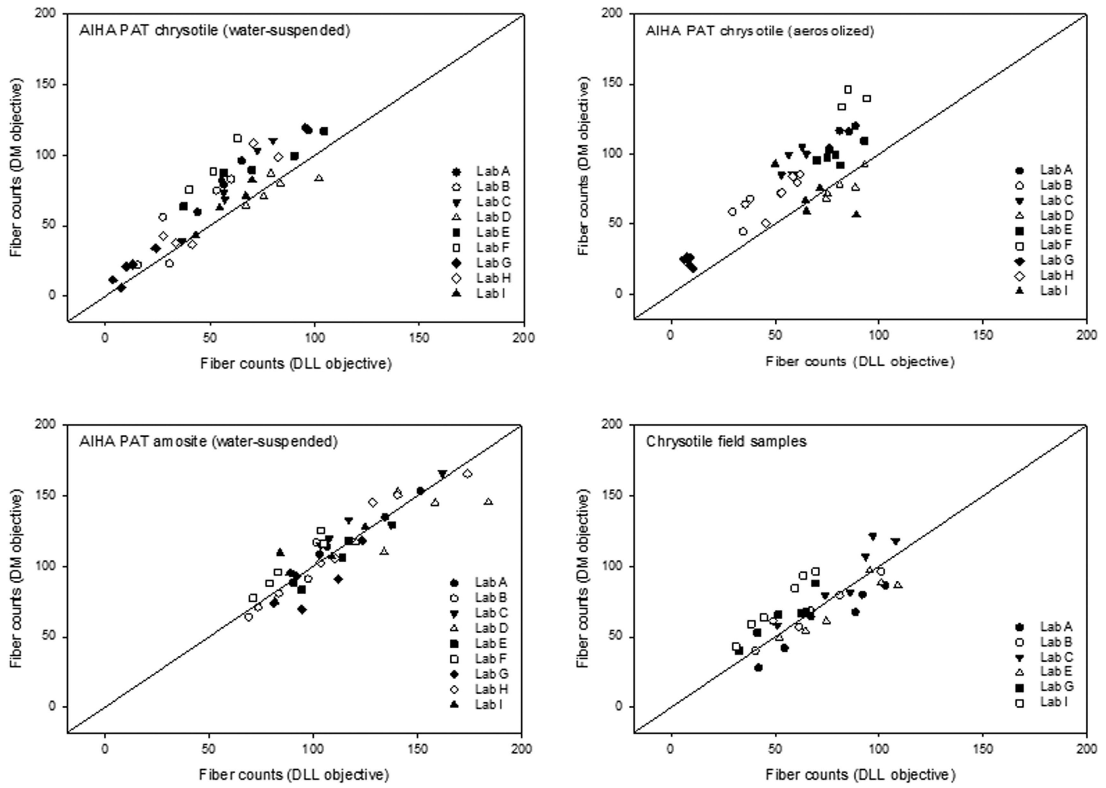

Figure 1.
Comparison of fiber counts between the DM and DLL objectives (all data). The diagonal line represents 1:1 relationship.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

