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SAMPLE PREPARATION – polypropylene

I'm trying to microtome various polypropylene pieces that are too narrow to place in the vise on the microtome. I need to slice the surface, not the cross-section. So, I was wondering if anyone has advice on the best way to mount this sample, in what kind of resin? And what kind of mold? The samples are going to be all shapes and sizes. Judith Ruiz <judith_a_ruiz@whirlpool.com> 22 Apr 2005

I have been fairly successful with similar samples by embedding them in a flat embedding capsule in an epoxy resin such as LX112. Before the advent of those capsules I used hinged BEEM capsules by turning the capsule upside down and cutting the pointed end off. With either one, you'll be able to cut the surface of the material. Mary Engle <mgengle@uky.edu> 22 Apr 2005

This can be tricky since you need to slice surface vs. cross-section. I'll be interested in what suggestions others might have as this has also been a problem for me. If the samples are not thin films you can use a room temperature cure epoxy such as epo-fix, fill a flat mold, wait until is has nearly cured and then press your sample into the epoxy oriented as need be; this way it shouldn't move during remaining cure. You have to be careful that the sample doesn't pop out when trimming which happens to me if the sample is to thin. One way I've gotten around this is to epoxy the thin film to a smaller epoxy block and then embed this into regular flat mold. Other ideas? By the way I assume you are using a cryo-ultramicrotome. Stephen McCartney <stmccart@vt.edu> 22 Apr 2005

Although this might not help in cutting polypropylene across the surface, I thought I'd mention a tip I learned last week for making cross-sections. Dip the polypropylene in isopropyl alcohol, used as a wetting agent, then into water, then into liquid nitrogen, and then snap it. I have yet to try this, but I am told that the cross-sections turned out extremely well for SEM. Lou Ross <rosslm@missouri.edu> 25 Apr 2005

I've done lots of microtoming of polypropylene. Normally, I've mounted the specimen directly on a dry ice bucket using freezable acrylic compounds. These are obtainable from your friendly local microscopy supplier. After cutting, the material can be washed from the sections or stub with water. I'd like to ask, are you trying to prepare sections for polarizing optical microscopy, or surfaces for electron microscopy? I've had years of work with polypropylene using POM, SEM and TEM - if you want further details feel free to ask. For polypropylene TEM morphology you might like to look at: http://www.personal.rdg.ac.uk/~spsolley/Picture_Gallery/new_pgal.html and click on the tabs "Impact Polypropylene" and "Row Structures". R. H. Olley <r.h.olley@reading.ac.uk> 25 Apr 2005

SAMPLE PREPARATION – Polyethylene

I have questions of how to prepare polyethylene film thin sections to be used for TEM. What kind of embedding materials should I use for polyethylene film? Do I have to use cryo-microtoming? How should I stain the samples? I found that in the literature, some one stained the thin sections while others stained the trimmed face off before microtoming? Which one is better? For each case, how long is appropriate for the staining? Mingzong <mingzong@ualberta.ca> 27 Apr 2005

I strongly recommend the following approach: Cryo-face the sample using a glass or diamond knife in a cryo-ultramicrotome. Stain faced sample in RuO4 vapors. Cut <100 nm-thick sections from the stained face using a diamond knife and ultramicrotome at ambient temperature. Have fun in the TEM. This procedure, as well as one for the preparation of samples for low voltage SEM analysis of the domain morphology of blends, is well documented in the paper referenced below. Our lab uses it exclusively, over other techniques for polyethylene, polypropylene, their blends and copolymers, with excellent results. Detailed instructions can be found in the appendix. The reference is: G. M. Brown and J. H. Butler (1997) *New method for the characterization of domain morphology of polymer blends using ruthenium tetroxide staining and low voltage scanning electron microscopy (LVSEM).* Polymer 38(15):3937. Gary M. Brown <gary.m.brown@exxonmobil.com> 27 April 05

SAMPLE PREPARATION – Insect cuticle

I was wondering if anyone has any suggestions for softening insect cuticle for histological work. I am having much difficulty sectioning insect ears as the cuticle is so hard. Any ideas or suggestions? Shannon Mahony <shaenon@hotmail.com> 27 April 2005

Believe it or not, I saw somebody successfully use Nair, the cosmetic hair remover product, for this very purpose. As I recall it was a project at the Southern Illinois University EM facility years ago involving serial LM sections through the abdomens of flies. Randy Tindall <tindallr@missouri.edu> 28 Apr 2005

