Quantitative serial sectioning analysis

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I. SUMMARY

A method for serial sectioning is presented that allows one to take ca. 20 sections per hour with spacings in the range of 1 and 20μm between sections. The alignment of the cross-sections is done with an LVDT (Linear Variable Differential Transformer), and thus independent of the microstructure of the sample and does not rely upon markers implanted in the sample. The alignment errors as well as tilts and rotation errors between sections associated with the new method are found to be negligible. Once all the sections are captured in a computer a three-dimensional image can be constructed. This image can be viewed interactively and rotated, thus allowing the direct observation of three-dimensional shapes. It can further be used to determine a vast array of microstructural parameters including those that can not be determined from planar sections. The technique is illustrated through the reconstruction of the microstructure of a cast standard aluminum alloy specimen.

II. INTRODUCTION

In 1983 R.T. DeHoff pointed out the necessity for using serial sections for the evaluation of metallic microstructures (DeHoff, 1983). The reason is that although some microstructural parameters like volume fraction or area of interfaces can be obtained directly from planar sections and others, such as particle size distributions or degree of anisotropy, can be determined from planar sections with simplifying assumptions, there is a group of parameters that can only be obtained from a three-dimensional representation of the microstructure. This group includes the number of features per unit volume (particles, cells, etc.), connectivity of features, size distributions, spatial distribution information, and the detailed shape of an element of the microstructure. The most practical method to obtain three-dimensional information of micron-sized microstructures is serial sectioning. It has been used several times (Bower *et al.*, 1966; Wolfsdorf *et al.*, 1997; Mangan *et al.*, 1997; Li *et al.*, 1998 and 1999) but has the very awkward quality of being extremely tedious. At most 10 cross-sections per day can be prepared and photographed. Since 50-150 cross-sections are necessary to get good shape information (DeHoff *et al.*, 1972) the preparation of one single sample can take weeks. DeHoff predicted that these problems will be overcome and the technique of serial sectioning will become standard in future studies of metallic microstructures. The method presented here brings this future one step closer. Described here is a technique which allows to prepare and photograph ca. 20 cross-sections *per hour.* The basic method has already been developed and described in 1998. It has, however, not been published until now. A patent application has been filed.

Once all the cross-sections are made and recorded in a computer, they can be reconstructed to give a three-dimensional image of the microstructure. The combination of serial sectioning and three-dimensional reconstruction is called *microstructural tomography.* Examples of such an approach given are the work of Wolfsdorf *et al.* (1997), Mangan *et al.* (1997), and Li *et al.*(1998 and 1999). Another example is given in this article, but with a more advanced technique of serial sectioning.

III. METHOD

The usual method for serial sectioning works by cyclic grinding/polishing of a sample and then photographing the planar sections. The largest problems with this method are ensuring that the distance between two sections is known accurately, the alignment of the images with regard to horizontal displacement and tilt, and the tedious time consuming nature of this approach. The first problem can be solved by using automated polishing machines and keeping the polishing times constant. Alignment of pictures can be done with markers put into the microstructure such as microindents, which also allow the distance between the sections to be determined. Tilts between sections are usually negligible. The time required to obtain the sections using this method is a major problem, see Mangan *et al.* (1997) as an example.

The method of Wolfsdorf *et al.* (1997) employed a micromiller (*Reichert-Jung (Leica) Polycut E* microtome with a micromilling attachment) to prepare the samples, which were subsequently etched and photographed using a microscope. The micromiller was used because a very soft material (Pb-Sn alloy) was studied and milling, instead of polishing, gives nearly scratch-free cross-sections. It also does not require a lot of time to prepare a cross-section. The images were aligned using the microstructural features of the individual cross-sections. This alignment procedure as well as uncertainties introduced by the repeated mounting and unmounting of the samples from the milling machine were major limitations of this technique. The method described here, however, builds upon their work.

