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Review

# Some trends in microscope image processing

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**Abstract**

The present review tries to identify some trends among the multitude of ways followed by image processing developments in the field of microscopy. Nine topics were selected. They cover the fields of: signal processing, statistical analysis, artificial intelligence, three-dimensional microscopy, multidimensional microscopy, multimodality microscopy, theory, simulation and multidisciplinarity. A specific topic is dedicated to a trend towards semi-automation instead of full automation in image processing.


*Keywords:* Microscope image; Image processing; Statistics; Multidisciplinarity; Simulation; Multidimensional microscopy

**Contents**



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## 1. Introduction

The origin of image processing can be traced back the middle of the 20th century. The application of this activity to microscope images started with trials to improve the quality of images (improvement of the signal-to-noise ratio and of contrast, image restoration) through frequency filtering. Of course, as for any application, the real developments in microscopy started when analogue image processing could be replaced by digital image processing, and especially when computers became sufficiently powerful to apply sophisticated algorithms to large images within a reasonable amount of time.

The aim of this review is not to describe the state-of-the-art in this discipline,[1] nor to take stock of image processing in microscopy. It is, rather, to give an overview of some current trends, and to describe how image processing will evolve during the next decade(s). Of course, although some views described below will probably be shared by many people working in this field, this presentation is necessarily personal and subjective.

For this presentation, I have selected nine topics, which I will briefly enumerate in this introduction, before examining them more deeply in the following sections. These trends are those in which I am personally involved (except number 6). There are doubtless others that could also be listed and developed.

### *1.1. Trend number 1: signal processing*

Some image processing tools will be based on much more elaborate methods of signal processing than those employed in the past. More specifically, local methods (such as wavelets) will probably replace global methods. As a consequence, methods for performing image analysis and image improvement/restoration (in terms of signal-to-noise and/or contrast) will become adaptive, which is a great improvement over non-adaptive techniques.

### *1.2. Trend number 2: statistical methods*

Other image processing tools will be based on new statistical methods. Besides the Bayes' theory and the maximum likelihood procedures, robust statistics and order statistics will start to play an important role. Statistics based on the theory of information (maximum entropy, cross-entropy, etc.) will also increase in importance.

### *1.3. Trend number 3: artificial intelligence*

Some image processing tools will be based on methods originating from the fields of pattern recognition and artificial intelligence. Neural networks and expert systems will play an increasing part. Automatic classification, in the supervised or the unsupervised mode, will become more important when certain experimental techniques currently under development come into routine use. The same is true for data fusion, which consists in combining the information provided by two or more different microscope imaging modalities. Data fusion can be performed under the general framework of multivalued logic, including fuzzy logic and other variants.

### *1.4. Trend number 4: multidimensional imaging*

Microscope imaging is rapidly moving from a two-dimensional (2D) space towards a three-dimensional (3D) one. This fact tends to annihilate the main criticism of philosophers concerning imaging, which was considered as a flattening of the real universe. The 3D reconstruction of an object from a series of 2D images (serial sections or tilt series) can now be performed from most microscope imaging modalities (wide field optical microscopy, confocal microscopy, transmission electron microscopy, etc.). This constitutes one of the great successes of the image processing community. Now, the next step consists in generalising to 3D images the methods developed for processing and analysing 2D images: restoration, segmentation, quantification, etc. This is also a meeting point between the community of digital image processing and the community of scientists working on image synthesis, visualisation and modelling.

'Simple' imaging will be more and more often replaced by multidimensional imaging. Besides the 3D imaging mentioned above, this trend concerns the acquisition of images sequences, as a function of time (time-resolved imaging, or chrono-imaging) or as a function of energy/wavelength (multispectral imaging). The weakest form of multispectral imaging, colour imaging, has been used for a long time in optical microscopy. New forms of multispectral imaging are now appearing: spectrum imaging (or a variant of it: image-spectroscopy), where a complete spectrum is recorded for each pixel in the image, combines the useful properties of spectroscopy and imaging.

New tools for data processing/analysis have to be developed so that the huge amount of information contained in such data sets can be extracted and fully exploited. Multivariate statistical analysis, for instance, will be more and more used.

### *1.5. Trend number 5: multimodal imaging*

Simple microscope imaging is also evolving towards multimodality imaging. This approach, sometimes called collaborative microscopy, consists in trying to investigate a complex reality through different complementary microscope imaging approaches. The combination may

[1] Very few attempts have been made to do it, except perhaps in electron microscopy (Hawkes, 1980, 1988).

concern different scale levels (from optical microscopy to electron microscopy through confocal microscopy), different points of view of the specimen (topography with near-field microscopy, interior with transmission electron microscopy) or the capture of physical versus chemical information (electron microscopy/microanalysis, phase contrast optical microscopy/fluorescence microscopy).

### *1.6. Trend number 6: on the side of the theory*

Several theories have been developed during the youthful period of image processing. Some of these theories were based on linear signal processing theory while others were highly non-linear (mathematical morphology is one example). Nowadays, we are witnessing a unification of some of these theories into a more general framework. Image algebra is one result of such a unifying process.

### *1.7. Trend number 7: simulation*

Besides image processing/analysis, another way of extracting information from images consists in comparing experimental images and image series to simulated images/image series. This approach, which we can name a modelling approach, is not new in some fields of physics, chemistry and biology. For imaging, this approach is relatively new and becomes more and more necessary because the models that can be studied through imaging are becoming more complex and direct image analysis no longer capable of providing the parameters of these models. High-resolution transmission electron microscopy of materials and video microscopy of cell populations are two examples where the simulation/modelling approach plays an increasing role.

### *1.8. Trend number 8: from complete automation to semi-automation*

All the trends listed above describe a tendency towards more and more complex imaging procedures and image processing algorithms. Besides this kind of tendency, I think it is worth noting that, sometimes, an inverse tendency can also be observed. As one single example in this category, I have chosen the question of image segmentation, i.e. the partitioning of an image into several disjoint regions. From the very beginning of image processing, this task has been considered as one of the most difficult. Although a lot of work in this field has led to some progress in the direction of automatic segmentation, it must be recognised that good results are obtained in favourable situations only. Thus, there is a trend to abandon fully automatic segmentation in favour of semi-automatic segmentation. Succeeding in performing image segmentation with a limited amount of user interaction is of course better than failing to obtain a correct segmentation without user interaction. This approach will be illustrated through two examples of semi-automatic procedures.

### *1.9. Trend number 9: multidisciplinarity*

Besides being an autonomous discipline, image processing has also many links with other disciplines: artificial intelligence, physics, infography, to name the most important of them.

## 2. Signal processing

The beginning of image processing was in fact the generalisation of 1D (temporal) signal processing to 2D (spatial) signals. At that time, linear signal processing was dominant. The Fourier transform (FT) was the tool most often used for analysing and processing signals. The forward FT was used for analysing the frequency content of images. Then, filters could be applied to the signal spectrum in order to attenuate or enhance some selected frequency bands and finally, the backward FT was applied to go back to the real space and obtain the processed signal. This approach (or, equivalently, the convolution approach in the real space) has been applied extensively for analysing images, and more particularly images of crystalline specimens (Stewart, 1988). It was also applied for image pre-processing: improvement of the signal-to-noise ratio by low-pass filtering, contrast enhancement[2] by high-pass filtering, image restoration[2] by inverse or Wiener filtering.