SAMPLE PREPARATION - membranes of cultured cells

I have recently been having problems getting good staining of the membranes of cultured cells with osmium. The cells were fixed with 2% glutaraldehyde and post fixed in 1% osmium tetroxide. I have tried 0.1 M phosphate buffer, pH 7.4 and 0.1 M cacodylate buffer, pH 7.3. When using these, the membranes were not visible at all. I also tried osmium with potassium ferricyanide, which worked well with phosphate buffer but left a black precipitate. With cacodylate buffer and potassium ferrocyanide, the membrane definition was a little better but still not adequate. If anyone has any suggestions as to how to improve the membrane staining, they would be greatly appreciated. Robert Temkin <rtemkin@mtsinai.on.ca> 31 May 2005

Getting good contrast when embedding a cell monolayer is often a problem for us too. I have always suspected extraction, during dehydration. Are you using "*en bloc*" staining with uranyl acetate? It might be considered optional by some, but I find it is necessary for good membrane contrast when working with monolayers. Marc Pypaert <marc.pypaert@yale.edu> 01 Jun 2005

I had problems with my monolayers and suspensions for a while. I reviewed my procedures and found two things. 1. I had changed from acetone to ethanol dehydration. I'm not sure, but I believe I first saw this in Pease's procedures book – 2nd edition. It referred to the ongoing problem with loss of membranes when ethanol extraction is used. Subsequent studies had shown that osmium did not adequately stabilize membranes against extraction. When I went back to acetone, I solved all the problems. Unfortunately you cannot use acetone all the time. 2. *En bloc* staining with uranyl acetate, as Marc Pypaert suggested. This is going back to the original paper on uranyl acetate staining by Stempek and Ward. It is certainly outlined in Dan Pease's book, where *en bloc* staining with uranyl acetate is a solution for membrane extraction. The explanation was that the uranyl acetate not only stained, it stabilized the internal membranes by addition of density so that they were not extracted by ethanol. In a sense it is a form of fixation. Now I use both wherever possible and get perfect membranes. When I can't (e.g., with LR White), I use uranyl acetate and have no problems. Unfortunately, it is a matter of old literature that has been lost today. Some of the old books from the '60's still have very good basic information. Hayat's 1st edition of Principles and Techniques is still excellent, and his two books on 1. fixation and 2. positive staining are, sadly, long out of print. I am lucky to have both, and they are borrowed

regularly by students. Paul R. Hazelton <paul_hazelton@umanitoba.ca> 01 Jun 2005

You don't mention whether you scrape the cells before processing or fix and process them in situ. This may affect the contrast because a pellet can be less easily penetrated by your chemicals. Neither do you say which resin you are using. Spurr resin will give you less contrast than Epon or an Epon substitute. There is a method used by Lou Tilney that supposedly produces good contrast when used on cells in culture dishes (see Tilney, L.G. and D.A. Portnoy (1989) Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, Listeria monocytogenes. J Cell Biol 109:1597-608 for details). I think he mixes glutaraldehyde with the osmium tetroxide in a buffer at pH 6.2, but maybe Pat Connelley could supply more details on this. However, the cells have to be processed in the dish. Other, less drastic things you can try are to use uranyl acetate as an *en bloc* stain, as suggested by Marc. It works really well when used at 0.5% to 1% in 50mM maleate buffer. Alternatively, make up a saturated solution of uranyl acetate in 70% methanol and leave your cells in this overnight. If you like the contrast produced by the reduced osmium, just work at removing the black precipitate. It is caused by a reaction between the phosphate buffer, glutaraldehyde and osmium. Wash the aldehyde and/or the phosphate buffer away and all will be well. Of course, you can also manipulate contrast with the electron microscope. A smaller objective aperture will give you more specimen contrast, but less resolution. However, if you are working for good specimen contrast, there is a high probability that you have low specimen resolution anyway - you are washing away and aggregating the intracellular components. Paul Webster <pwebster@hei.org> 01 Jun 2005

