Imaging Soil Mesofauna

The Land in Between

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This chapter will try to make some generalisations on current approaches to the imaging of small specimens. By that, we mean those with sizes lying typically between that of microphotography and that of macrophotography. These constraints do not necessarily mean a requirement for taxon-specific techniques. Rather, mainstream imaging procedures can be successfully used, although it is advisable to consider certain characteristics of the taxa in question. A case, or rather 'bookcase', study (i.e. different taxonomic groups) depicting our laboratory's procedures will act as a basis for discussing several issues.

Assemblages of the largest specimens can be pictured, though rarely, with SLR cameras fitted with a good macro lens. However, mesofauna are usually imaged under the stereomicroscope or by transmitted light microscopy. Preserved specimens are typically mounted on slides and imaged at low power under optical or scanning electron microscope, and details are captured at higher magnifications. Surfaces, and superficial features, are targeted more often than internal structures, and optical techniques are much more common for contrast improvement than chemical stains.

The main drive should be directed at ensuring a good scientific usability of the images. Thus, compromises will be needed where feature visibility, enhancement, and fidelity conflict with aesthetic qualities.

Soil Mesofauna

Soil mesofauna is the most diverse component of the soil ecosystem. Animals living among the litter and inside the microscopical crevices of the soil have a fundamental role as processors and translocators of the organic matter that ends up forming the humus. Many taxa are represented, including several orders of insects and their larvae, as well as Myriapoda, Crustacea, Thysanura, Tardigrada, and others. But three of them (Acari, Collembola, and Nematoda) dominate in terms of numerical abundance and diversity. A typical soil sample from a Mediterranean forest may contain several hundreds of different species, many of which have yet to be described. The least diverse of the three mentioned groups, Collembola ('springtails'), includes more than 7,600 known species: more than all mammal species, or three quarters of the known number of bird species. About fifty new species are described each year. And there are about twice as many known free-living species of Nematodes. It is suspected that the number of undiscovered species is very high in these groups (there are about two orders of magnitude less invertebrate taxonomists per invertebrate species than plant taxonomists per plant species), which warrants a high probability of many new type series needing to be imaged per year.

The wide spectrum of soil mesofauna means that a variety of handling and preparation procedures, as well as several observation and imaging techniques, are applied even within a group. Thus, the proposed "bookcase" study will have several tiers at times, where techniques differ for specific groups.

Case Study

Taxon Group: Main Soil Mesofauna Components (especially Phylum Nematoda; Phylum Arthropoda: Subclass Acari, Class Collembola)
Institution: Department of Zoology and Ecology, University of Navarra, Spain
Persons Responsible: Enrique Baquero and Rafael Jordana
Number of Person Hours per year devoted to imaging: Varies
Number of Images captured and stored each year: Varies

Purpose of Image

In most cases, images are for scientific use. Whole specimens and parts thereof are depicted that have taxonomic significance. Images are produced as they are needed to illustrate textual descriptions for taxonomic research papers; to build an iconographic bank for visual identification keys; to exchange information and queries amongst researchers; to detect and research taxonomically relevant features; to measure specimens or their features for taxonomical and ecological purposes; and to serve as basis for scientific drawings.

Target Audience

Mainly scientific, especially systematists/taxonomists, ecologists and field biologists.

Form

Originally intended for research, documentation and paper publications, conforming to scientific literature standards. However, copies of the primary pictures are also post-processed differently (i.e. resampled, resized and/or enhanced) for web use.

Choice of imaging technique

The imaging techniques to be used in soil mesofauna are, of necessity, varied both because of the large size range of the specimens and their taxonomic features, and their different tegumentary characteristics.

Stereomicroscopy provides the lowest level of detail, but is often the choice for living, large specimens. It allows colour pattern, which would otherwise be lost, to be retained (see fig. 2) and provides "natural" aspects of the specimen. However, even at maximum magnification it cannot reveal great detail for most mesofauna (see fig. 9 for a typical acari). Artificial enlargement of the image, i.e. by using a dense CCD, may aid visualization but perhaps not resolution, as the latter is fraught with other problems such as narrow depth of field and chromatic aberration, discussed elsewhere in this manual (ARIÑO & GALICIA, "Taxonomic-grade Images")].

Choice of imaging technique (cont'd.)

Optical microscopy is the technique used most often for soil meso-fauna. The usual set of objectives in most microscopes allows for almost the full range of features to be observed, from whole specimen to minute details less than one micron across. Good images, however, are notoriously difficult to obtain for the following reasons:

- *Specimens are observed by transmitted light but are mounted complete. Attenuation and dispersion of light is intense. Much tissue lies between the focal plane and the surface that obscures the observation. Also, many taxonomic features, such as setae, may lie along the Z-axis, making observation at a single focal plane pointless.*
- *Observations are usually made by juggling the focus knob to make a mental picture of the objects in 3D space, but the effect of this dynamic play can only be approximated by automontage techniques.*
- *As magnification increases, the definition of the image relative to the field size decreases as it approaches the dimension of the Airy function. High magnification images show poor detail.*
- *The frequent lack of contrasting features, staining, or other light-absorbing characteristics in the specimens forces the use of contrast enhancement techniques on the optical system. These almost always mean that the width of the light beam entering the objective has to be reduced, which has the effect of reducing the numerical aperture of the lens and, consequently, the image sharpness. There is a trade-off between the gain in feature visibility by contrast enhancing techniques and the loss of detail, which is normally much biased towards the former.*
- *The same contrasting techniques tend to increase the dynamic range of the images to the point where the sensors can have many pixels saturated, whereas other remain below response threshold. Once the full dynamic range is achieved, additional contrast visible to the eye is lost.*

Scanning electron microscopy (SEM) overcomes many of the above limitations but introduces others. It dramatically extends the depth of field above optical microscopy, and pushes the resolution power several orders of magnitude beyond (see fig. 7). But it can only be used on surfaces, under high vacuum, and requires special preparation of the specimens. It is often not possible to spare a type for this special preparation, which cannot be undone. In all, however, SEM should be attempted whenever possible to fully characterise a type.

Microarthropods
Collectively known as 'microarthropods', Acari (mites) and Collembola (springtails) along with other groups such as Thysanura or Tardigrada are extracted from soil samples by Berlesse-Tullgren funnels or high-gradient behavioural methods. Heptane flotation is our method of choice for soil samples already preserved by neutralised formalin (see BELASCOAIN et al., 1998 for a detailed description), although we have also used other flotation methods. In all cases, specimens are ultimately transferred to ethyl alcohol-based fixative fluid. Suction micropipettes, micropins, or single-bristle pencils are used for handling.

Acari	Collembola
Many Acarina, such as Oribatida, possess strongly chitinized, dark integuments that prevent observation by transparency at different depths. Thus, they are often bleached, usually with chloral hydrate or Nesbitt solution, to observe features at different planes by transmitted light.	Colour pattern can be a taxonomic character for some groups of springtails. As colours may fade in time if animals are preserved in the usual ethyl alcohol medium, pictures can be taken from unmounted specimens from the series at this stage during the type description process.