Besides the linear[3] signal processing methods, some non-linear methods were also used. The median filter was used for the improvement of the signal-to-noise ratio. This filter is a special case of rank order filters (Pitas and Venetsanopoulos, 1992). Mathematical morphology constitutes another important class of non-linear filters based on the set theory (Serra, 1982; Dougherty, 1992; Dougherty and Lotufo, 2003; Soille, 2003). It forms the basis for many kinds of applications in image pre-processing, image segmentation and image analysis.

[2] Image enhancement consists in improving the image quality (especially the contrast) independently of the characteristics of the imaging instrument. Image restoration consists in taking into account the characteristics of the imaging process (especially the contrast transfer function) and trying to eliminate their effects a posteriori.

[3] The linearity of the process described above can be more easily understood when the equivalent convolution approach is used. The filtered signal is then computed, for each pixel, as a linear combination of the content of neighbouring pixels.

From my point of view, the main characteristic of these analysis/processing methods is that they are global[4] methods. This means that the (1D or 2D) signal is analysed as a whole. The power spectrum, for instance, represents the frequency content of the whole image. As a consequence, the filters applied to the spectrum (or, equivalently, the convolution kernels applied in the real space) work on the whole signal in the same way. This fact has a number of important consequences, which constitute the drawbacks of global signal/image processing approaches. When a low-pass filter is applied in order to improve the signal-to-noise ratio, a degradation of contrast and resolution is observed concomitantly. When a high-pass filter is applied in order to enhance contrast, a simultaneous degradation of the signal-to-noise ratio cannot be avoided. Applying band-pass filters instead of low- or high-pass filters improves the situation a little bit, but the drawbacks remain present. It should be recognised that these drawbacks come from the fact that the applied filters are not adaptive: if smoothing filters could be applied in regions of the image without contrast (flat regions) and not in highly-contrasted areas (edges, spots), the degradation in contrast and resolution would be highly reduced. If contrast enhancement could be limited to areas with existing contrast, the degradation of the signal-to-noise ratio could also be reduced.

Thus, the question of local[4] methods for signal analysis/processing is raised. In other words, the main drawback of the FT is its lack of localisation: computing the FT is equivalent to analysing the signal with series of sine and cosine functions with a varying frequency. Since these analysing functions are not limited in space, there is no way to analyse the signal locally (Bonnet and Vautrot, 1997).

Attempts to overcome the limitations of the FT in terms of localisation can be traced back to Gabor (1946, 1965):[5]

(a) The first attempt led to the concepts of windowed Fourier transform (WFT) and of spectrograms. Limiting the analysing functions in time (or space) allows us to study the signal locally. The result of the analysis, expressed as a function of time (or space) and frequency is called the spectrogram.

(b) Another attempt is the concept of Gabor filters. Gabor filters are periodically modulated Gaussian kernels in real space, which can be written as: $G(\underset{\sim}{k}, \underset{\sim}{u}) = \mathrm{e}^{-u^2/(2\sigma_u^2)}\mathrm{e}^{-j\underset{\sim}{k}.\underset{\sim}{u}}$, where $\underset{\sim}{u}$ represents a real space vector ($\underset{\sim}{u} = (x, y)$ for images) and $\underset{\sim}{k}$ represents a vector in the reciprocal (frequency) space. The first exponential term performs the real space localization, which allows local signal analysis[6]. Selecting a set of values for $\sigma_u$ and a set of orientations for $\underset{\sim}{k}$ allows us to sample the frequency space efficiently. Since this filtering approach is local, the analysis/filtering approach can be performed for each image pixel, and several filtered images can thus be produced. Since Gabor filters are complex ($G(\underset{\sim}{k}, \underset{\sim}{u}) = G_r(\underset{\sim}{k}, \underset{\sim}{u}) + jG_i(\underset{\sim}{k}, \underset{\sim}{u})$), amplitude and phase information can be obtained concomitantly.

As an example of an application of Gabor filters, I will select the determination of the local image phase in high-resolution transmission electron microscopy (HRTEM) (Hytch, 1997; Hytch and Potez, 1997; Hytch et al., 1998). In periodic (and quasi-periodic) images of crystals, much information is concentrated close to the spots observed in reciprocal (frequency) space. Filtering this information with a Gaussian filter centred on the selected spot is equivalent to convoluting the original image with a Gabor filter. The width of the Gaussian filter is inversely proportional to the localisation in the real space. A complex filtered image is obtained, from which the local amplitude and the local (geometrical) phase can be estimated. The visualisation and the quantification of the local phase allow the defects in periodicity (dislocations, grain boundaries, etc.) to be studied very precisely.

It should be stressed that this approach is not limited to periodic or quasi-periodic specimens. The local geometrical phase is an important intermediate clue for solving different problems in computer vision, such as optical flow or stereopsis.

(c) Wavelets:

Wavelets constitute another attempt to perform the local analysis of signals. They differ from Gabor filters in the definition of the analysis function. Whereas the width of the Gaussian analysis function (related to $\sigma_u$) is not related to the frequency studied ($k$) for Gabor filters, it is related for wavelets. The justification for this is that low frequency components do not need to be localized as precisely as high frequency components. Moreover, the shape of the wavelet may not be Gaussian and can be adapted to specific purposes.

One way to explain the wavelet analysis simply is the following: the original signal is smoothed (convoluted with a low-pass wavelet) and the difference between the original signal and the smoothed signal is computed. This difference represents the high frequencies (small details, edges, etc.) of the original signal. Then, the smoothed signal is smoothed again, i.e. convoluted with another wavelet deduced from the mother wavelet, and the difference is calculated again, providing another level of analysis (at intermediate frequency), and so on. Thus, we get a series of filtered signals, which are linked hierarchically: this is a multiscale approach. In most cases, separable wavelets are used. So, at each scale, three filtered images are produced, providing information in the horizontal, vertical and intermediate directions.

Wavelet-based multiscale analysis has proved to be very powerful for performing different tasks in computer vision:

[4] By global algorithms, we mean algorithms that perform the same task over the whole image, and are thus non-adaptive. On the contrary, local algorithms adapt their effect to the local content of the image, the local contrast for instance, and are thus adaptive.

[5] An interesting comment on Gabor's contribution to image processing, and especially local image processing, can be found in Lindenbaum et al. (1994).

[6] It can be shown that the Gaussian shape constitutes the best compromise in terms of simultaneous spatial and frequency localisation.

denoising, contrast enhancement, texture analysis, and pattern recognition. Its use in microscope imaging has been more limited than in other fields. A few applications can however be cited:

(a) denoising and enhancement of electron micrographs and electron diffraction patterns (Gomez et al., 1992)
(b) texture analysis (Livens et al., 1996; Van de Wouver et al., 2000)
(c) estimation of the resolution in near-field microscopy (Barchiesi and Gharbi, 1999)
(d) spot detection in immunomicroscopy images (Olivo-Marin, 2002)

I have no doubt that wavelet-based local methods for analysis/processing microscope images will play an increasing role in the near future.