With tissue cultured cells I have found it critical to fix them as soon as possible after they come out of the incubator. Do not wash them! Simply decant the growth medium then immediately and gently flood the cells with the fixative. The fixative that works well on many different types of cultured cells as well as pelleted material and small tissue pieces is as follows: 1% glutaraldehyde + 1% osmium tetroxide + 0.05 M phosphate buffer, pH 6.2 pH, on ice and kept dark. Usually 45 minutes is fine. Yes, this fixative will start to oxidize. To avoid this have all the components on ice; mix in the glutaraldehyde immediately before the fixative is needed and allow sufficient volume to fix both the cells and the small amount of protein that is left on the cells after decanting the medium. With the phosphate buffer one needs to wash well with cold (best available) water at least 3 times over 20 - 30 min. time and remember to rinse the entire vessel (top too) at least once before adding cold 1% uranyl acetate in water overnight in the refrigerator to avoid the dreaded uranyl-phosphate crystals. If the cells are to be fixed in the flask/Petri dish for face-on sections, an ethanol dehydration is used and Ladd's LX-112 as an Epon substitute. These do not melt the plastic. All other cases or cells grown in "Permanox" dishes are acetone dehydrated and any Epon substitute can be used. If this fixation does not show what you wish with the membranes, try using an objective aperture that is a size smaller than is usually used in the TEM. Pat Connelly <psconnel@sas.upenn.edu> 01 Jun 2005

I had problems with osmium fixation of cell cultures when they grew on plastic membranes, and a nice lady from this list server suggested that I should reduce the osmium tetroxide with potassium ferrocyanide because the osmium reacts with the plastic polymer. We now grow the cells on "Aclar film" and get good membrane contrast, with or without reducing the osmium tetroxide. This film is supposed to be chemically insensitive and can withstand dehydration and embedding. The film has to be pretreated with poly-L-lysine to prevent the cells from floating away, though. Gerd Leitinger <gerd.leitinger@meduni-graz.at> 01 Jun 2005

You have gotten some good suggestions so far. I might add a few things: You should try the reduced osmium - potassium ferricyanide with cacodylate buffer instead of phosphate and you can get rid of the precipitate problem. Leave your cells in serum until you are ready to fix and keep them warm until ready to fix (right out of incubator). Sick cells will never look good no matter what you do. I embed cultured hippocampal neurons in Chang embedding molds then dissolve away the whole glass coverslip after embedding. Dissolve the coverslip with hydrofluoric acid (under hood) - that way you have the whole coverslip and lots of cells. If the coverslips are coated with Matrigel, they are harder to work with the nitrogen method. For thin sections, I stain in 5% aqueous uranyl acetate for 20 minutes or longer. Follow with a shorter (1-2 min) in fresh lead stain. I do not do a prolonged uranyl acetate stain *en bloc* - I microwave everything. Use a lower kV or smaller aperture to image cells. JoAnn Buchanan <redhair@stanford.edu> 01 Jun 2005

Try adding 1% tannic acid to your glutaraldehyde and possibly the osmium tetroxide. This helps with the membrane preservation. Philip Oshel <peoshel@wisc.edu> 01 Jun 2005

For membrane preservation, I use a fixation protocol given to me years ago by someone who studied photoreceptors (tons of membrane). Her recipes were as follows: Primary fix: 2.5% glutaraldehyde, 4% paraformaldehyde and 0.2% picric acid in 0.1M cacodylate buffer, pH 7.3. Post fix: 1% osmium tetroxide, 1.5% potassium ferricyanide (aqueous). I also *en bloc* stain with uranyl acetate. For the picric acid (my office of environmental health and safety just loves me), I keep the smallest jar available with the crystals fully covered with water. I use the resultant saturated solution in my primary fix (2 ml picric acid solution to 40 ml fix). The primary fix is based on one published by Somogyi and Takagi (1982) Neuroscience 7(7):1779-1783. Leona Cohen-Gould <lcgould@med.cornell.edu> 03 Jun 2005

SAMPLE PREPARATION – lung tissue

I have to embed some lung tissue into plastic and also prepare some for cryo-ultramicrotomy and immuno-gold labeling. I was wondering if I need to take some precautions to prevent collapse of the tissue during embedding and sectioning (especially cryosectioning). Marc Pypaert <marc.pypaert@yale.edu> 04 May 2005

What you need to do depends on what your concerns with collapse are. If you want to maintain the more than 80% of the lung that is air as empty space, you can freeze the lung while it is inflated. Freezing will be relatively slow, however. If you want to examine the structure of the cells and tissue components, you will get smaller ice voids if you allow the lung to collapse (become nearly airless) and freeze then. For plastic embedding, you can inflate the lung with liquid fixative; we use 2.3% glutaraldehyde in sodium cacodylate paying attention to the osmolarity, pH, and inflation pressure. Jacob Bastacky <jbastacky@chori.org> 05 May 2005