Dead specimens are almost invariably observed and imaged at this stage under the dissecting microscope while submerged in low-grade ethyl alcohol, unless selected for scanning electron microscopy (SEM) mounting.

Nematoda
Behavioural methods for nematodes include wet extraction on water funnels (FLEGG & HOOPER, 1970). Formalin preservation is done after killing the specimens by heat (HOOPER, 1986). Centrifugation in sugar gradient of already-preserved samples (CAVENESS & JENSEN, 1955) is the physical alternative, often carried out on samples from which microarthropods have been previously extracted by heptane flotation method (BELASCOÁIN et al., 1998). Specimens are generally not stained but treated with Nesbitt solution to improve their transparency, as in this case internals are taxonomically important. All handling involving individual transfers between treatments or onto slides is done with micropins or single-bristle pencils under the stereomicroscope.

Table 1. Isolation procedures for soil fauna.

Wish-list (unlimited budget and unlimited staff)

− to have all specimens from the type series optically sliced and digitized at several magnifications;
− to have a fine series of al least one specimen optically digitized with enough z-precision and contrast so as to be able to produce a workable, deconvoluted 3D-model;
− to have at least one specimen from each series imaged by scanning electron microscopy; and
− to have an image bank of all known and relevant taxonomic characters from the pooled type series.

Capturing

Handling Specimens

As most types are preserved, images can rarely be obtained from live soil mesofauna belonging to the type series. Extraction of live specimens is achieved by behavioural methods, with specimens usually collected on or in water, or taken to the laboratory together with their substrate or nutritious material (plants, fungi) when feasible. Imaging is then done directly under high-power macro lenses for masses of the largest specimens on their natural medium, or under the dissecting microscope as single organisms. Non-living extraction can be subsequent to behavioural methods, or is effected directly by physico-chemical procedures. Whichever mode of extraction is adopted, specimens are mounted on slides for detailed observation, and frequently for final storage as well. Table 1 describes the handling methods used for various taxa.

Mounting Specimens / Specimen Preparation

Methods depend on the type of observation procedure to be used (optical or electron microscopy) and the taxon type. Table 2 explains the possible mounting choices.

Optical microscopy
Specimens, often dehydrated by the Seinhorst procedure, are mounted on microscope slides in any of several mounting media (glycerin, glycerogelatin, Hoyer fluid, etc.) according to taxon type, and covered with a glass cover. These mounts are permanent or semi-permanent and constitute the basic storage and handling unit.

Microarthropods	Nematoda
Pre-mounting triage of the specimens under the dissecting microscope generally allows the preparation of slides of single species that can be accessioned once. Mapping the slide by establishing grid co-ordinates, or by encircling the specimen with a pen, is useful on the smallest mites, as these are often too small to be seen by the naked eye.	Mapping the slide by establishing grid co-ordinates, or by encircling the specimen with a pen, is most useful on nematodes, as they are not visible to the naked eye. The storage unit usually contains several specimens from various species; thus, the type series may be spread across different slides and each slide can contain both type series and other material. Generally, several accessions refer to the same storage unit.

Scanning electron microscopy
Specimens are cleaned in an ultrasonic water bath from residuals clinging to their surface. The adjustment of timing and power is delicate, as many thin structures such as setae may become loose.

Microarthropods	Nematoda
Microarthropods are dehydrated in ethyl alcohol series and dried by critical point drying (CPD) with carbon dioxide. Anti-static cloths are used to prevent dehydrated specimens from "jumping".	Nematodes have been treated by various methods, including critical point drying (CPD) with carbon dioxide and DMP method (WEYDA, 1992) followed by resin inclusion.

Specimens are mounted on aluminium SEM stubs with two-sided sticky tape. Large microarthropods can also be glued to the tip of micropins in order to separate them from the stub's surface. Temporary storage is effected inside a dehydrating jar.
Specimens are finally metal-coated by evaporated gold or gold-palladium.
Final storage is effected for each stub separately inside a plugged glass vial partially filled with dehydrating resin

Table 2. Mounting procedures for soil fauna.

Capture Devices

A number of combinations and techniques are in use (Table 3).

Optics	Macro	Optical Microscopes			SEM
Digitizers	Nikkor AF Micro ED (7)	Zeiss DV4 (8)	Leica MZ6 (9)	Olympus BX50 (10)	Zeiss DSM 940 A
Nikon D100 (1)	(A)				
Nikon Coolpix E995 v1.6 (2)		(B)	(C)	(E)	
Canon Powershot S45 (3)			(D)	(E)	
JVC KY-series (4)				(F)	
Point Electronic DISS 5 (5)					(G)
Agfa Arcus 1200 (6)	Digitising of chemical prints and slides				
HP Scanjet 5550c, 8200 (6)	Digitising of chemical prints				
(1) Single Lens Reflex (SLR) digital camera; 6.1 megapixels 23.7 x 15.6 mm CCD					
(2) Digital camera with integrated 8 – 32 mm (38 - 152 mm as 35mm equivalent) aspherical lens; 3.2 megapixels 7.2 x 5.3 mm CCD					
(3) Digital camera with integrated 7.1 – 21.3 mm (35 – 105 mm as 35mm equivalent) aspherical lens; 4 megapixels 7.2 x 5.3 mm CCD					
(4) 752-line video camera with ½" CCD sensor without lens; linked to Scion LG3 frame grabber through single (green) channel					
(5) Digital sensor for SEM, feeding directly from SE- and RE-detectors at preamplifier output for maximum 16k (x) by 16k (y) pixels					
(6) High resolution desktop scanners					
(7) 70-180 mm (105 – 240 mm as 35mm equivalent) f/4.5-5.6 macro lens					
(8) Stereomicroscope with 0.63x-4x aspherical lens system					
(9) Stereomicroscope with 0.63x-4x f/13 lens					
(10) Optical microscopes with phase and differential contrast systems					
(A) Masses or colonies of specimens on their substratum					
(B) Unmounted specimens shot through eyepiece tube with adapter					
(C) Unmounted specimens shot directly through phototube					
(D) Unmounted specimens shot through phototube with optical (eyepiece) adapter					
(E) Fixed specimens on slides shot through eyepiece tube with mechanical adapter					
(F) Fixed specimens on slides shot through phototube with optical (eyepiece) adapter					
(G) Carbon or carbon/gold-coated specimens					

Table 3. Combinations of imager and optics in use at the laboratory.

In addition, a number of chemical 35mm, 6" by 9", and 4" by 5"-Polaroid holders were used and still some are on various microscopes. Prints, slides or negatives are scanned with the appropriate adapters.

Optical microscopy images

See ABRAMOWITZ & DAVIDSON, 2003, for an excellent tutorial on basic microscopy techniques. Much of what is discussed here in terms of general optics comes from this tutorial.

Although heavily chitinized animals such as Oribatida can be observed with ordinary bright field techniques, high-magnification images of most soil mesofauna, which seldom absorb light, benefit from contrast enhancement by dark field, phase contrast or differential contrast microscopy at the time of capture.