FIG. 1. Reichert-Jung Polycut E micromiller

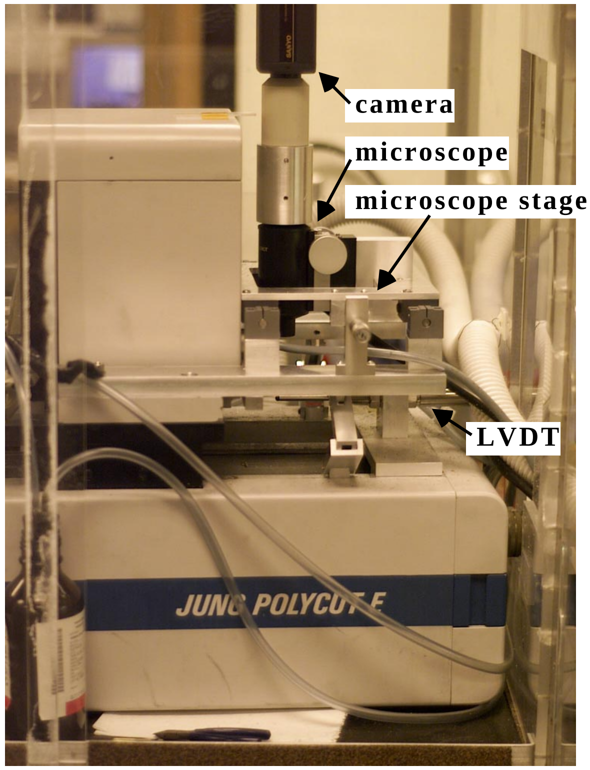


FIG. 2. Attachment to the micromiller to make serial sections

The new procedure is quite simple, see figure 3: The surface of the sample is milled off with a step-size between 1 and 20μm. Next the sample is translated to the microscope, which is directly attached to the milling machine. The samples do no longer need to be removed from the milling machine between different cuts. All sample movements are done by using the stage of the micromiller. Etching of the samples can be done in a position between the milling area and the microscope. An LVDT (Linear Variable Differential Transformer) is used to obtain the necessary alignment information. A discussion of the alignment and the involved errors, a matter of crucial importance if the technique is to be used quantitatively, is given below. After the image is taken the sample is moved back and the surface is milled off again and so on. Since the sample remains attached to the micromiller at all times the speed is increased dramatically. As a result ca. 20 cross-sections per hour can be prepared and photographed. It should be noted, however, that the micromiller is not well suited for steels, since the milling is done with a diamond blade and the diamond will react with the iron to quickly form iron-carbides. Relatively soft materials, like most aluminum alloys and solders, are ideal materials for this procedure, but polymers, bones, and many other materials can also be prepared.

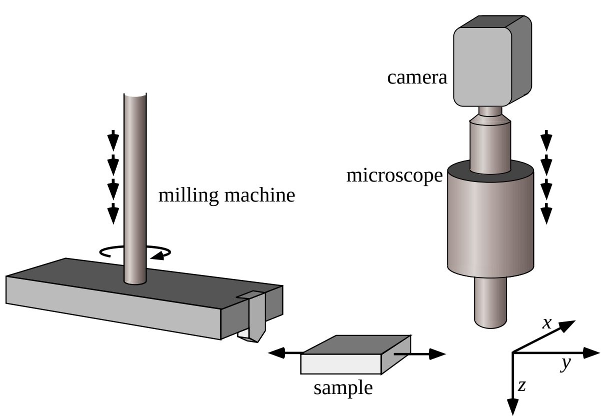


FIG. 3. Schematic drawing of the serial sectioning procedure. The sample translates from the miller to the microscope in the y-direction. The sections are taken in z-direction. The two lines of four short arrows indicate the simultaneous movement of the miller and microscope in z-direction between the individual cuts.

IV. ALIGNMENT AND MEASUREMENT ERRORS

The resolution of the images in the current configuration of the facility is 0.96μm in x- and y-direction. The resolution can of course be changed by exchanging the microscope objectives or the camera. The value given above correspond to a 10×-objective and our specific microscope and camera. This is the configuration that was used in the test procedure and the example given later in this article.

A series of tests were performed to determine the error in the sample position perpendicular to the direction of movement (x-direction). These errors may result from the translation of the sample over distances of ca. 30cm between milling area and microscope. By comparing the microstructures after translating the samples forward and back in y-direction without cutting no translation in x-direction was found. This implies in addition that there is no rotational misalignment of the samples in the x-y-plane.

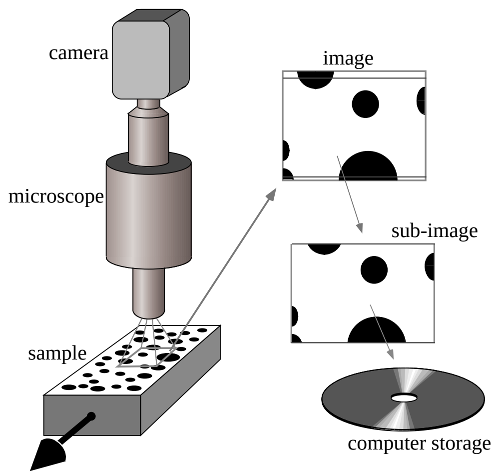


FIG. 4. Microscopy of the surface. The image of a section of the sample surface is captured. A sub-image is then created based on the position information given by the LVDT (see text) and saved to disk. The large arrow at the end of the sample denotes the direction of translation.