SAMPLE PREPARATION – hydrogel

I have a student who wishes to "measure the mesh size of a gelatin/maltodextrin hydrogel crosslinked with genipin. The sample is mostly water and has the texture of Jello." We have a Hitachi S-4700 FE-SEM, and are wondering what is the best way to prepare his samples to get the results that he wants. We don't have a critical point dryer, but do have access to a gold sputter coater. Patricia Scallion <pscallio@dal.ca> 25 May 2005

The best way to do this is with cryoSEM. You don't mention if you've got the equipment for this, but I suspect not -- most materials facilities don't. The next best choice is freeze-drying. Be sure to freeze small enough samples. If you don't have access to a high pressure freezer, then another good way is plunge-freezing into slush nitrogen, made by pulling a vacuum (with a high capacity pump) on liquid nitrogen. Then vacuum sublimate starting at about -90 °C. Leave until the pressure is <~6 microns Hg (or whatever the vapor pressure of water is at the temperature and vacuum you use. Vacuum should be ~10^{-5} torr -- diffusion pump or big rotary pump range. Make sure the vacuum system has a big throat and short, direct path to the pump, or better, a liquid nitrogen cold trap. Once you get below the vapor pressure of water, slowly raise the temperature, stopping if (when) the pressure goes above the vapor pressure of water

at that temperature. Continue to about -60 °C. Around here, the water of hydration and other bound water will start to come off. Be careful, this is where most specimen collapse happens. Once the pressure is again below the vapor pressure of water at this temperature, continue until about -40 °C. Pause if needed. Work you way up to -20 °C and let warm. This will likely take 24 to 48 hours. Critical point drying can be useful, if done carefully and correctly and thoroughly and the water is gotten out and the ethanol doesn't affect the gel and all of the ethanol is exchanged away in the critical point dryer with enough cycles of soaking and purging (meaning most manufacturers' directions I've seen are wrong). But cryoSEM is best and freeze-drying next best for true structure preservation of hydrogels. Philip Oshel <peoshel@wisc.edu> 25 May 2005

IMMUNOCYTOCHEMISTRY – ethanol permeabilization

I know that fixation in acetone permeabilizes tissue pieces so that one can do whole mount immunocytochemical staining of tissues measuring about 3 mm x 3 mm x 3 mm. I can't use acetone for a particular experiment and want to fix in ice cold ethanol. Does anybody know from experience if ethanol fixation permeabilizes the tissue enough for antibody access to intracellular epitopes? I don't want to use aldehyde fixes with Triton x-100 or saponin. Tom Phillips <phillipst@missouri.edu> 26 May 2005

There was a paper in the Journal of Histochemistry and Cytochemistry some time ago about using graded ethanols, buffered with phosphate, to permeabilize tissue for immunostaining. Eldred, W.D. et al (1983) J Histochem Cytochem 31:285-292. Versaux-Botter and Nguyen-Legros (1986) J Histochem Cytochem 34:743-747. Llewellyn-Smith and Minson (1992) J Histochem Cytochem 40:1741-1749. I tried the graded, buffered ethanol method some time ago on sections destined for pre-embedding immuno for TEM. It worked well and ultrastructure was tolerable. eoff McAuliffe <mcauliff@umdnj.edu> 27 May 2005

FLUORESENCE – Fiber-coupled light guides

I bought an old microscope that I am attempting to get in good working order. It came with the original epi fluorescence lamp house with a 200 W mercury lamp. Is it possible and would it make sense to remove the original lamp house and use a fiber coupled light source instead? Kathleen McMillan <km602223@comcast.net> 25 May 2005

There are advantages to using a fiber-coupled or liquid light guide light source; the primary benefit is a more even illumination of the field of view. The output coupling into the illumination system does need to be done correctly, however, and there is a drop in illumination intensity across the fiber. The reference below is pertinent: Zvi, K. et al. (1993). Design and construction of an optimal illumination system for quantitative wide-field multi-dimensional microscopy. Bioimaging 1:71-81. Karl Garsha <garsha@itg.uiuc.edu> 25 May 2005

DIGITAL IMAGING - Rolling shutter cameras

Can someone explain the how "rolling shutter" digital cameras are different? I'd especially like to know how this difference affects practical use for applications in microscopy. Michael Shaffer <michael@Shaffer.net> 15 Apr 2005