Besides wavelets, new tools for local analysis, such as curvelets and ridgelets, are currently being investigated and promising (Donoho and Flesia, 2001).

It should also be stressed that adaptations of the Fourier transform are not the only attempts to perform local processing: anisotropic diffusion and local contrast enhancement, among others, can be cited (see Bonnet, 1997, for more developments on these topics).

## 3. Statistical methods

Besides the signal theory mentioned in the previous section, statistical theories also play an important role in image processing. In microscope image processing, these methods did not play a very important role until now, but this role is also increasing. Briefly speaking, what is used now can be mainly categorised into probabilistic data analysis. Two recent reviews were given by Taupin (1998) and Skilling (1998).

An increasing role can be expected for several aspects of statistics: multivariate statistical analysis, robust statistics, and entropy-based statistics, to name a few.

### *3.1. Multivariate statistical analysis (MSA)*

MSA, first developed at the beginning of the 20th century, is not a new technique. But the type of data recordings now available in different fields of microscopy and microanalysis (see trends 4 and 5) makes their use more and more necessary.

MSA allows us to characterize a multidimensional data set as a whole, and to put into evidence the different sources of variation (i.e. of information) contributing to the set. Multivariate statistics encompass a large group of techniques, ranging from linear decomposition (Principal Components Analysis (PCA), Correspondence Analysis (CA), Karhunen-Loëve transformation (KL), etc.) to non-linear transformations (neural networks).

These techniques were first introduced in microscopy as tools to manage the classification of images of macromolecular assemblies prior to 3D reconstruction (van Heel and Frank, 1981; Frank et al., 1982).

Examples of these techniques, when applied to different topics in microscope imaging, are given in Geladi (1992); Van Espen et al. (1992); Bonnet et al. (1992); Bonnet and Zahm (1998), among others. Partial reviews are given in Bonnet (1998, 2000)). I will only insist here on the limitations of existing methods for orthogonal linear MSA (OLMSA), which are characterized by a decomposition of the data set into orthogonal components. This may be sufficient for a qualitative characterization of the data set, but not for a quantitative analysis. The reason is that, owing to the orthogonality constraint, the abstract components found by the decomposition process cannot be identified with the real components in the system. An attempt to obtain the true components, which are not orthogonal in general, goes through oblique analysis (Malinowski and Howery, 1980). Attempts to perform oblique analysis (also called factor analysis) in microscope imaging can be found in Kahn et al. (1997, 1999)): they concern the analysis of multispectral, dynamic, three- and 4D image sequences recorded by confocal microscopy. Other attempts concern quantitative elemental mapping by X-ray imaging (Trebbia et al., 1995; Trebbia and Ferrar, 1996; Vekemans et al., 1997).

An alternative approach to estimate the true sources of information is independent components analysis (ICA) (Hyvärinen and Oja, 2000). Although the concept of independence is clearly related to the concept of orthogonality for Gaussian processes, this is no longer true for non-Gaussian processes. The interest of ICA is clearly growing in different fields of application. I imagine that it will be introduced soon for applications in microscope imaging.

### *3.2. Robust statistics*

When analysing/modelling a large data set, we are often faced with the problem that the majority of the measurements obey some model but a few of them do not obey the same model. They are called inliers and outliers, respectively.

When fitting the data to the model, we must examine the importance of the outliers on the results of the fitting process. If the outliers do not play a significant role, we say that the method is robust. Alternatively, if the presence of outliers disturbs the result, we say that the method is not robust. The breakdown point of an estimation method is defined as the percentage of outliers that can be tolerated before this method fails. Unfortunately, most of the commonly used optimization methods are not robust (the least squares fitting method, where one single outlier may deteriorate the estimation result, is a typical case). The usual way of coping with this problem is to ask the user to select from the whole data set the part of it that has to be fitted to the model. A more objective way to proceed would be to replace non-robust optimization criteria by robust ones, which allows the outliers to be detected and discarded automatically. This is the aim of robust statistics (Rousseeuw and Leroy, 1987). Some examples of robust estimators are:

M-estimators (Huber, 1981): the residues are weighted in such a way that outliers do not strongly contribute to the estimation process

the least median of squares (Rousseuw, 1984): the average value of the residuals (or of the squared residuals) is replaced by their median value

the number of sign changes criterion (Venot et al., 1984): the number of sign changes obtained when scanning the residuals is computed and maximized.

Some applications of robust statistics are concerned with:

(a) image registration (Bonnet and Liehn, 1988; Van Dyck et al., 1988)
(b) parameter estimation in curve fitting (Zhang, 1997)
(c) robust computer vision (Meer et al., 1991)

I expect that robust statistical methods will be used more and more in place of the classical least squares method in microscope image processing.

### 3.3. Entropy-based statistics

Different sorts of entropy have long been used to characterize the content of an image (Fan, 1988). More importantly, the maximum entropy principle, first proposed by Jaynes (1982), has been used to perform image and spectrum restoration (Skilling and Gull, 1984). This principle states that the best solution that one can find to a restoration problem is the one that employs as little information as necessary to fit the data.

The main applications have concerned:

(a) the approximation of missing cone data in 3D electron tomography (Barth et al., 1988; Lawrence et al., 1989)
(b) the enhancement of scanning tunnelling microscopy and the estimation of atomic positions (Böhmig et al., 1994)
(c) the reconstruction of compositional depth profiles from electron probe microanalysis data (Smith et al., 1995)
(d) focus tuning in exit-wavefunction reconstruction in high resolution electron microscopy (Van Dyck et al., 1996) the deconvolution of high resolution transmission electron microscope images (Pennicook et al., 1992; Fu et al., 1994; Chen et al., 1999)
(e) the a posteriori correction of uneven illumination (shading) in optical microscopy (Likar et al., 1999)

In the future, we will probably witness the generalization of the maximum entropy principle, i.e. the minimization of the Kullback-Leibler cross-entropy, applied to microscope image processing problems.

## 4. Artificial intelligence

Originally, image processing techniques grew as extensions of the 1D signal processing techniques. They were soon complemented by tools originating from set theory, such as mathematical morphology. As it becomes more mature, the image processing/analysis activity has to explore other disciplines in order to enrich its own catalogue. Artificial intelligence is one of these disciplines able to enrich image analysis. Since this connection between artificial intelligence and pattern recognition techniques and microscope image processing was reviewed recently (Bonnet, 2000), I will only summarise it briefly. The ingredients of artificial intelligence I will consider are: fuzzy logic, automatic classification and neural networks.

One of the most important concerns of artificial intelligence is the way classical methods and algorithms behave in the presence of uncertainty. Several information theories have been developed during the last 30 years, dealing with this problem in different ways:

(a) probability theory, and the associated Bayes decision theory (Duda and Hart, 1973)
(b) fuzzy set theory, with the concept of membership function (Zadeh, 1965)
(c) evidence theory (Schafer, 1976), with the belief and plausibility functions
(d) possibility theory (Dubois and Prade, 1988), with the possibility and necessity functions.