In bright and dark field techniques, condenser settings, including its numerical aperture ('condenser diaphragm') and position, and field diaphragm settings, do not differ from the ones that would afford good observation contrast at the chosen magnification without the appearance of diffraction artefacts. It should be noted, however, that the amount of light should be better regulated by the use of filters, or the shutter speed of the camera, as opposed to the other alternative of manipulating the voltage of the filament. (It is not regulated by condenser or diaphragm settings, which influence the sharpness, numerical aperture and contrast ratio). Changing the voltage also changes the colour temperature of the image.

Phase contrast converts differences in refractive index into differences in phase of the light passing through the object, in such a way that the transmitted light and the diffracted light originating at the object can interfere destructively. The final result is that the image of the object acquires amplitude contrast, showing features darkened against a bright background. Good phase contrast images require a correct alignment of the phase annuli of condenser and objective, and benefit also from adequate selection of the amount of light illuminating the object.

Fig. 1. *Ypsilonellus similis*, female holotype, shot with four microscopic techniques. Clockwise from top left: ordinary brightfield; darkfield; differential interference contrast; phase contrast. Observable features vary among techniques. Field width 0.23 mm; pixel size 0.11 µm. White 5000°K light, unfiltered, Olympus BH50 microscope (except darkfield: Olypus Vanox); 40x objectives. Nikon E995 on phototube. Post-processing: colour balance; image resizing to SVGA; contrast enhancement and gamma adjustment. JPEG format.

Lighting

Images taken with SLR cameras are shot, whenever possible, with natural sunlight. Otherwise, either an electronic ring flash or a set of cold, full spectrum (three-phosphor) fluorescent lamps at 5000ºK are used. See ARIÑO & GALICIA in this book for details.

Objects under the stereomicroscope are illuminated depending on whether or not colour capture is necessary. Colour patterns are generally lost during the fixation procedure, and should be captured beforehand if taxonomically important. In this case, halogen-tungsten

filament dichroic lamps at high temperature setting (i.e. 5500°K) are used. Light is spread over the object by optic fibre channels. This produces a rich spectrum that is additionally filtered for excess red and infrared by light green glass.

Optical microscopy images (cont'd.)

The main limitations of phase contrast imaging in soil mesofauna arise from the thickness of the specimens. The technique works better with thin slices, as phase contrast from planes above or below affect the current focal plane. Also, bright artefact halos appear surrounding the image details that may saturate the sensor, erasing captured detail.

Differential interference contrast (DIC) virtually eliminates these halo artefacts. Contrast in DIC is produced by evaluating the length gradients of the optical path through the specimens resulting from density differences (the rate of change in the direction of wavefront shear), whereas in phase contrast the different densities that result in different optical path lengths yield different light magnitudes (denser objects appear darker). The optical gradients in the specimens are converted into intensity gradients. Polarized light and prisms are used, and the light spectrum is altered in such a way that specimens appear both with a shadowing that confers a 3D appearance and bright, selectable interference colours. The numerical aperture reduction is less than that of phase contrast, yielding better detail; but specimens having bi- or multi-refringent features such as mineralised parts cause interference with the polarized light, affecting resolution and creating artefacts.

Another unwanted effect of DIC is that since the tri-dimensional effect observed does not correspond to the actual 3D geometry of the specimen, its reconstruction by deconvolution techniques is hampered.

See fig. 1 for an image comparison between contrast enhancement techniques.

When colour is not important, objects are illuminated with 23-W PL-type compact fluorescent lamps in reflective aluminium mounts. These mounts are also used for routine work. Transillumination is used concurrently if appropriate for the specimen being pictured.

Shadows are often reduced by using at least three light points (optic fibres), six-point concentric lamps, or large-surface lamps (fluorescent and compact) and diffusive elements. See ARIÑO & GALICIA (this book), figure 18 (left), for a typical macroscopical stage with stereomicroscope, two fiberoptic lighting systems (ring and spotlights), camera mount and workstation.

Fig. 2. Live *Orchesella* (above, halogen white spotlight) and after decoloration (below, diffused compact fluorescent light, colour-corrected). Image pixel is 2 μm. Taken with Coolpix mounted on Zeiss stereomicroscope. Cropped from the original field width (4 mm).

Resolution

Camera images are invariably taken and stored at their maximum size and resolution, except for SEM digital images that are produced at 1 Mpx (square 1k by 1k pixels) size. Images from D100 are 3028 x 2018 pixels; Coolpix produces 2048 x 1536 pictures, and Powershot

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Size Range and Resolution

The size of soil fauna can spread across five orders of magnitude. The largest epiedaphic Collembola are about 10 mm long, although typically sizes range from 0.5 to 5 mm. Edaphic acari range from 0.2 to 1.5 mm, and free-living Nematoda from 0.1 to 2 mm in length and about one-tenth wide. Taxonomic features are generally much smaller but often can be of greater interest than the image of the whole animal. Microarthropod sensory setae can be less than 10 μm in diameter, and Nematoda taxonomic features or Collembola cuticle details may be less than one micron across. Thus, it is important to select an image size that is commensurate with the object being imaged (figs. 4 and 5), which in turn may influence the choice of observation technique (Table 3).

The degree of detail achieved will depend on the macro- or microscopic technique selected and the relative size of the pixel in the image. Conventional optical microscopy using white light has a resolution limit of about 250 nm (the Rayleigh limit, one-half the average wavelength). Although near-field scanning optical microscopy (NSOM) can increase the resolution power to ?/60 through the avoidance of the diffraction limit, in practice it does not seem to suit the heavily tridimensional nature of specimens. Laser scanning confocal microscopy could also achieve resolutions below the Abbe's limit in some fluorescence-dependent novel implementations, but seems perhaps little suited to fixed specimens where subcellular features are not of particular interest.

However, the diffraction limit is seldom reached in soil fauna. Even with a perfectly focused image plane, artefacts, chromatic aberration, dust, dispersion in thick specimens, or the necessary aperture reduction in several techniques, reduce the image definition. Numerical aperture (f-stop) in macro or stereomicroscopy must be used judiciously in a delicate trade-off between image definition at the focal plane, which increases with larger apertures, and depth of field, which increases with smaller apertures.

yields 2272 x 1704 pixels. Chemical prints are scanned at variable resolution, from 300 dpi minimum for plain 4x5 Polaroid plates up to a maximum of 4800 dpi for 35mm slides. The frame grabber captures video output from the G channel at 752 x 512 pixels. These resolution settings try to capture as much detail as it is necessary to characterise the specimen adequately, without excess, unnecessary information.

Size Range and Resolution (cont'd.)

Relative pixel size is the measure that the image pixel represents in the real object plane, and is directly dependent on the number of pixels into which the image is divided. The image of a one-millimetre line that completely and exactly fits the frame and is one thousand pixels across, will have an one-micrometre relative pixel size. Denser sensors (more megapixels) imaging the same field of view render smaller relative pixel sizes.