An electronic rolling shutter is similar to a drop-curtain shutter on a film camera. With a Rolling Shutter, only a few rows of pixels are exposed at one time. The camera builds a frame by reading out the most exposed row of pixels (and ceasing exposure of that row), starting exposure of the next unexposed row down in the Region of Interest (ROI), then repeating the process on the next most exposed row and continuing until the frame is complete. After the bottom row of the ROI starts its exposure, the process "rolls" to the top row of the ROI to begin exposure of the

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next frame's pixels. The exposure down each frame, and from frame-to-frame, remains consistent due to this continuous read-out. The row read-out rate is constant, so the longer the exposure setting, the greater the number of rows being exposed, or integrated, at a given time. Rows are added to the exposed area one at a time. The more time that a row spends being integrated, the greater the electrical charge built up in the row's pixels and the brighter the output pixels will be. As each fully exposed row is read out, another row is added to the set of rows being integrated. Rolling Shutter provides evenly exposed image data with the greatest possible speed. Because of its speed, a camera in rolling shutter mode is programmed to free-run, that is to sends frames across the bus as fast as it can. Each row of pixels has a slightly different exposure start and end times from the adjacent rows, so Rolling Shutter can produce a distorted effect when imaging fast moving subjects, even with very short exposure times. The distortion is due to the comparatively lengthy process of readout compared to exposure. As an example, if using a camera with a 6.6 megapixel sensor, to readout the entire frame requires approximately 250 milliseconds. While a short exposure may stop a moving object, the same object can move appreciably in the quarter second that it takes to readout the frame resulting in distortion in the direction of motion. The distortion will be less noticeable on sensors with faster readout times, smaller resolutions (fewer rows in the ROI) or if strobe/flash illumination is used. For best results, Rolling Shutter should be used with constant illumination and with a static subject. Michael McKay <mike.mckay@pixelink.com> 15 Apr 2005

EDS - Accuracy

What accuracy should I expect from my EDS system when analyzing non-conducting samples? I have consistently been getting a ratio of 1:2.5 for Si to O when analyzing a well-grounded piece of quartz. The applications guy for our system told me even if I coat the outside I will still get charging in the bulk which will skew my results. I tried to take into account the charging by looking at where the bremsstrahlung tailed off and adjusting the accelerating voltage by an appropriate amount. Still the results show an oxygen-to-silicon ratio greater than two. Pat McCurdy <pmccurdy@lamar.colostate.edu> 25 April 2005

I suspect that your experience illustrates more the problems with quantitative oxygen analysis (by EDS) than with the analysis of nonconducting samples. EDS can and does give very good quantitative analytical results, for elements from sodium up, for all sorts of silicates, most if not all of which are nonconducting. What oxygen standard(s) are you using? Ritchie Sims <r.sims@auckland.ac.nz> 27 Apr 2005

The quantification of the EDS results on an SEM is complicated and very dependant on many factors of the SEM, EDS system, and sample. The quantification of the very light elements is further complicated by the very soft nature of the x-rays, which means that not all of them are detected, and the very large correction factors that are calculated for atomic number and absorption for the elements below sodium on the periodic table. If your sample charges, then the apparent electron beam voltage drops as the sample builds up a negative charge, which changes the calculation of the correction factors. As a final problem, if the EDS detector gets contaminated with a film of oil from the SEM pumping system, the softer x-rays from the lighter elements get preferentially absorbed. I used to go from an oxygen peak half the height of the silicon on my SiO_2 standard, when the window was dirty, to an oxygen peak twice the height of the Si, after I had cleaned the window. Some questions: What is your EDS take-off angle? What is your EDS window material? Is your SiO_2 sample polished flat and exactly perpendicular to the beam? Is it coated with a thin layer of carbon to prevent charging or are you using variable pressure? Is your EDS window clean or can it be cleaned? Sometimes it is better to use your sample as a standard in the EDS system, than to try to get the EDS to produce the right numbers (standardless) for these materials containing very light elements. In answer to your question, not much accuracy. Mary Mager <mager@interchange.ubc.ca> 28 Apr 2005

Along these lines, I was recently asked if you could get an idea about stratification of different elements in a sample using EDS by adjusting the kV so that the beam would penetrate to different depths and then comparing the resulting spectra. The investigator expects that when a particular material (primarily light elements with some Zn and Mn of interest) dries down, some of the components will settle at different rates based on particle size and composition. He would be content with some very general data that would confirm or reject his theory. Is this possible or reasonable to get the desired information? Would Monte Carlo simulation be able to predict this type of information and help in determining the necessary sample thickness to make the results meaningful? Debby Sherman <dsherman@purdue.edu> 28 Apr 2005
Debbie, this is an inadvisable approach and will be potentially fraught with problems and inaccuracies. I certainly would only try this as an absolute last resort. There are much better and more accurate approaches. The simplest would be for your user to make a cross-section of the sample, (s)he can then image and analyze the respective strata by XEDS using conventional approaches, geometries and correction factors. Nestor J. Zaluzec <zaluzec@microscopy.com> 28 Apr 2005