The alignment in the translation direction (y-direction) is done with the help of an LVDT. The idea behind the procedure is that instead of repositioning the sample after each cut within 1μm, which proves to be a challenge, using a LVDT it is possible to easily obtain the position of the sample in y-direction with an accuracy better that 0.5μm and use this information to obtain afterwards a set of perfectly aligned images. The sample position, however, may scatter from cut to cut over 50μm or more. The method, by which the images are aligned, is depicted in figure 5. From each image one can obtain a sub-image by erasing a number of lines, according to the position information, at the top and at the bottom of the image. As an example, we assume a resolution of 1μm per pixel, an image size of 1000×1000 pixel, and that our positions do not scatter more than 50μm. Then, for the first section, we take a sub-image of 1000×900 pixel that we obtain from the original image by erasing the top 50 lines (50μm) and bottom 50 lines (50μm). Let's assume the next section has a placement of -11μm relative to the first one. Then we reduce the corresponding 1000×1000 pixel image to a 1000×900 pixel image by erasing the top 39 lines (39μm) and bottom 61 lines (61μm). The other sections are treated similarly. This procedure gives a stack of 1000×900 pixel images that are aligned to within one pixel – no matter what the pixel resolution – assuming the LVDT measures with sufficient accuracy. It should be noted that the method of positioning images can easily be used to create montages of overlapping images in one or two directions (x or y) by two LVDT's. Furthermore, using a motorized stage this process can be fully automated. It is thus an improvement over common methods of creating montages that involve matching the features of two overlapping images. The current design of the serial sectioning facility allows, with only minor modifications, the creation of stacks of montages instead of stacks of individual images. This, however, is in general of no particular interest since the amount of data created by the current setup is already extremely large and since the z-dimension (cutting direction) is typically significantly smaller than the x- and y-directions.

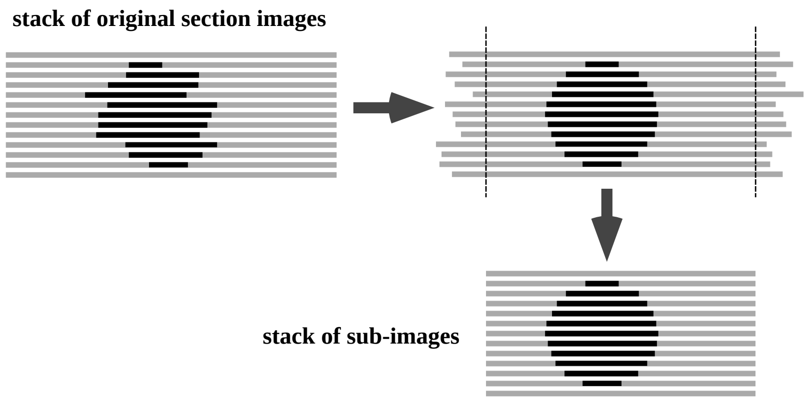


FIG. 5. A set of perfectly aligned sub-images can be obtain from a set of images from the different sections if the displacement between the different sections is known

The milling is done with a micromilling attachment on a microtome table. The miller is moved down a certain distance following a cut. This distance is then removed from the sample surface. The micromiller allows full micron steps between 1 and 20 microns. One problem that can affect the accuracy of the distances between cuts are changes in the temperature during the serial sectioning process. Since the milling attachment of the micromiller is ca. 30cm tall and made mostly from steel, changes in the temperature of $1K$ can result in up to 5μm displacement of the milling tool. This can easily be observed when the milling machine is started and warms up. The displacement of the milling tool within the first few minutes was found to be >10 microns. The exact value depends on the room-temperature and other factors. Other thermally related problems are changes of the temperature of the sample and sample holder. Since alcohol is used as a cutting fluid and lubricant, this cools the sample and sample holder significantly in the first minutes of the procedure. Typical temperatures for the samples during cutting were found to be 10°C whereas the room-temperature was 21°C. After running the milling machine and the cutting fluid (alcohol) for 45 minutes, however, changes in the displacement due to temperature changes were found to be negligible.