The interplay between resolution power at the optical system, and pixel size, can be worked out in terms of the digital image "sampling" the contrast of the analog image (SPRING et al., 2004). Features in the focal plane can be thought of as alternate areas of light and shadow. The smallest possible separation of these areas corresponds to the diameter of the Airy disk, which in turn is a representation of the diffraction limit. The Nyqvist theorem can thence be invoked by considering the Airy disks as sine waves to be sampled in the spatial domain instead of the temporal domain. It can be boiled down to having to sample the image with a "probe" which is at least twice as small as this resolution limit in any one dimension (2.5 times is a practical figure in microscopy), the 'probe' being the pixel size, to allow for a faithful reconstruction of the analog image on the sensor array. ampling at smaller frequencies (e.g. with a pixel size comparable to the Rayleigh limit) may introduce aliasing artefacts masquerading as real features.

Thence, we postulate that **digital images cannot show details finer than the resolution limit of the technique used, or twice the relative size of the pixel, whichever is greater.** *Figure 4 can aid in this selection for optical techniques. At high optical magnifications, having a large number of pixels in the CCD becomes irrelevant and 1 Mpx cameras may suffice.*

Although SEM images can capture huge amounts of data in a single frame, in practice shots at various magnifications are taken. See figure 6 for a comparison between optical and electron microscopy on mesofauna taxa.

Software

Capture software is dependent on the camera being used. Normally, cameras or internal camera software are directly controlled from workstations and their local storage facilities are disabled, the signal being transferred via USB or RGB to the workstation. These are in most cases Compaq EVO D510 with Windows NT4 Workstation or Windows XP Pro operating systems.

−   Nikon D100: Nikon Capture 3.0.0, USB transfer.
−   Canon Powershot S45: Canon Utilities RemoteCapture 2.6.0 with WIA controllers, USB transfer.
−   Nikon Coolpix E995: Images are captured directly on the camera CF card and read with a CF reader. Image control is done on the camera. Image monitoring is done on a SONY 15" Trinitron monitor fed by PAL composite video from the camera.
−   JVC analog camera/Scion LG3 FrameGrabber: Scion Image for Windows Beta 3B.
−   Point Electronics image system for SEM: DISS-5 for image capture and DIPS for processing.

Scale

Images from macro lens normally need an explicit scale, as the distances are variable. The scale is photographed at the same focal plane as the focused plane of the specimen, or, more generally, on the plane where the specimen lies. Pixel width is deducted from the scale by measuring it at post-processing. For fixed lenses, such as those of microscopes, a pre-tabulation of the objectives by a microscopic scale suffices for all pictures provided the objective used is recorded and the image is not resized.

Specimens that are imaged in 3D (i.e. SEM images of whole specimens at some angles, see fig. 7) cannot be scaled correctly at all points without parallax correction (see ARIÑO & GALICIA, this book).

Scales can only approximate dimensions, which must be calculated from landmark placement.
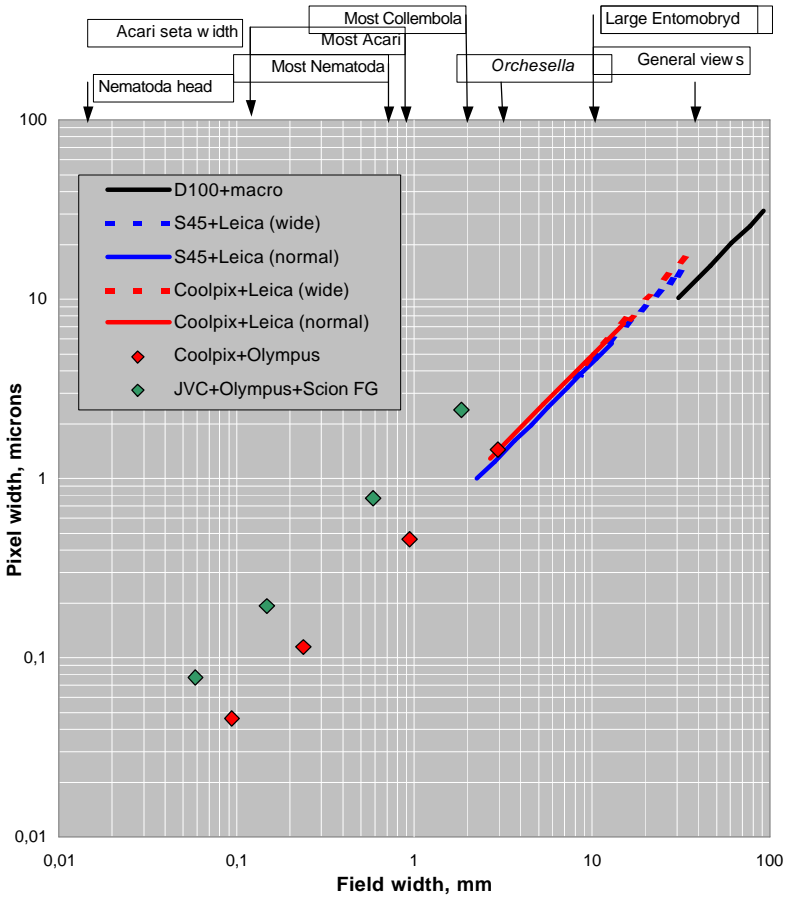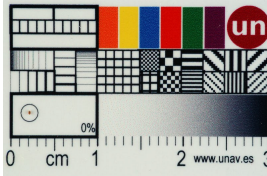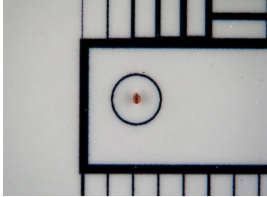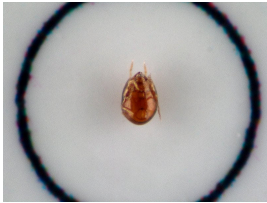

Fig. 4. Image width and ultimate pixel width for various combinations of imager and optics used in the case study. Note logarithmic axes.

Nikon D100 + 180 mm macro, 30.8 mm across, pixel 10.25 µm

Canon S45 + Leica stereomicroscope 10x, 10.5 mm across, pixel 4.64 µm

Canon S45 + Leica stereomicroscope 40x, 2.26 mm across, pixel 0.99 µm

Nikon E995 + Olympus BX50 microscope 100x, 1.03 mm across, pixel 0.5 µm. Insert corresponds to next image at current magnification.

Nikon E995 + Olympus BX50 microscope 400x, 0.23 mm across, pixel 0.11 µm (just below resolution limit of conventional optic microscopy).

Nikon E995 + Olympus BH50 microscope 1000x, 93 µm across, pixel 45 nm. Detail is limited by the microscope, not the camera.

Fig. 5. Images at various scales pertinent to mesofauna. Animals depicted are an oribatid mite (Acari) and   a nematode.