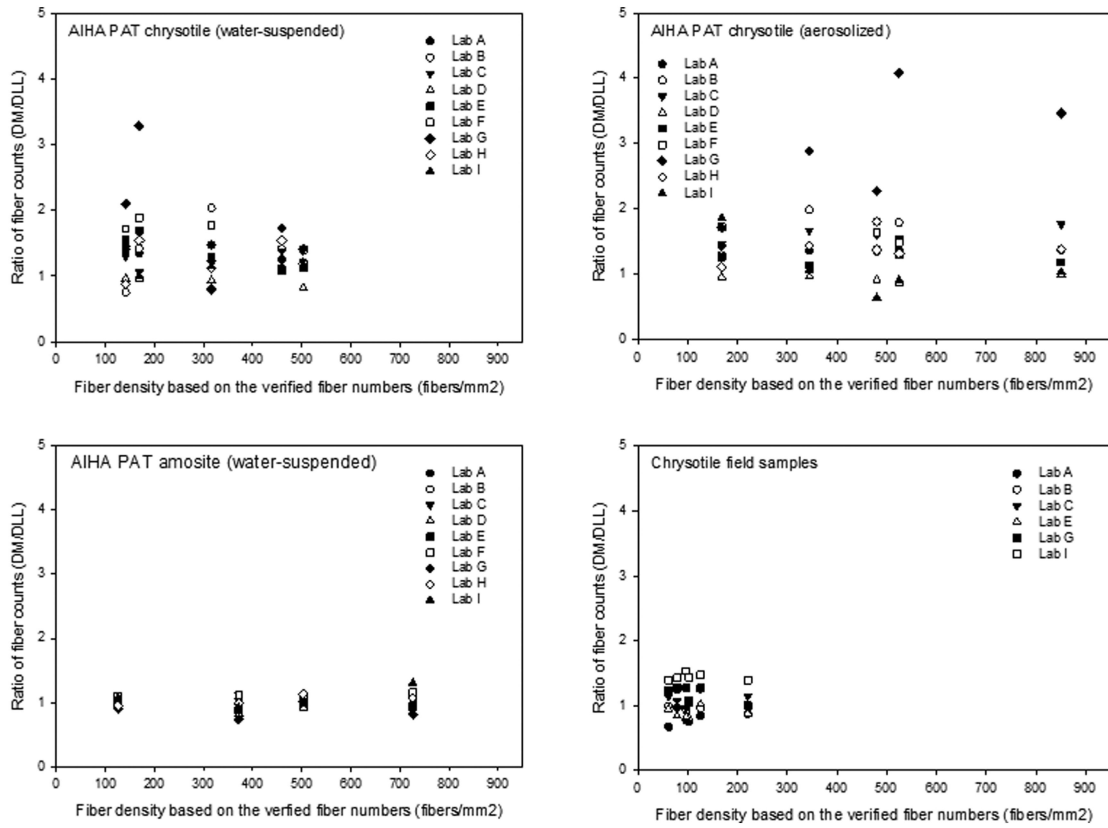

Figure 2.

Ratio of fiber counts (DM/DLL) versus fiber density based on the verified fiber numbers (all data).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 1

Requirement of microscope setup and test slide by national and international standards.

Method	Microscope requirement				Visibility of HSE/NPL Mark II test slide		
	Phase contrast	Magnification	Numerical aperture (NA)	Phase-ring absorption	Blocks 4 and 5	Block 6	Block 7
NIOSH 7400	Positive	×8 to ×10 eyepiece and a ×40 to ×45 phase objective (total magnification ca. ×400)	From 0.65 to 0.75	—	Partially visible	Invisible	Invisible
OSHA ID-160	—	×40 phase objective and ×10 eyepieces	From 0.65 to 0.75	—	Partially visible	Invisible	Invisible
WHO	Positive	×40 objective, ×400–600 magnification (preferably ×500 corresponding to a ×40 objective and a ×12.5 eyepiece)	From 0.65 to 0.75 (preferably between 0.65 and 0.70)	From 65% and 85% (preferably from 65 to 75%)	Completely visible	Partially visible	Invisible
HSG 248[a]	Positive	×40 objective (preferably ×500 magnification)	From 0.65 to 0.70	From 65 to 85%	Completely visible	Partially visible	Invisible
ISO 8672	—	×10 and ×40 parfocal phase-contrast achromatic objective (total magnification of ×400 to ×600)	0.65	From 65 to 85%	Practical detection limit for Block 5[b]	—	—
ASTM D7200	Positive	×8 to ×10 eyepiece, and a ×40 to ×45 phase objective (total magnification of approximately ×400 to ×450)	From 0.65 to 0.75	—	Partially visible	Invisible	Invisible
Quebec IRSST 243	Positive	×8 to ×10 eyepiece, and a ×40 to ×45 phase objective (total magnification of approximately ×400)	From 0.65 to 0.75	—	Partially visible	Invisible	Invisible

— not described.

[a]HSG 248 also recommended HSE Mark III test slide issued with a red certificate requiring Block 4 visible, Block 5 partially visible, and Blocks 6 and 7 invisible.

[b]Instead of clearly defining the visibility (e.g. one of completely visible, partially visible, or invisible), the standard described it as ‘practical detection limit for Block 5’.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 2

Ratio of fiber counts between the DM and DLL, *P*-values, and model estimates from ANOVA.