A series of tests was performed to evaluate the precision of the distance milled off in z-direction (the step-size). Aluminum samples were cut and the milling procedure was stopped with the surface half-milled. This resulted in samples which included the step-size in the surface profile. This step-size was measured for several samples with a surface-profilometer. The uncertainty in the step-size was determined to be smaller than 100nm for a 5μm step. From the same measurements tilts in the x-y-plane between cuts were found to be negligible. If they exist they are smaller than 0.002°. It is important that the normal to the cutting plane is parallel to the optical axis of the microscope. A deviation here would result in systematic errors of the reconstructed volume. However, the errors introduced by small deviations are small and the alignment of the microscope is not difficult. The peak-to-peak roughness of the milled unetched surface was found to be about 200nm. As will be seen later, a different test with a 3-D reconstruction showed that a nominal 5μm step corresponds to a 4.75μm step. This discrepancy is ascribed to the different thermal environment that is present after the machine and sample reach thermal equilibrium.

The microscope is attached to the milling tool. It thus moves with the tool and refocussing after a section is made is not necessary.

V. THREE-DIMENSIONAL RECONSTRUCTION

The three-dimensional representation is simply the combination of all the two-dimensional images, see figure 6, with the appropriate scaling factors to account for different sizes of the voxels (volume-pixels) in the x-, y-, or z-directions. The interfaces in three dimensions can be determined by thresholding the complete three-dimensional image. Handling and visualization of three-dimensional data sets is straightforward with image analysis software such as *IDL* (*Interactive Data Language*, Research Systems Inc.). Basically all microstructural features can be determined from the three-dimensional data sets. See for example (DeHoff, 1983; DeHoff & Rhines, 1968; Weibel, 1979; Weibel, 1980).

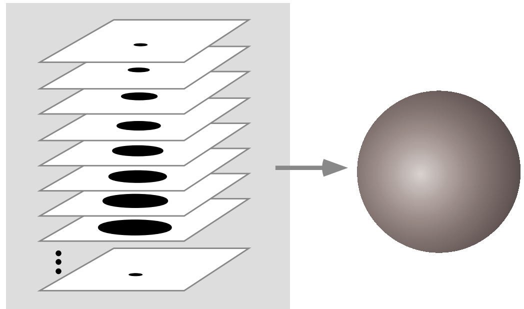


FIG. 6. The three-dimensional shape is reconstructed from the collection of cross-sections.

VI. TEST

To test the method and obtain possible errors we created a sample made of pure aluminum mounted in Buehler Transoptic hot-mount material with a flat smooth interface between the two materials that is tilted in x- and y-direction as shown in figure 7. This specific sample was chosen because by looking at the sample-surfaces one can determine the structure inside the sample. 40 sections of the sample were prepared spaced 5μm apart. Afterwards pictures of the x-z- and y-z- surfaces of the sample were taken, which now allow to measure the tilt angle of the interface between the two materials with respect to the cut surface (see figure 8a and 8b).

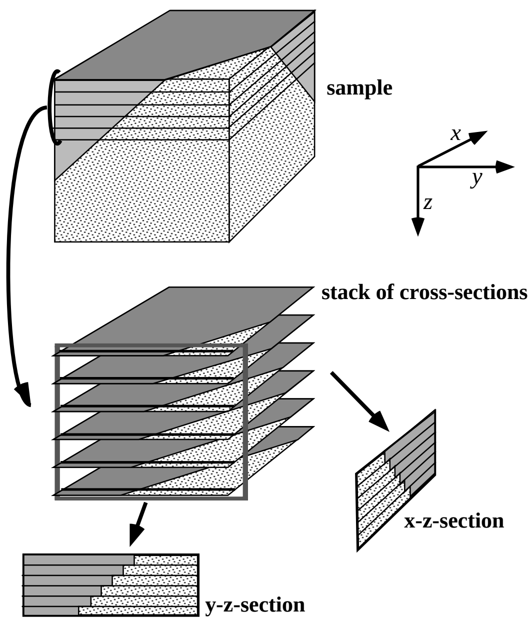
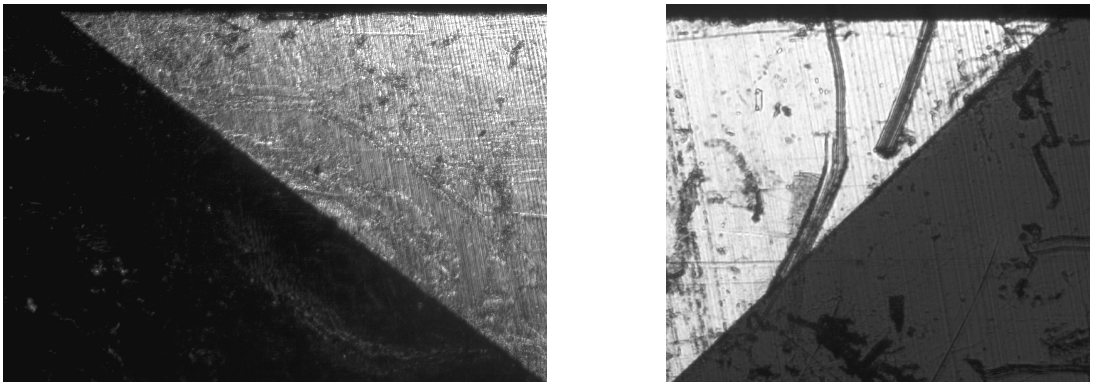