Wish-list (unlimited budget and unlimited staff)

As imaging of these animals involves a variety of observational techniques, future developments and ideal setups vary greatly.
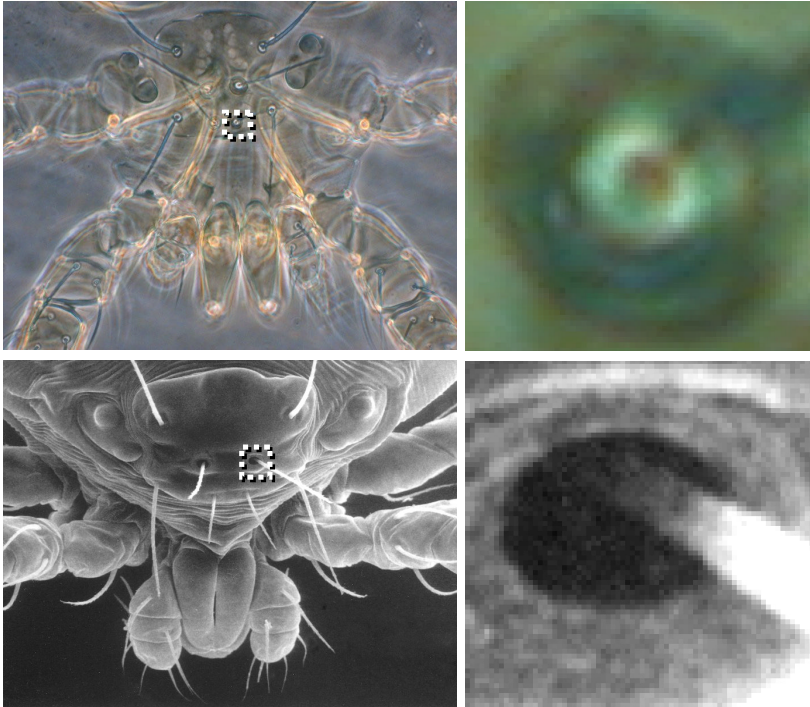

Fig. 6. *Polydiscia deuterosminthurus* (types), parasitic acari from a Collembola. Above: Nikon Coolpix E995 on Olympus BX50, 40x objective, phase contrast. Image 0.21 mm across, pixel size 101 nm. At right, blow-up of marked ROI, 66 pixels across. The smallest discernible features (bothridium) appear to be about five pixels (ca. 500 nm) across, which is congruent with the Abbe limit of the technique. Below: Same region captured on Zeiss DSM 940A SEM, Polaroid plate scanned with Agfa Arcus 1200. Image 0.15 mm across, pixel 74 nm. At right, blow-up of same ROI, 86 pixels across. Resolvable details are about 2-3 pixels (150-210 nm) for similar magnification.

Stereomicroscopy could benefit from fully planapochromatic optical trains with extreme high quality lenses. At typical maximum magnifi-

cation of 40x at the microscope, chromatic aberration may easily appear near the fringes of the image if the camera is used together with its own lens at full zoom in order to enlarge typically small specimens. Lens-less cameras with large CCD sizes coupled with high-magnification ocular lenses could greatly reduce the effect, at the cost of less definition in terms of pixel size.

SEM specimen preparation

Scanning electron microscopy images are becoming a very useful tool for the taxonomy of soil fauna. The ability to produce images at a very wide range of magnifications, with very fine detail and a large depth of field, enables the examination of taxonomically important surface features impossible by light microscopy. The technique, however, is still quite expensive and complicated compared with light microscopy. This prevents its routine use, but not its use for imaging type specimens, which, naturally, should warrant enough investment.

Notwithstanding special cases (live specimens at very low magnification in the so-called "environmental SEM"), successful SEM images can only be obtained if the full process of sample preparation and observation is done carefully. Clean (often ultrasonicated) specimens must be prepared to withstand high vacuum both during coating and during observation. Except for heavily chitinized taxa such as Oribatida, which can be dried directly, or morphologically compact animals having relatively impermeable cuticles, such as some Nematoda, that can be prepared by internal inclusion into resin, most soil fauna specimens have very fragile or soft teguments, and direct exposure to vacuum collapses them both from turgescence loss and from surface tension effects that appear when the water evaporates. The specimen must be completely dehydrated before entering the vacuum chambers; it could otherwise explode and/or prevent or delay the high vacuum to be achieved. But this must be achieved while ensuring that the specimen does not lose its morphological or taxonomic detail. A good, canonical histological fixation of soft specimens significantly contributes to good imaging.

A motorised, automatic stage would also ease the task of obtaining the frames of the stack. Although accurate Z-movement can be achieved by using the vernier markings of the focus knob, and auto-

montage algorithms are more robust against differences in slice position than deconvolution algorithms, a precise, repeatable positioning could improve the final performance.
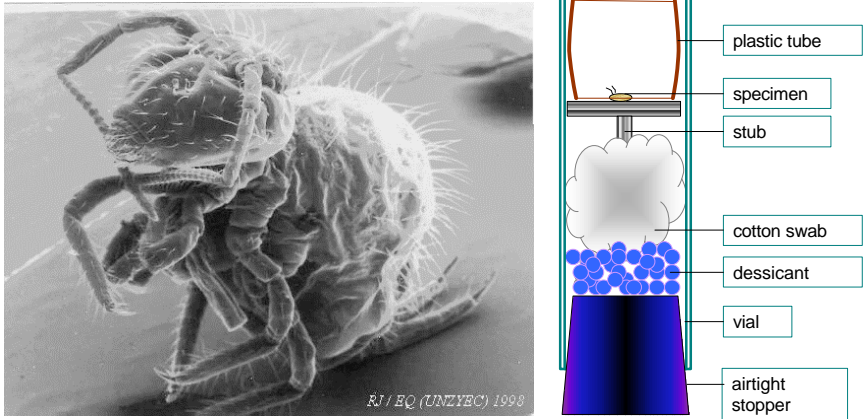

Fig. 7. A specimen of the Collembolan *Sminthurus viridis*, placed on the SEM stub at the angle adequate for imaging the feature of interest. Length of the animal about 1.5 mm. At right, individual desiccation and storage chamber for this type of mounts.

Alternate microscope techniques could be explored for new, better, or complementary imaging. Currently, observation and imaging of specimens is mostly dependent on contrast-enhancement techniques at the microscope: phase contrast and differential interference contrast. A move to confocal microscopy could potentially render well-focused images or 3D models of the internal structures of the animals, perhaps bridging conventional microscopy and scanning electron microscopy. It remains to be seen whether these techniques, which are best suited to fluorescence microscopy, could be widely used in the taxonomic field (where most imaging is done on already preserved and often mounted thick specimens) at an advantage over scanning electron microscopy. At least, image processing based on deconvolution techniques related to the ones used in these two fields might potentially be investigated in order to remove the strong brightness artefacts that badly hamper current automontage algorithms.

SEM specimen preparation (cont'd.)