Apart from the Bayes theory, it appears that only fuzzy set theory has diffused a little way in the community of microscope image processing.

An example is given in Hillebrand (1998) for the analysis of the local composition of III–V compounds from high resolution electron microscope images: combining neighbouring image cell similarities, the underlying chemical composition is evaluated by applying fuzzy logic criteria of inference.

Automatic classification is an activity which is generally considered as pertaining to artificial intelligence. Its importance in microscope image analysis is growing and concerns:

(a) the segmentation of multicomponent images, i.e. the classification of pixels, described by a vector of several signal intensities: $\underline{I}(x,y) = (I_1, I_2, \ldots, I_N)^t$, into an unknown number of classes. These different classes of pixels may represent different phases of the material (in material sciences) or regions of the specimen incorporating differently the various fluorochromes (in fluorescence microscopy for applications in Biology). the classification of sub-images representing individual views of macromolecular assemblies. These different views may represent several points of view of a unique 3D object or several conformation states of an object family. In both cases, grouping the views into several nearly-homogeneous classes is a pre-requisite to achieving meaningful 3D reconstruction. The same type of classification of sub-images may be useful in HRTEM of crystalline specimens, where the sub-images correspond to unit cells across an interface.

(b) the classification of objects detected in images, such as microscopic particles, defects, or textured regions.

Automatic classification techniques can be divided into supervised and unsupervised ones (Duda and Hart, 1973). The former requires a training set in order to learn how the different classes can be discriminated. Learning and discrimination can be performed by means of numerous techniques developed in the artificial intelligence community: the Bayes decision theory (after learning the probability density function (pdf) of each class), the $k$ nearest-neighbours (kNN) technique, the multilayers feed-forward neural networks (MLFFNN) or expert systems, to name a few.

Examples of application of supervised classification to microscope image processing are:

(a) the segmentation of multicomponent images in X-ray microanalysis of minerals in scanning electron microscopy (Tovey et al., 1992)
(b) the classification of corrosion defects on the basis of their texture features (Livens et al., 1996)
(c) the classification of unit cells in HRTEM of crystalline interfaces (Aebersold et al., 1996)

Unsupervised techniques for automatic classification do not require any training set: the different objects that constitute the data set are clustered into different classes, according to some similarity criterion. Many techniques are available, ranging from hierarchical ones to partitioning ones. Although the former techniques have been mainly used in microscopy (especially for the classification of 2D views of 3D macromolecular assemblies before 3D reconstruction), there is a growing interest for the latter techniques. These include:

(a) the $k$-means technique (clustering of objects around the centres of classes),
(b) the fuzzy C-means technique, a variant of the previous one incorporating the concept of fuzzy membership to each class,
(c) methods based on the estimation of the global pdf and the partitioning of the parameter space according to the estimated pdf, using mathematical morphology approaches (skeleton by influence zones or watersheds) (Postaire et al., 1993; Herbin et al., 1996; Bonnet, 1998) neural networks working in the unsupervised mode: the self-organizing map (SOM)(Kohonen, 1984) and its variants (Pascual-Montano et al., 2001), the fuzzy learning vector quantization (FLVQ) also called fuzzy Kohonen clustering network (FKCN) (Bezdek and Pal, 1995) or neural networks based on the adaptive resonance theory (ARTNN) (Carpenter and Grossberg, 1987).

All these techniques have found preliminary applications in microscope image processing (Van Heel, 1984, 1989; Frank, 1990; Marabini and Carazo, 1994; Bonnet, 1995, 1998; Wu et al., 1996; Zuzan et al., 1997; Sherman et al., 1998; Guerrero et al., 2000; Pascual et al., 2000). However, it should be stressed that very few comparative studies were performed in order to test whether different methods produce similar results and, if not, which one is the most appropriate to solve one class of problems. From this point of view I would say that the application of automatic classification methods in microscopy is still in its infancy.

## 5. Multidimensional microscopy

Besides conventional (2D) imaging, microscope imaging is expanding towards many other variants. I will consider first the case of 3D imaging, which is the most developed one at the present time, and then other variants.

### 5.1. Three-dimensional reconstruction and processing

For a long time, microscope imaging, like most imaging techniques, was mostly limited to 2D images. The 3D reconstruction of objects was mainly based on the technique of physical sections. Structures of interest were delineated in each image and virtually stacked in the computer. The technique was very cumbersome.

However, starting 35 years ago, an ineluctable tendency towards 3D imaging can be observed. It reached all forms of microscopic imaging, from the cellular level to the macromolecular level in biology for instance.

In transmission electron microscopy, owing to the large depth of field,[7] changing the focus alone does not allow us to perform 3D reconstruction. It is thus necessary to rely on tilt series to record the complementary pieces of information. Two images of the specimen viewed along different directions allow analogue or digital stereoscopy to be performed. But many more views are necessary to perform microtomography, the analogue of medical tomography at the cellular and sub-cellular levels. As an alternative to

[7] Sometimes called the depth of focus.

recording many views, images of a specimen containing a large number of equivalent objects in two directions may be recorded. Microtomography (with its different variants), launched at the end of the 60s, has evolved very rapidly, for crystalline structures as well as for isolated objects (Frank, 1996). It has now attained a high level of sophistication and of automation, allowing 3D reconstructions at the cellular level as well as at the macromolecular level to be performed (Koster et al., 1997). Tilts series can also be combined with focus series in order to correct the contrast transfer function of the microscope, allowing the 3D density distribution of macromolecules to be reconstructed with a resolution better than 10 Å, which compares favourably with X-rays and neutrons (Henderson, 1995). A partial list of very interesting results includes those published by Böttcher et al. (1997), Baker et al. (1999), Van Heel et al. (2000), and Grünewald et al. (2003).

In the field of optical microscopy, the development of confocal microscopy can be considered as a revolution as far as 3D reconstruction is concerned. Confocal systems allow us to select very thin slabs of a thick specimen for producing an image. Thus, slicing optically the specimen and scanning it along its vertical dimension yield series of images (sometimes called Z-series) that can be stacked in the computer to reconstitute the 3D specimen afterwards.

Progress made in deconvolution techniques also makes it possible to perform 3D reconstruction from non-confocal optical microscopes: although the experimental depth of field is too large to obtain 3D reconstruction directly, deblurring methods allow us to reduce this depth of field a posteriori and to perform 3D reconstruction with sufficient vertical resolution (note that these deconvolution techniques (McNally et al., 1999) can also be used to improve the resolution of confocal-based 3D reconstructions (Van der Voort and Strasters, 1995; Verveer et al., 1999)).

These different possibilities for performing the 3D reconstruction of an object from a series of 2D images constitute an undeniable success of the microscope image processing community. In parallel, this community is also tackling the problem of 3D image processing and analysis. The aim is to extend the possibilities of 2D image visualisation, processing, analysis and quantification to the 3D reconstructions obtained from 2D image series.