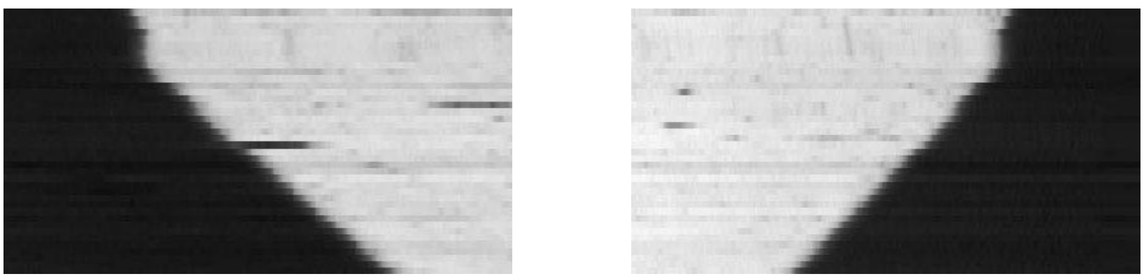
FIG. 7. A cube of aluminum and mounting material with a flat but tilted interface between the materials is sectioned. From the sections an x-z- and an y-z-cross-section can be extracted.

The serial sections were combined to create a three-dimensional representation of the sample from which x-z- and y-z-sections were extracted, see figure 9. One can clearly see how the process of reaching the thermal equilibrium affects the sections: the top 15 cuts seem somewhat shifted while the bottom 25 are quite regular and show the correct flat interface between aluminum and mounting material. When the three-dimensional representation is constructed, we assume that the sections have specific predetermined distances from another. In the beginning, before thermal equilibrium is reached, however, this assumption is wrong and results in a distortion of the reconstruction in z-direction. In a cross-section perpendicular to the cutting-plane this gives the effect as seen in figure 9.



(a) x-z-surface of the sample (b) y-z-surface of the sample

FIG. 8. The surfaces of the sample after the sectioning procedure. The aluminum appears bright and the mounting material appears dark.



(a) x-z-section (b) y-z-section

FIG. 9. Sections calculated from the three-dimensional data set. The aluminum appears bright and the mounting material appears dark. The steps in the interface correspond to the cutting steps.

Due to the specific sample chosen these sections should be identical to the corresponding surface images - particularly the tilt-angles should agree. The actual angles measured using the images of the surface were 38° for the x-z-surface and 43° for the y-z-surface. The angles obtained from the reconstructed sections are 40° and 45°, respectively. This means that the assumption of 5μm per cut is slightly to high. Thus, the actual section distance 5% lower.

This test showed that the procedure works perfectly in aligning the images since the interface in the x-z- and y-z-sections is perfectly flat once thermal equilibrium is attained. It shows further that under working conditions a slight deviation between nominal and real cutting distances of 5% exist, which has to be taken into account when doing serial sections. This, however, might vary between different micromillers even of the same type. The third and very important fact the test shows is that warming up the facility is crucial in obtaining accurate serial sections. One should run the machine for at least 45 minutes before starting the serial sectioning process or even better discard the first 15 images.

VII. EXAMPLE

As an example we took 40 sections of a standard Al-Si alloy with a section spacing of 3μm. The sections were treated in the described manner and x-z- and y-z-sections were prepared. Figure 10 shows a view of the cross-section with reconstructed x-z- and y-z-sections at the sides. The image gives a good idea of the possibilities of the new method. The 40 cross-sections were obtained in ca. 2 hours plus another hour of set-up and warm-up time. No adjustments of brightness or contrast were made. The alignment is based solely on the LVDT-data.

FIG. 10. Reconstructed Al-Si alloy. The bright phase is aluminum and the dark phase is silicon. The microstructure shows aluminum dendrites and a aluminum-silicon eutectic phase. Shown is a tilted view of a reconstructed volume. The upper area is one of the original cross-sections. The two sides are the x-z- and y-z-sections that were reconstructed fron the data set.

VIII. ACKNOWLEDGMENTS

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