Members of most taxa must undergo histological dehydration through the usual alcohol series. Critical point drying usually follows this process. The specimens' alcoholic fluids undergo a new change into liquid CO_2, either directly or through an intermediate change in acetone. This is done inside a pressure chamber at 40 bar. Once all alcohol (or acetone) has been thus removed, the chamber is heated (and pressure increased correspondingly) until the critical point of carbon dioxide is surpassed. At this moment, liquid becomes gas by phase transition, and no internal pressure or surface tension develops in the specimens. The chamber is flushed slowly, maintaining the gaseous phase, until atmospheric pressure at which point it can be opened. It is of paramount importance to prevent the gas phase reverting to liquid while flushing.

At this point, specimens are extremely light and are very delicate and sensitive to static charges. A static-free environment helps avoiding the specimens from "jumping" and being lost. Free air manipulation must now be kept to a minimum, and specimens must be stored inside dehydrating chambers. This allows for permanent archiving of the specimen itself, rather than just the pictures, as it was common in the past.

Dry animals are glued to an aluminium stub in a delicate operation performed under the stereomicroscope. Good observation can be guaranteed only if the interesting features are well exposed to subsequent coating and scan beam, and do not lie towards the stub surface. Often a small pole, such as the tip of an entomological pin or a deformable aluminium tape, is planted on the stub and the specimen is glued to the tip to ensure the widest possible coating and observation angle. The specimen is coated with a 8-20 nm layer of gold or gold-palladium in a sputter-coater. The thickness of this layer can be controlled, and contributes to the quality of the image. A thick layer can obscure details, but can also help the specimen to withstand thermal damage by the electron beam and improve conductivity. Although coated specimens are less prone to damage by environmental humidity, they are also stored in individual desiccation chambers (see fig. 7).

Another approach worth exploring would be any novel development in NSOM-based imaging for very small features. Currently, the

need for the probe to stay within a few nanometres of the sample seems to prevent its use with mounted or 3D complex specimens.

If storage space and time were not an issue, current SEM systems could produce extremely detailed slow scans up to 256 Mpx. However, other scan parameters such as voltage, coating thickness and beam alignment can affect image quality much more than the mere pixel size.

Working with Images

Storage Formats

Two basic image formats are used. Master images are stored as they are captured, in raw format (.NEF, .CRW, .TIFF) without any postprocessing, and copied onto removable media (CD-R and DVD-R). Secondary (working) images are produced from masters, at various levels of compression and resolution depending upon the intended use, and stored online. Table 4 shows the formats according to their source and purpose. Typical file sizes are about 4-9 MB for compressed masters, 1-2 MB for working files, and 400 KB, 100 KB, 40 KB and 10 KB for web varieties.

Software

Working software falls into three categories: post-processing, calculations, and storage/databasing management.

− Post-processing (cropping, enhancing, colour correction, filtering, automontage) is done with standard image packages such as Photoshop®, or specific-purpose analysers such as Image-J or CombineZ.
− Calculations on images are done with morphometrics packages such as MORPHEUS.
− Bulk managing and processing (batch resizing, trans-formatting, compressing, renaming), as well as examination, is done with an image database application (ThumbsPlus) or image managers (IrfanView). Metadata about images are captured by database managers directly from the database files created by image database (ThumbsPlus) or by EXIF extractors (ImageMagick).

Images are mounted generally with document editors in the context of their intended output or production document (Word, PowerPoint, Publisher, Dreamweaver, Flash, Acrobat).

Digitizer	Mas-ter	Working	Publica-tion	FTP download	Web-inline	Catalog thumbnail (large)	Catalog thumbnail (small)
Nikon D100	NEF	Com-pressed TIFF	TIFF	95% JPEG	80% JPEG resized to SVGA (800x600)	70% JPEG resized to QVGA (320x240)	GIF resized to 1/8 (160x120)
Canon Power-shot S45	CRW	Com-pressed TIFF	TIFF	95% JPEG	80% JPEG resized to SVGA (800x600)	70% JPEG resized to QVGA (320x240)	GIF resized to 1/8 (160x120)
Nikon Coolpix E995	TIFF	Com-pressed TIFF	TIFF	95% JPEG	80% JPEG resized to SVGA (800x600)	70% JPEG resized to QVGA (320x240)	GIF resized to 1/8 (160x120)
JVC + LG3	BMP	GIF	TIFF	95% JPEG	80% JPEG	70% JPEG resized to QVGA (320x240)	GIF resized to 1/8 (160x120)
DISS	TIFF	Com-pressed TIFF	TIFF	95% JPEG	80% JPEG resized to SVGA (800x600)	70% JPEG resized to QVGA (320x240)	GIF resized to 1/8 (160x120)

Table 4. Storage formats for the images produced. NEF: raw sensor output from Nikon DSLR; CRW: raw sensor output from Canon DC.

Linking to/within Databases

Images are not included in a database, but kept in directories under standardised file names including the accession number of the items. Two separate file systems are used: a NTFS namespace for the raw and working files, and a SMBFS that an Apache webserver uses for serving web-versions of the images.

Metadata from images, including pointers to the actual files, are included in a database that links to the specimen database of the Museum through the specimen's ID which is part of the file name of each image.

Imaging in SEM

Virtually any taxonomic work with the scanning electron microscope requires imaging. This instrument was originally designed to capture the image on film by slow scan, and the imaging techniques are well known. Good manuals exist for general SEM.

A good SEM image in taxonomy should show clearly the features of interest and not lack sharpness or quality, should have no extraneous noise, and should not show deformation or distortion of the specimen. Many factors may lead to the undesirable effects. A partial list would include inadequate photographic settings (focus, focal depth, contrast, brightness); improper accelerating voltage, probe diameter and current intensity, or astigmatism correction, equivalent to bad lighting conditions in optical microscopy; improper or off-centred objective aperture; instabilities on accelerating voltage or gun emissions (inadequate heating of filament); discharge of detector and column interior charge-up which may induce image drift; improper positional relation between specimen and detector or specimen tilting; excessive photomultiplier gain; or mechanical vibration.

These glitches are applicable to all types of objects. However, some factors seem to be particularly relevant in soil fauna, such as deformation of a specimen during its preparation, avoidable by careful dehydration and handling procedures (especially after critical point drying); dirtiness; charge-up of specimen surface, which may result from uneven metal coating due to the very complex spatial structure (hairs, bristles, scales) allowing for poorly coated spots that may break the electrical continuity of the specimen; or electron beam damage, due to local heating. Poorly transmitting heath, specimens with inadequate coating frequently do not resist damage caused by the well-focalized, high-voltage, intense beams that allow for fine detail or focus. It is often necessary to reduce voltage and current, and/or to scan rapidly with a shorter exposure time, in order to avoid damage to the specimen. This is of paramount importance when imaging type series specimens. But all these operations have the effect of reducing the overall quality of the image, resulting in poorer definition, sharpness and contrast, as well as more noise. Ideal images from these animals, thus, are usually below the standards for more robust insects.