Fiber type		N [a]	Ratio of fiber counts (DM/DLL)			*P*-value (Estimate: DM versus DLL) [c]
			Median	Range	RSD [b]	
AIHA PAT	Chrysotile_water	9	1.30	0.75–3.29	0.32	0.0066 (70 versus 58)
	Chrysotile_aero	9	1.37	0.63–4.08	0.43	0.0001 (81 versus 63)
	Amosite_water	9	1.00	0.74–1.30	0.11	0.9778 (112 versus 112)
Chrysotile field samples		6	1.03	0.67–1.53	0.21	0.5058 (71 versus 68)

[a] N = number of laboratories participated in the round-robin study.

[b] Relative standard deviation (RSD) = standard deviation divided by average.

[c] Estimate of least square means from analysis of variance (ANOVA) test (unit: fiber numbers).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 3

Summary of fiber counts divided by 'verified fibers' across the labs (all data).

Fiber type		Median		Range		RSD[a]	
		DM	DLL	DM	DLL	DM	DLL
AIHA PAT	Chrysotile_water	0.62	0.47	0.05–0.90	0.04–0.81	0.30–0.48	0.37–0.52
	Chrysotile_aero	0.64	0.53	0.14–1.18	0.05–0.83	0.33–0.49	0.42–0.47
	Amosite_water	1.00	1.05	0.71–1.51	0.73–1.53	0.17–0.22	0.18–0.21
Chrysotile field samples		0.92	0.91	0.55–1.39	0.51–1.31	0.13–0.25	0.16–0.29

[a] Range of RSD across the labs.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 4

Summary of visibility under different test conditions.[a]

Comparison	Group 1			Group 2			*P*-value[b]	
	Eyepiece	Objective lens	Test slide[c]	Eyepiece	Objective lens	Test slide[c]	3 Days[d]	All[e]
DM versus DLL	×10	DLL	II	×10	DM	II	0.4338	0.3504
	×10	DLL	III	×10	DM	III	0.9606	1.0000
	×12.5	DLL	II	×12.5	DM	II	0.4183	0.4562
	×12.5	DLL	III	×12.5	DM	III	0.8813	0.6882
×10 versus ×12.5	×10	DLL	II	×12.5	DLL	II	0.6042	0.5477
	×10	DLL	III	×12.5	DLL	III	0.6635	0.6554
	×10	DM	II	×12.5	DM	II	0.4719	0.5488
	×10	DM	III	×12.5	DM	III	1.0000	1.0000
Test slide (Mark II versus Mark III Green)	×10	DLL	II	×10	DLL	III	0.0021	0.0001
	×10	DM	II	×10	DM	III	0.0231	0.0029
	×12.5	DLL	II	×12.5	DLL	III	0.0052	0.0001
	×12.5	DM	II	×12.5	DM	III	0.2797	0.0530

[a] Only four blocks (from Block 3 to Block 6) were considered, and the results of Lab I were excluded due to a misunderstanding of guidance.

[b] *P*-values from the Fisher exact test including eight laboratories.

[c] II = HSE/NPL Mark II test slide and III = HSL/ULO Mark III Green-certified test slide.

[d] *P*-values considering results of 3-day mandatory exam except for one laboratory performed a 2-day exam.

[e] *P*-values considering all results provided by the laboratories.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 5

Summary of the 8-form diatom test plate examination.

Species image	Analyst's response[a]							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1: *Gyrosigma balticum*	Equiv	Equiv	Equiv	Equiv	Equiv	Equiv	Equiv	Equiv
2: *Navicula lyra*	Equiv	Equiv	Equiv	Equiv	Equiv	Equiv	DLL	Equiv
3: *Stauroneis phoenicentron*	Equiv	Equiv	Equiv	Equiv	DM	DM	DLL	Equiv
4: *Nitzschia sigma*	Equiv	Equiv	DLL	Equiv	DM	DM	Equiv	Equiv
5: *Surirella gemma*	Equiv	Equiv	DM	Equiv	Equiv	Equiv	Equiv	Equiv
6: *Pleurosigma angulatum*	Equiv	Equiv	Equiv	Equiv	Equiv	Equiv	DM	DLL
7: *Frustulia rhomboides*	Equiv	Equiv	Equiv	Equiv	DM	DM	DLL	Equiv
8: *Amphipleura pellucida*	Equiv	Equiv	Equiv	Equiv	DM	DM	Equiv	Equiv

[a]Equiv = the determination of lines and/or dots was equivalent for both DM and DLL objectives (i.e. equal resolution), DM (or DLL) = the DM (or DLL) objective was better to determine lines and/or dots.