All the fields of image processing/analysis are concerned:

(a) Image restoration: 3D reconstructions are often far from perfect. They suffer from several drawbacks such as: the remaining depth of field in focus series reconstructions (even in confocal microscopy), the missing cone problem in microtomography, the microscope transfer function problem in high resolution electron microscopy 3D reconstruction. All these problems can be solved (at least partly) through 3D restoration procedures, applied after or during 3D reconstruction.

(b) Image segmentation: As for 2D image analysis, segmentation is an important step in 3D quantification. Three-dimensional segmentation, like its 2D counterpart, constitutes one of the most challenging tasks of image processing, but some progress in the field has nevertheless being made. Interactive methods as well as automatic ones have been improved.

Besides contouring the objects or zones of interest within each consecutive section, interactive (or manual) procedures offer new tools for manipulating the 3D image reconstruction and delineating 3D structures. Some examples of such systems are described in Rodenacher et al. (1997), Einstein et al. (1997), and Lockett et al. (1998). More importantly, techniques for automatic segmentation have been extended from 2D to 3D. Besides simple thresholding of the grey levels (Umesh Adiga and Chaudhuri, 2001), more sophisticated techniques are becoming available: mathematical morphology tools, for instance, were extended to the third dimension (Preston, 1991; Meyer, 1992). The detection of small objects can be done using the top-hat transformation using mathematical morphology tools. Another morphology approach useful for the automatic segmentation is the watershed procedure (Beucher and Meyer, 1992; Ancin et al., 1996). Another approach for 3D segmentation starts from the concepts of algorithmic geometry: the Voronoï diagram and the Delaunay triangulation (Bertin et al., 1993; Eils et al., 1995). Clustering methods followed by relaxation have also been suggested (Kett et al., 1992).

Although these techniques have been mainly developed for applications in confocal microscopy, there are few doubts that they will also be applied to 3D reconstructions of sub-cellular systems and of macromolecular assemblies, where they will replace the simple thresholding technique used until now with very few exceptions.

(c) Volume measurements after segmentation: Once the segmentation of the 3D reconstruction is done, quantitative measurements can be performed, in order to determine the number of objects, their individual characteristics (volume, surface, shape parameters, orientation…) or their collective characteristics. For instance, the spatial relationships exhibited by a set of objects can be computed through statistical approaches (König et al., 1991), computational geometry concepts (Dussert et al., 1987; Marcelpoil and Usson, 1992), fractal approaches, ad-hoc procedures such as the coefficient of margination (Höfers et al., 1993; Parazza et al., 1995; Beil et al., 1996), the anisotropy in the orientation of objects (Usson et al., 1994; Mattfeldt et al., 1994). An analysis of measurement accuracy can be found in Delorme et al. (1998).

(d) Volume measurements without segmentation: In two dimensions, this group of methods concerns texture analysis, fractal analysis, co-localisation approaches and the analysis of image series. Only a few attempts have been done to extend texture and fractal analysis in three dimensions (Strasters et al., 1994; Beil et al., 1995). This is still a challenge for the future.

Co-localisation approaches, involving the construction of 2D or 3D histograms have been extended to 3D data sets (Beltrame et al., 1995; Demandolx and Davoust, 1997). Another co-localisation approach consist in classifying the pixels or voxels, on the basis of their different intensity values, and building a 'map' of the different classes.

Some suggestions related to the 3D reconstruction of macromolecular assemblies at high resolution are given in Herman et al. (2000). According to these authors, three multidisciplinary approaches have the potential for improving 3D electron microscopy in this field:

(a) incorporation of realistic image formation models into new reconstruction models
(b) incorporation of knowledge regarding the specimens. This knowledge could be obtained by means other than electron microscopy, such as atomic force microscopy
(c) improvement of the rendering and the analysis of the reconstruction volumes by the development of more accurate segmentation and visualization algorithms.

We will see in Section 10 that these multidisciplinary trends are not limited to the 3D reconstruction of macromolecular assemblies but are much more general.

### 5.2. Other variants of multidimensional microscopy

For a long time, imaging was a 2D process: a signal was recorded as a function of two spatial coordinates ($x$ and $y$). This process was found useful and complementary to 1D signals such as time-dependent signals and frequency/wavelength-dependent signals found in spectroscopy. Nowadays, we are witnessing the development of more complex acquisition procedures that combine two (or three) spatial coordinates and one or several other variables, such as time and/or wavelength.

The combination of spatial coordinates and time gives rise to time-resolved microscopy, a dynamic imaging process that allows us to record images of living cells (Tvarusko et al., 1999) and/or of mechanical/chemical dynamical processes, in environmental scanning electron microscopy or near-field microscopy.

The combination of spatial coordinates and wavelength allows multispectral imaging, a very promising combination of imaging and spectroscopy, to be performed. The simplest and oldest form of multispectral imaging is probably colour imaging, i.e. recording of data within three different energy windows in the visible domain (Red, Green, Blue components). But full multispectral imaging (sometimes called deep multispectral imaging) can also be performed when many images are recorded in energy windows close together, giving rise to image-spectroscopy (Lavergne et al., 1994; Körtje, 1994; Mayer et al., 1997). As an alternative, a large part of a spectrum can be recorded for any pixel of the image, giving rise to spectrum-imaging (Hunt and Williams, 1991; Balossier et al., 1991; Colliex et al., 1994; Tencé et al., 1994). These two modes do not differ from a conceptual point of view: in both modes, a data cube is recorded as $I(x,y,\lambda)$ (Jeanguillaume and Colliex, 1989). They differ only from the instrumental point of view, which may have some incidence on the data sampling: larger images and less energy channels in the latter case, less pixels and more energy channels in the former case.

The recorded data cube $I(x,y,\lambda)$ contains a lot of information, which may be partly hidden and has to be extracted a posteriori. Of course, classical image processing/analysis methods may be applied to the set of stacked images $I(x,y,\lambda_k)$ and classical signal processing/analysis methods can be applied to the set of spectra $I(x_i,y_j,\lambda)$. The coherence and completeness of the data cube is partly lost however. Thus, multivariate methods, working on all the data at once, are to be preferred. For instance, multispectral image segmentation is generally more efficient than mono-component image segmentation followed by a combination of the different binary images (Bonnet, 1995). The same is true for image pre-processing tasks (improvement of the signal-to-noise ratio, local contrast enhancement, etc.). Factorial filtering, for instance, has been demonstrated to be very efficient (Trebbia and Bonnet, 1990). It consists in submitting the whole data set to multivariate statistical analysis, principal component analysis or correspondence analysis. Then, the eigen-components that contain only noise or artefacts are discarded and the data set is reconstructed using useful components only. Applications of this procedure can be found in Quintana and Bonnet (1994) and Quintana et al. (1998).

However, I consider that tools for processing and analysing multidimensional data sets have not attained a sufficient level and have still to be developed. Even the simple visualization of these data sets necessitates specialized tools that are still in their first stage of development (Mountain et al., 1996; Kenny et al., 1997).