Enhancing Images

Original ('master') images are always stored without any enhancing or post-processing, in raw (i.e. direct sensor output) format, normally off-line because of their large size. Several masters are taken and stored from one object, although only selected ones proceed to the working stage. From each master labelled as working, copies are produced that are enhanced if necessary, as long as this enhancement does not degrade the scientific validity of the image These include:

-   Cropping. Image is cropped to eliminate irrelevant regions and reduce online size, especially when using fixed focal length optics such as microscope objectives that capture fixed-width fields.
-   Generally, grey scale images are expanded to the full dynamic range by histogram equalisation. In addition, gamma values are adjusted subjectively.
-   Colour correction is often not necessary, as monochrome or green-channel images are widely used.
-   Multi-image stacks can be occasionally deconvoluted or FFT-filtered to remove random noise. However, filters are used very parsimoniously. They may enhance differences in detail, but also introduce artefacts: all filters change the brightness or colour values of each pixel. Conversely, some filters may erase details altogether. There is no given rule as to which filters produce a better view of already-existing features; trial and error (and careful cross-examination of the resulting image against its master) are necessary for each image being enhanced.
-   Some images, especially SEM scans, are artificially coloured to highlight interesting features. This is done on 24-bit RGB copies of the raw, greyscale TIFFs. The specimen's background is negatively masked and the image is assigned a given hue, without any change of lightness. A wand-type selection tool is then used to encircle the ROI, and its colour is changed to a contrasting (or complementary) one, by inversion or any additive or XOR filter. Finally, the masked background is often filtered with edge-sensitive median filter and assigned a third, neutral hue. Figure 8 shows an example. It should be noted, however, that we generally publish the original, greyscale pictures in scientific papers

and only colour them for more popular outlets or web site posting.
- Publication images are finally resized and compressed according to the intended destination (web, paper).

Fig. 8. *Vesicephalus europaeus* ARDANAZ & POZO, 1985 enhanced to show features of interest, suitable for popular publication. Masking and darkening have neutralized the background, and the specimen has been colorized to further detach it. The newly discovered photosensitive vesicles are shown in yellow to mark their location on the head relative to the known eyes (in blue). Scanned from a Polaroid plate. Image width is 1018 pixels spanning 0.73 mm (pixel width 0.71 µm).

Automontage

As automontage algorithms are not standard, different programs (ImageJ Stack Focuser class; CombineZ v.4.6, or AstroStack 3) are used. A stack is produced of several (in practice, up to a dozen) images from evenly distributed focal planes, i.e., fixed positions in the focus vernier knob. Images in the stack are checked for alignment, magnification and orientation, and matched and corrected if necessary by landmark placement. Several patch sizes between 4 and 25 pixels are tested, and the best overall result is selected.

This procedure is restricted to a few cases ideally suitable for the technique; in most cases, specific focal planes are preferable as images, for individual details are often more important than the overview. There are certain limitations to the technique that are very apparent in soil mesofauna specimens, especially related to the relative position of taxonomic features.

Quality Control

On-screen inspection of the master images against the visual counterpart at the microscope, and comparing the masters and the final deliverable images, are still the best QC check in use. Comparisons need to be made especially regarding the accuracy and visibility of taxonomically relevant characters. In automounted images, QC also involves correct alignment of patches. With SLR or compact cameras using their own lenses (D100, S45, E995) EXIF data are checked in order to ensure a correct focal length match between the image and that of the tabulating ruler.

Naming Images

Current naming conventions for images include the accession number of the specimen and various prefixes and suffixes denoting species, optics, magnification, and feature. These data are also included as metadata in the database.

Automontage in soil mesofauna

Montage techniques attempt to select well-contrasted patches from a stack of optical slices, generally by running Sobel filters, and assigning them a given depth in 3D space. A particular set of pixels in the image has to be chosen from the available slices, according to either its inferred depth or its relative contrast. Patches are stitched together in the final image, which is essentially a mosaic formed from well-focused pieces chosen from the slices forming the stack. Refinement at the position of the stitches, somewhat sophisticated in high-end packages such as Syncroscopy's AutoMontage, can result in a smooth, almost seamless image if the specimen is adequate for this technique.

High-magnification images of soil fauna are often inadequate for automontage. The imaging techniques used, especially phase contrast and differential contrast, produce very large lighting and position differences between focal planes, easily confusing this algorithm.

In addition, most soil mesofauna specimens are observed by transparency at different focal planes. Phase contrast and DIC allow for good focusing and separation of features: the ones on the focal plane being observed, i.e. the underside of the specimen, are clearly visible whereas features in the opposite side of the comparatively thick specimen remain totally blurred and are not discernible. After automounting, patches from all focal planes collapse into one, and features on one side 'seem to appear' along others in the opposite side. This can be confusing, especially in animals where the relative position and placement of the hairs and bristles (the chaetotaxy) is important for their characterisation. See fig. 9 for an example.

However, high-contrast specimens imaged with standard microscopy at medium or low magnifications can be automounted after some preparation of the master images, especially size matching and alignment, often by selecting a small portion (e.g. from dorsal view to middle view) of the stack (see fig. 10).

Metadata Information

Metadata should include all relevant data about the picture object and circumstance that are not to be found in other tables of the database such as the specimen table or taxon table. A partial field list includes:

− ID: Filename; GUID.
− Object: Accession number; Series number of the specimen (in multi-specimen accessions); Aspect, if applicable (i.e. dorsal, ventral); Target feature or part.
− Optics: Type of scope; Lens; Objective; Zoom; Contrast mode; Aperture; Voltage (SEM); Eyepiece; In-camera zoom.
− Camera: Type of camera; Mount position; EXIF Table Camera Fields.
− Take: EXIF Table Take Fields.
− Image: Size; Pixel Width; Original Colour Depth; Gamma.
− History: Parent filename (for derived images); Series number (for stack frames); Original size; Post-processing sequence (filters, adjustments, feature enhancing, colour correction).
− IPR: Photographer; Date of take; Post-processor; Date of change; Copyright date; Release policy; Permissions.

IPR policy for Images

There is no common IPR policy for images, although some rules apply. Copyright is always enacted for the photographer. Images for publication on paper are released to the publisher as mounted copies of the masters and IPR retained in all cases. Downsampled images released through the web server retain IPR but their copy or reposting is allowed, provided that the IPR notice is kept and a backlink is included to the original location, for scientific or educational purposes. Any commercial usage of any image is subject to a written agreement and fee or royalty payment.