Quantification of strata by HT variation does work but you must know some details about your sample ahead of time. Of course it is not an easy job and cross sections (on SEM or TEM) give a direct look at the specimen stratification. But a cross section is destructive! RBS or X-ray reflectometry are also useful methods but each with its own limits. To do that work using HT variation, one needs a way to theoretically model the interactions in the stratified sample. Software is available that can simu-

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late such situations, and with which one can determine the appropriate energies to pick data and fit these data. The software programs use $\Phi = f(\rho Z)$ models (ρ = density; Z = atomic number) to simulate what happens in a stratified sample and are able to calculate thickness of layers and composition of these layers. One must measure the k-ratio from the elements that are present. An unknown parameter, which can sometimes be difficult to evaluate, is the real density of film layer, which may be quite different from the bulk one. One can have the same element in different layers. The interfaces between layers are supposed to be steep, but in case of diffusion, or a gradient in composition, one can introduce one or a series of supplementary alloy layers. A flat sample, typically MBE or sputter coated layers on a polished substrate, give the best results but I know people in France that have successfully done such work on rough samples made by wet chemistry. The thickness range and the Z of the elements will determine the energy range to be used. What is easy is a comparison between samples in a series, where the absolute values can be verified by another method, e.g., cross section. One limitation is that the software can not yet model situations with particles included in a layers, like what can be done in Monte Carlo simulation. The steps of such analysis are the following: First, one describes the sample in the software, and calculates the k-ratio versus HV curves, which describe the variation of X-ray emission with the primary energy. One then selects the right energies to do the acquisitions. Of course, the better the description of the sample, the easier it will be to chose the conditions, and the more accurate the results will be. Two or three energies are enough. The application engineer of my software company says one energy is enough, but I prefer to use 2 or 3. Secondly, one acquires the spectra at these energies, and calculates the real k-ratio, using standard reference samples of the elements. This is a lot of work, because one needs accurate standards, which is not always easy. As Ritchie asked, what sample is a good standard for O, in particular when one must work at 3 or 5 keV, where a carbon layer on an oxide will be easily seen and will give a bad background shape on the low energy side of O-K? Using WDS will give much better results, but one must have one (!) and it's possible to do nice work with EDS too. (By the way, I work with EDS and cold FE-SEM, the most difficult situation!) One must only work in drastic conditions, with a long "time constant" of the acquiring chain, a clean detector, monitoring the beam current, re-polishing often each standard that could have an oxide layer, counting 300, 500 seconds or more at low energy to have a good signal to noise ratio, etc. Third, one puts the k-ratio into the software and runs the fit calculation. It's an iterative procedure, which will stabilize more or less quickly, depending of the good "tuning" between the description and the reality of the sample, and the accuracy of the measurements. But what is interesting, is that if one starts with different "false" descriptions of the sample, good measurements will converge to the same final situation. I've done such work for example on series of magnetic multilayers such as $Fe_{25}Ni_{25}Pt_{50}$ (nominal) 50 nm layers on MgO, after annealing. Another case was with FePt alloys on MgO, with a Pd or Pt cover layer and with or without a Pt buffer between the MgO substrate and the alloy. Thicknesses were 5 nm cover, 50 nm layer and 5-10 nm buffer. The results in one case were interesting: the sample should be 5 nm Pt, 50 nm PtFe, 10 nm Pt buffer, on MgO. The results were: one 61 nm layer of Fe_{43}-Pt_{56}! The buffer and the cover mixed during the annealing with the alloy layer. Of course, a gradient couldn't be seen. One must perform RBS for that. X-ray reflectometry was unable to detect that. In such examples, the density varies much with the Pt concentration. OK, that all is a lot of work, time consuming, and in some cases, when the combination line-energy/primary energy/depth don't match, or when one have multiple line superpositions (Pt-N with Pd-M and C-K!), it doesn't work. Last but not least, these software packages are expensive, and with a quite "relative" ergonomy! But they work, and it's what we need! Jacques Faerber <jacques.faerber@ipcms.u-strasbg.fr> 02 May 2005