Higher dimensional imaging (also called hyper-dimensional imaging) is still in its infancy, but will, without any doubt, become more common in the future. Among the different possibilities, I can cite:

(a) $I(x,y,z,t)$ : time-resolved 3D imaging is already in use in 3D video microscopy,
(b) $I(x,y,z,\lambda)$ : multispectral 3D microscopy,
(c) $I(x,y,t,\lambda)$ : time-resolved multispectral microscopy,
(d) $I(x,y,z,t,\lambda)$ : time-resolved multispectral 3D microscopy.

## 6. Multimodal microscopy and data fusion

Modern microscopes (and new prototypes) are not only able to produce higher and higher quality images. Some of them are also capable of producing signals and images in different modes. Some examples of these possibilities are—not exhaustively—listed below:

the optical microscope was certainly the first instrument to offer different working modes: bright field (BF), dark field (DF), phase contrast (PC), differential interference contrast (DIC), fluorescence microscopy (FM). An illustration of this multimode light microscopy for the study of the dynamics of molecules, cells and tissues was given by Farkas et al. (1993). Another illustration was given by Glasbey and Martin (1996) who tried to explore the complementary information content of multimodal images (BF, DIC, PC).

the confocal microscope maintains most of these possibilities with, in addition, the facility to perform optical sectioning and easier 3D reconstruction. A description of this 3D versatility was given by Jovin et al. (1990). Beltrame et al. (1995) combined transmitted light and fluorescence images in a confocal microscope. They also developed some tools (such as the 3D scatterplot) for dealing with this multimodal data set and classifying pixels into different clusters.

the transmission electron microscope (TEM) also offers different imaging capabilities (bright-field, dark-field, convergent beam diffraction, etc). When coupled with a spectrometer, the imaging and the analytical possibilities are combined. The scanning transmission electron microscope (STEM) is even more versatile in the sense that the image-forming electrons can be selected more easily (Burge et al., 1982).

(a) the scanning electron microscope (SEM) also allows different signals to be recorded (back-scattered and secondary electrons) and can be combined with analytical systems allowing microanalytical information to be collected.
(b) versatile analytical instruments, with which several analytic signals can be recorded simultaneously, have also been built. An example is the multispectral Auger microscope built in York (Prutton et al., 1996, 1999).
(c) some small instruments can also be put inside others, allowing a large magnification range to be covered: near-field microscopes have been put inside SEMs.

Generally speaking, the aim of such multimodality techniques is to gather several types of information about the object, in order to better explore its complex reality. Different possibilities are:

(a) to study the specimen at different scale levels (from light microscopy to electron or near-field microscopy through confocal microscopy)
(b) to study the specimen from different points of view (topography with a near-field microscope and interior of the specimen with a TEM)
(c) to capture chemical information in complement to physical information (electron microscopy and microanalysis; phase contrast light microscopy and fluorescence microscopy)
(d) to combine chemical information with low and high atomic numbers (electron energy loss imaging and X-ray mapping).

These different possibilities give rise to what is sometimes called collaborative microscopy.

From the point of view of image processing, the multimodal data sets require specific image processing techniques if we really want to combine the different pieces of information. Very few attempts have been made to set up the necessary tools.

One significant attempt is due to Glasbey and Martin (1996) who tried to explore the data set composed of BF, PC and DIC images recorded from a sample of algal and bacterial cells. They mainly used PCA to explore the information content in the triplet of images. They found that the three principal components could be interpreted. The first principal component, which represents 74% of the total variance, expresses the correlation between BF and DIC images and their anti-correlation with the PC image.

But this type of analysis, which is well suited to multispectral or time-dependent image series (because all the images are of equivalent nature) becomes largely insufficient for multimodal image series. For this type of image series, other tools have to be developed and used in order to elicit information unavailable from any single modality. This process can be named image fusion, in relation with the more general data fusion[8] principles. Image fusion may concern the production of a resultant image by merging the whole set of individual images. With a series of two or three images only, the technique of pseudo-coloured composite image can be used, and has been used extensively in multifluorescence imaging or in X-ray microanalysis (Razdan et al., 2001). The scatterplot technique can also be used (Bright and Newbury, 1991; Kenny et al., 1994; Demandolx and Davoust, 1997). But several new possibilities are available. As an example, the combination of images recorded at different resolution can be performed through wavelet merging (Nuñez et al., 1999; Scheunders and De Baker, 2001).

[8] Data fusion consists in considering several data sets as a whole and combining the different pieces of information they contain in order to produce new information that could not be extracted from any individual data set.

Besides simple merging, the main problem in image fusion concerns the way the different sources of information can be fused in order to provide an answer to a given problem. For doing this, one has to define: (i) which measure of belief is chosen for the individual sources of information, (ii) how the different measures of belief are combined. This subject is slightly more developed in Bonnet (2000). The interested readers may also consult Bloch (1996) for an extended theoretical study.

## 7. Theory

As stated by Ritter et al. (1990), 'vast increase in image processing activities in the military, industrial and academic communities have resulted in a deluge of different image processing techniques, notation, and operations that all too often perform similar or identical tasks'. At the beginning of the 1980s, some people started to think that this chaotic situation could be transformed and they decided to work on 'the development of a highly structured mathematical foundation for image processing and image analysis with the intent that the fully developed structure would subsequently form the basis of a common image processing language'.

This unified system is supposed to work on any type of image, and particularly with multivalued as well as single-valued images, and to support non-linear as well as linear transformations.

Limitations of space prevent me from describing in detail the results of this research, which has taken the terminology of image algebra(s). Only a very brief summary is given below.

Image algebra consists of images, templates (images whose pixels are themselves images), mathematical operations (addition, multiplication, sup, subtraction, division, inf, etc.), the sets F and X of types of values (integer, real, complex, etc.) and of types of coordinates (Cartesian, polar, …), respectively.

It was shown theoretically (and verified in practice) that any known (linear or non-linear) operation on images and templates[9] could be described in terms of a few operators of image algebra. This is the case of: the Fourier transformation, all linear convolution routines, mathematical morphology basic operations (erosion, dilation, opening, closing) and extensions, such as adaptive morphology, histogram equalization, median filters and their generalizations such as rank-order filters, restoration, including the Wiener filter and iterative procedures such as the Gerchberg-Saxton algorithm, singular value decomposition and multivariate statistical analysis, 3D reconstruction with the filtered back-projection method or the projection onto convex sets (POCS).

[9] Image-image, image-template or template-template.

The concepts of image algebra were introduced in the microscope image processing community by Hawkes, who also showed that the images which can be recorded in microscopy may be of numerous types but all can be pretty well managed using the concepts of image algebra.

Hawkes (1992) deals with the question of image restoration.

Hawkes (1993) concerns the algebraic manipulation of sets of electron images and spectra. As an example, he shows that the different possible representations of a multivalued image can be used as alternatives to standard techniques for multivariate statistical analysis.

Hawkes (1995a, 1998) presents a review of image algebra for electron images and shows that all known image processing operations (divided into four classes: image acquisition and coding, enhancement, restoration and analysis) can be described using the framework and notations of image algebra.