Wish-list (unlimited budget and unlimited staff)

Some good automontage software packages run into the K$s (i.e. AutoMontage by Syncroscopy). Although less expensive, or even free, software (i.e. AstroStack, CombineZ, ImageJ) can attain similar effects, the routine usability and throughput of the more expensive programs seems better. Any automontage program, however, cannot be used successfully in many microscopy images of the types adequate for soil animals without prior extensive retouching and masking of the frames, which must be done by hand. Thus, adequately

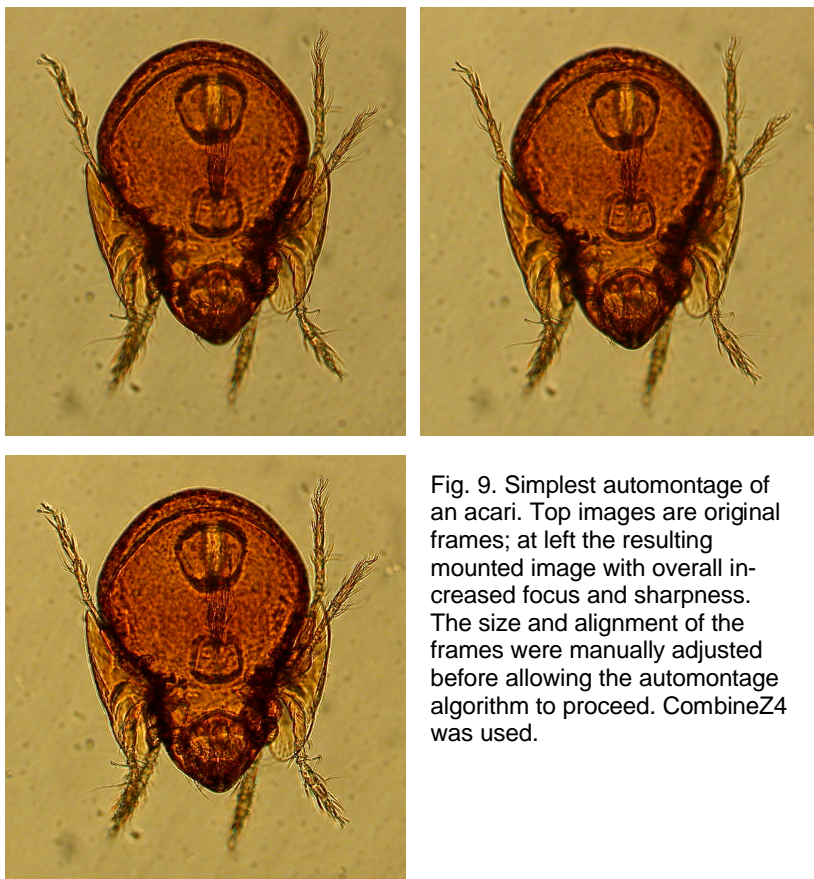trained staff are probably the most interesting investment in the production of quality images.

Fig. 9. Simplest automontage of an acari. Top images are original frames; at left the resulting mounted image with overall increased focus and sharpness. The size and alignment of the frames were manually adjusted before allowing the automontage algorithm to proceed. CombineZ4 was used.

Another immediate development should probably deal with image format. The relatively new JPEG-2000 standard allows a much more versatile image management in a distributed environment (see MORRIS, this book). Both the ability to define and hyperlink regions of interest within the picture, and the "lossless" option of this format, could lead to a new concept of image storage and annotation.

An affordable image server having the ability to produce and serve on-demand both scaled-down versions of the full images and full-quality ROIs based on that format could also be of much interest.
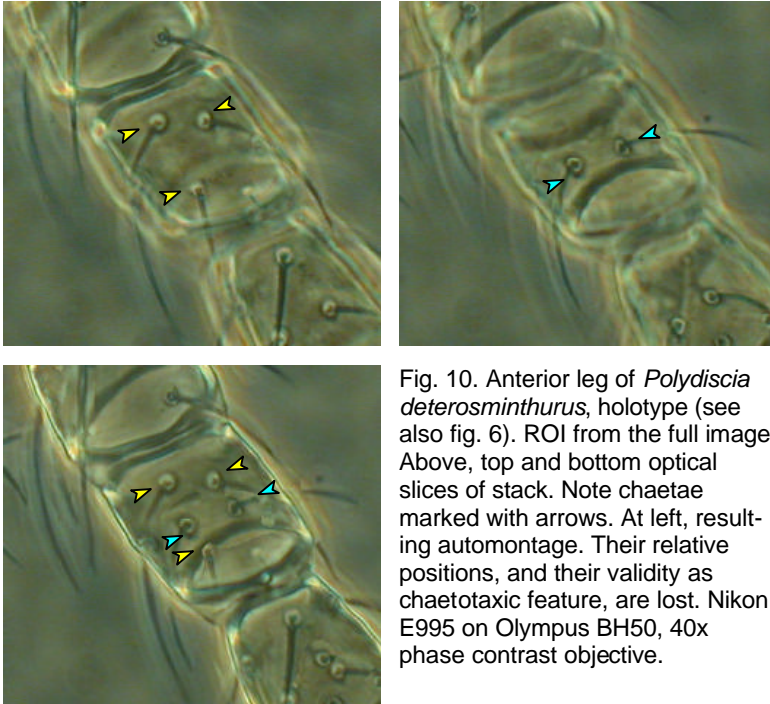

Fig. 10. Anterior leg of *Polydiscia deterosminthurus*, holotype (see also fig. 6). ROI from the full image. Above, top and bottom optical slices of stack. Note chaetae marked with arrows. At left, resulting automontage. Their relative positions, and their validity as chaetotaxic feature, are lost. Nikon E995 on Olympus BH50, 40x phase contrast objective.

Maintaining databases

Master databases are kept in-house, although copies are integrated within the general server system of the Institution. In general, photographers are the researchers themselves and they are also in charge of entering the metadata into the database. However, design, maintenance, backup and migration of the databases is done on a more comprehensive, general level by the systems administrator and DB manager.

The master DB resides in an NTFS and requires authorised access.

Back up

Master databases are fully and automatically copied by pro-grammed tasks twice a day via LAN into four alternating backup servers. At any moment, an original and four copies with a half-day lag between them are available on-line in order to prevent logical damage, breakdown or error propagation. Discretional copies are also made irregularly to a fifth backup server before major changes. Copies of the masters for web publication are maintained in a RAID system that also follows its own daily copy routine. Database status and access is logged daily. Deletions and additions are journalled, and changes are ID- and time-stamped. Additionally, permanent, weekly snapshots of the entire modified tables are produced and stored both online and off-line. Current off-line backup method is CD-R, although MFM and ZIP were also used. Weekly CDs are stored outside the premises of the laboratory in order to prevent loss by local, physical disaster. Every six months a full copy of the DB is burned onto removable media and sent to a safe place in a distant city in order to prevent loss from natural disaster.

Images themselves are also stored at a central NTFS file server, and copied daily into two alternate backup servers. Every file that is created or modified is also copied into CD-R and stored outside. Master, raw images are also copied into DVD for primary storage after working copies have been produced.

Storage capacity

Currently the system reserves 160 GB from each of the primary and backup servers, but this includes the ongoing imaging of speci-mens as well as that of type series. Off-line storage is essentially unlimited. At the time of writing the off-line accumulated storage mass (including all filetypes) is 237 CD-R in compressed format.

Maintaining links

A policy of dead link search is enacted irregularly as a part of the QC check.

Linking to other systems

The intradepartmental network is linked to the general, institutional network through routers. A copy of the database and files is fed to a separate file server that includes a web server for outside access. In addition, partial copies of the databases are fed to the GBIF node in Madrid, Spain.

Wish-list (unlimited budget and unlimited staff)

The overhead of maintaining and cleaning-up the databases and image files increases with the size and number of files. Dedicated staff, including DB managers, would be a good, though not cheap, investment.

Acknowledgements

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