Hawkes (1995b) shows that the very specific images which can be recorded with a STEM are in fact templates and can be processed as such within the framework of image algebra.

Undoubtedly, from a theoretical point of view, image algebra constitutes a progress towards a unification of image processing techniques. However, it seems difficult to affirm that image algebra has led to a real practical progress, in the sense that the unification has not produced new tools that were not available previously. Image algebra being only twenty years old, we can expect that only the first steps of its development have been achieved and that future developments will provide new practical tools that will help to cope with presently unsolved problems (Ritter and Wilson, 2001).

## 8. Simulation

In general, imaging is considered as self-consistent in the sense that the images obtained are supposed to capture the essential information contained within the object under study. This means that this information can be extracted directly from the image, perhaps after image processing and/or analysis. However, situations exist where, although the image still carries some information concerning the object, it is not possible to infer this useful information directly from the image. The reason for this may be that the transfer function of the imaging system, which carries the information from the object to the image(s), is rather complicated and cannot be inverted easily. When this situation occurs, one possibility is to make use of simulations as intermediate steps for recovering the hidden information from the complicated images.

The general process can be described as follows. First, two paths are followed in parallel: experimental images are produced with the imaging system and simulated images are computed according to a preliminary model of the object and a model of the imaging system. The two sets of images are thus analysed/quantified in order to produce two comparable data sets. Then, these two data sets are submitted to a comparator. If they do not agree, a feedback loop is activated in order to modify the model of the object, the model of the imaging system, or both. If the feedback loop is correctly designed, the process should converge and, after some iterations, the data sets originating from the experimental and the simulated images will agree. At this moment, we can say that the object and imaging models agree too. We can either stop here, considering that the information obtained about the object is sufficient. We can also try to design new experiments, which, following the same principle, can help to gain even more information (experimental design).

Below, I describe briefly three situations in microscopy where simulation proved useful.

### 8.1. Simulation in HRTEM

At very high resolution, TEM images no longer reproduce the magnified image function directly. The complex transfer function of the microscope interacts with the complex wavefunction emerging from the specimen to produce images that cannot be interpreted straightforwardly. Procedures have been developed for restoring the object structure from series of images (focus series, tilt series).

But the most often used approach to infer the specimen structure from the experimental images consists in performing many simulations of images from the expected atom positions, in specific experimental conditions, and fitting the experimental images to simulated ones. By playing with the unknown experimental parameters and the atom positions, the object structure may (hopefully) be determined (see, for instance, Self and O'Keefe, 1988; Möbus et al., 1998).

The simulation approach is already rather complex and sophisticated. It requires three steps:

(a) computation of the wavefunction at the exit face of the specimen, taking into account the presumed structure. This is generally done by the multislice approach (Van Dyck, 1997)
(b) simulation of the effects of the microscope on the wave travelling in the microscope (all microscope parameters have to be provided or checked)
(c) simulation of the recording process, i.e. transformation of a complex wavefunction into an intensity.

Some commercial software is available for performing these computations (Stadelman, 1987).

However, there remain some differences (especially in terms of contrast) between experimental and simulated images, which are not well understood yet (Boothroyd, 1998). This means that even more contributions have to be taken into account, so that HRTEM images simulations probably still have a brilliant future.

### 8.2. Simulations at lower resolution in materials science

Besides HRTEM of crystalline structures (with or without defects), another domain of application where image simulation is playing an increasing role is the domain of complex and disordered textures encountered in material science at lower resolution (metallurgical structures). Here again, structure or image synthesis may be of great help to understand real images and deduce some quantitative parameters. Following the work of Matheron (1975), Jeulin (1988, 1992, 2000) made several classes of models available for this purpose:

(a) stochastic point processes, simulating germination
(b) random tessellations, simulating granular structures
(c) random sets and multiphase random sets, simulating two-phase materials (such as porous media) or multiphase materials
(d) random functions, simulating rough surfaces.
(e) dead leaves.

Decker and Jeulin (1999) showed that complex 3D space-time textures can be simulated by reaction-diffusion models, suggesting that a reaction-diffusion mechanism is at the origin of these structures.

Boolean models have also been studied extensively by Handley and Dougherty (1996).

In any case, the comparison of quantitative values extracted from simulated and experimental images allows us to concentrate the recorded information into a few parameters of the models.

### 8.3. Analysis of the behaviour of cell populations (video microscopy)

Video microscopy (Inoue and Spring, 1997) allows us to record and visualise the behaviour of specimens as a function of time. This is particularly interesting in the case of living specimens, although the technique is not limited to this specific case. Cell populations, for instance, can be studied by optical video microscopy. Such populations often exhibit a complex behaviour, which can be described in terms of cellular sociology. By this terminology, we mean that cells in a population are not independent: they exchange information and their behaviour is the result of interactions with their neighbours. Among the consequences of such interactions is the different spatial distribution and space occupancy of different cell populations, which can be studied as a function of time with the help of videomicroscope recordings. Although some information can be gained directly from observation of these recordings, it is often difficult to deduce the modes of interaction between cells directly or from the set of spatial distribution parameters computed from these images. In this situation too, it is necessary to use simulations as an intermediate step. According to a pre-specified model of the interaction (the parameters of which are unknown), series of images of cell populations are simulated, according to the techniques of cellular automata (Wolfram, 1984) or multiagents systems. The parameters describing the spatial distribution of cells are then computed and compared to those computed from the experimental recordings. Finally, the parameters of the model, or the interaction model itself, are varied until a good agreement is obtained.

Different cell populations (for instance: invasive and non-invasive cells in cancerology) can be discriminated on the basis of the parameters that describe their interaction (Palmari et al., 1994; Bonnet et al., 2004).

## 9. From complete automation to semi-automation

One of the aims of image processing/analysis developers is to provide end-users with software tools capable of performing an automatic analysis of the image.[10] Achieving the general aim of automation requires that more and more complex imaging procedures and image processing/analysis algorithms are developed. In the previous sections, I tried to describe some of the tendencies of image processing that go in this direction of greater and greater complexity.

But, alongside this tendency, we also encounter some trends to abandon the aim of full automation and to limit the ambitions of image processing to semi-automation only. In a sense, this trend may appear as a setback to earlier ambitions, but in another sense, this can be seen to be more realistic taking into account the complex structure of images we have often to deal with.

As a single example to illustrate this trend, I have chosen the problem of image segmentation. From the very beginning of image processing/analysis, this task, which consists in partitioning the image into different regions (objects of interest versus background), has received considerable attention, because it is a central task for many applications. It has also been recognised as the most difficult part. Automatic image segmentation has constituted a challenge for several generations of developers in the field of computer image processing. It should be recognised that all these efforts have led to much progress and that sophisticated algorithms are now available for image segmentation, which I cannot unfortunately describe in this review (see Russ, 2002). But, at the same time, it must also be recognised that good results can only be obtained with fully automatic segmentation methods in favourable situations, where only one type of object is present within the scene. In many other situations, fully automatic segmentation appears unrealistic, and users prefer to use fully interactive segmentation (i.e. contour drawing) rather than having to modify the (wrong) results of automatic segmentation afterwards (Einstein et al., 1997).

One of the recently appearing trends in image segmentation is the awareness that, between these two extremes, i.e. fully interactive and fully automatic segmentation procedures, there is room for semi-automatic segmentation techniques. Here, a limited amount of user interaction is requested, which helps the computer to perform the remaining work. This user interaction may consist in telling the computer how many objects of interest are present in the scene and where they are (very approximately) located.

I will briefly describe two of these approaches: one approach based on the watershed technique we have developed and one approach based on the fuzzy connectedness concept, already in use in medical imaging, but not in microscope imaging (Cutrona and Bonnet, 2001).

(a) An approach based on the concept of watersheds:

The watershed technique is one of the central techniques used for segmentation in the community of mathematical morphology (Beucher and Meyer, 1992). When applied in a full automation context, the technique requires the automatic determination of seeds from which the different regions are grown. Using the local minima of the gradient modulus as seeds generally results in over-segmentation, the number of local minima being much larger than the actual number of objects or regions. Even grouping the regions, on the basis of their saliency (Najman and Schmitt, 1996), is often insufficient, in difficult situations. The approach we have developed consists in applying the watershed technique within a minimally-interactive context. The user interaction consists in telling the system how many objects of interest are present in the scene, where they are approximately located (one pixel per object is designated with the graphic mouse). The same is done for the objects or regions in the image that are not considered of interest by the user and constitute the background. The designated pixels (of the objects of interest and of the other objects) are then used as seeds for the watershed technique, which grows the different types of objects taking into account the regularized image gradient.

(b) An approach based on fuzzy connectedness (Udupa and Samarasekera, 1996; Carvalho et al., 1999):

This technique has already found applications in medical imaging but not yet in microscope image processing. I am convinced that this kind of technique will be useful when it will be imported in our field. The technique is based on the concept of fuzzy connectedness.[11] It relies on two considerations: (i) objects are not always defined by a constant grey level, they often display a graded grey level, (ii) the image elements (pixels or voxels) that constitute an object hang together in some way. These two properties

[10] Of course, it remains the responsibility of the users to choose among these tools those appropriate to the type of image to be processed and to the goal of the study.

[11] This is another example of using the concepts of artificial intelligence, here fuzzy geometry, in image processing and analysis.

(grey-level grading and 'hanging-togetherness') can be handled with the notion of fuzzy object or, more precisely, of fuzzy connected components.

First, the user designates (interactively) an image element belonging to the object to be extracted. Then, from the original image, a parametric image is built: for any pixel in the image, its affinity with the selected pixel is computed. This affinity takes into account the degree of adjacency of the grid points and the similarity of their intensity values.[12] Then, the affinity map can be segmented through affinity thresholding, more easily than through grey level thresholding.

This method was improved recently by incorporating the multiscale concept (Saha et al., 2000) and by incorporating competitive learning (Cutrona and Bonnet, 2001; Saha and Udupa, 2001), avoiding any thresholding.

Besides these two approaches, it may be useful to mention other approaches to semi-automatic image segmentation:

(a) intelligent scissors (Mortensen and Barrett, 1998)
(b) live wire and live lane (Falçao et al., 1998)
(c) computational geometry (Voronoï partition) followed by deformable contours, or snakes (Klemencic et al., 1998).

## 10. Multidisciplinarity

Image processing and analysis has become a very specialised discipline, at least so far as algorithmic and software development is concerned. This tendency will probably persist in the future, since new algorithmic procedures require higher and higher skills in mathematics and computer science. But, at the same time, image processing will probably get out of breath if better links are not established with other disciplines and communities, such as computer graphics (computer visualisation and animation, image synthesis), artificial intelligence or physics, for instance.

I will only describe two examples of links that appear as a necessary condition for passing beyond the present state of image processing/analysis.

### *10.1. Example 1: image processing and computer graphics*

Multidimensional and multimodality microscopes are now on the market but the means of visualising the recorded data are still relatively inefficient. Even visualising a 3D reconstructed object is not as trivial as might be expected and the surface rendering methods, applied after simple thresholding, are not sufficient. Herman et al. (2000), for instance, consider the improvement of the rendering as one of the three areas that have the potential for improving 3D electron microscopy.

Things are even more complex for other multidimensional data sets, such as 2D or 3D time-dependent or energy-dependent records.

Clearly, developing new approaches to visualization requires some collaboration with specialists of this domain, i.e. computer graphists (Foley, 1998). These people have been working for a long time on problems related to visualisation and some of the techniques they have made available have to be introduced in the field of multidimensional microscope image analysis. Volume rendering methods should be implemented in addition to the surface rendering methods (Diaspro et al., 1996; Lucas et al., 1996; Razdan et al., 2001). New attempts to model 3D shape and topology in the framework of the α-shapes approach can be found in De-Alarcon et al. (2002).

The use of tools originating from the world of virtual reality (Burdea and Coiffet, 1994; Sherman and Craig, 2002; Volino and Magnenat-Thalmann, 2000) and augmented reality (Behringer et al., 1999) could also bring something new, in terms of interaction between the user and the multidimensional data sets.

### *10.2. Example 2: image processing and physics*

Physicists have to play a role in image processing because the physical processes of image formation have often to be accounted for during the process of image restoration and analysis. This is more and more true when structures are studied at higher and higher resolution.

In addition, many image processing algorithms are already based on analogies with physical concepts. A few examples of these interactions are:

(a) the concept of energy: 'It is clearly possible to write any vision problem in terms of minimizing an energy function' (Yuille, 1987). Energy minimization is often used in segmentation problems, in stereoscopic correspondence and in motion problems, to name a few. the concept of entropy (see trend 2)
(b) the geometric moments, inspired by classical mechanics, are used in shape description and recognition
(c) isotropic and anisotropic diffusion concepts, originating from fluid mechanics, are used in order to perform local signal-to-noise ratio and contrast enhancement
(d) optical flow techniques, also originating from fluid mechanisms, are useful for the characterization of motion from image sequences
(e) concepts originating from quantum mechanics, such as the uncertainty principle, are used to describe space-time and multiscale approaches and fuzzy logic modelling
(f) concepts inspired of statistical mechanics, such as the partition function or phase transitions, are used to

[12] I do not reproduce formula and ask the interested readers to refer to original papers.

perform image segmentation and clustering
(g) models that have been defined in Physics, such as the Potts and Ising models, electrostatic and electromagnetic models, are also used as models in image processing applications.

We can expect that even more physical concepts could be imported in the general field of image processing and then in the specific field of microscope image processing.

As a whole, we can say that image processing is becoming more and more an interdisciplinary activity.

## 11. Conclusion

In this review, I have attempted to select and comment on some trends of microscope image processing. Some of them form part of image processing in general, while others appear to be specific to microscope imaging.

From this (personal) selection, it appears that image processing is not a stand-alone research activity, but is connected to a number of other fields, such as physics, artificial intelligence, statistics and computer graphics. This means that progress made in these other fields have to be followed by people working in image processing and incorporated as soon as possible.

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