Enhancing microscopic imaging for better object and structural detection, insight and classification

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1. Introduction

It is precisely 20 years of integration of digital imaging devices with optical microscopic systems, with the crucial CCD system component which had previous short and exciting history in astronomic discoveries. At the terminal end, the optical image was substituted with mathematical object in the computer – a matrix, with resolution defined by CCD chip, with dynamic resolution - the depth of individual pixels, i.e. integer corresponding to the number of different pixel states - light intensities, finally, the pixel size, which in the focus of the microscope optical system determines the size of an image atom. Matrix as a CCD output, in either monochrome or multiple CCD for color images, which is a peripheral input device of a computer, is a function on finite 2D domain, thus a 3D object, often a 3D manifold. With this remarks, we can state now that the original microscopic systems were expanded into new imaging systems with appropriate CCD device, computer and software for basic image processing and advanced software components which are introduced in order to support advanced quickly growing customer needs. The last items in this structure depend only on the developer’s creativity and implementation capacity. We can also note, that while the optical systems originally produced images that we percept or photograph, which was more or less the same thing, modern imaging systems incorporate a rich variety of interventions in the perception path, so that they diverged substantially from what originally image did mean, integrating broad presentations with highly sophisticated components. In this way, instead of our own perception direct amplification, we now days deal with complex mathematical models serving the same and expanded purpose.

At our Group for Intelligent Systems, GIS, at the Faculty of Mathematics in Belgrade, we have been involved in microscopic imaging since its beginning and have implemented a number of solutions aiming to support the operation of our colleagues in biomedical research at our institutes and departments, reaching broad number of foreign institutions ([1-2]).

Our developments in imaging were related to our works in other imaging domains, namely sonar and radar imaging, signal spectroscopy applications, integration of all sorts of sensors and specific applications, by means of exchange of useful ideas and functionality ([3-5]).

In the limited available space we will shortly review some of the methods in our implementations related to the scope of this chapter, presenting the basic methods with short descriptions and comments on the related issues. All presented ideas are rather simple and should be easily understandable by a general reader interested in the subject. Majority of the method is rather broadly spread now days. Unfortunately, even some rather simple methods have not entered the clinical use yet, as not supported by the most advanced microscopic system manufacturers supplying the medical market.

Material shown here has been mainly published in the cited articles, but part of it has remained unpublished so far. The growth of the research and number of methods and implemented solutions in microscopic imaging has been large and rich, extending to neighboring domains in a variety of ways. To some extent, this will be presented in other parts of this book. The ideas presented here are partly embedded into larger systems, some of which are converging towards partial or complete automatization. Many of the algorithms involved demand of more complex treatment and present distinguished problems in different parts of Artificial Intelligence ([6-13]).

As with all other subjects, the full integration of microscopic digital imaging systems needs links to the more abstract information processing, spanning the large scope from perception devices and systems to the large scale processing, which includes organized real time accessible memory functions with all sorts of property representation, retrieval, classification and maintenance, fused with formalized knowledge systems which would model the essential reasoning in research and related practice (e.g. genetics, general biology, clinical practice) (see e.g. [14-17]). We will not include discussion of these aspects here as transcending by large the scope of intentions for this chapter, but we will mention that the problems presented have to be resolved in any imaging system with interfaces to the higher complexity processing.
Our presentation form combines shorter explanations with selected visual material which should contribute to the fast and easy comprehension of the selected problems, their modelling and solutions. All images are from our experimentation and involve relevant biological contexts, situations and issues as present in both research and broad biological and medical practice.

2. Method – some examples

Mathematical modeling provides for numerous fruitful, straight asserted achievements. We selected some for this text, which are sorted in a natural way, with useful application within simplified short explanation.

2.1 Photo morphology

Images usually contain features or formations which we designate as objects, which are usually considered as objects in the real world. Two dimensional photometry presenting the distribution of intensities in the whole image or its part intended to be an object, is simply a graph of CCD matrix or its part, a 3D structure. In presentations we integrated full 3D navigation, enabling a user for a complete inspection, thus, avoiding hidden/masked details. This simple technique provides for inspection of structural details which could otherwise remain unnoticed. The images shown exhibit other features related to photo morphology, some rather specific, e.g. object selection, longitudinal photometric section at characteristic positions (here chromosome meridians), object normalization – here automatized object = chromosome reconstruction – as if it originally were straight (we also call it rectification), finally comparison of mathematical representations of normalized chromosomes, i.e. measure of similarity (for more details see e.g. [1-3], [17-23]).

![Fig. 1](image1.png)

**Fig. 1**  a) a pair of corresponding chromosomes, with no visible essential difference, when inspected photometrically shows the right chromosome with one dark band more, characterizing pathology – trizomy; b) arrow pointing to a chromosome which normally does not exist, whose photometric structure enlarged shows the merge of large leg of chromosome 1 with the Y chromosome on top, characterizing a rare hematologic syndrome.

![Fig. 2](image2.png)

**Fig. 2**  a) a part of mitosis with comparison of normalized photometric structures of two chromosomes; b) the step by step normalization of a right most chromosome along its central meridian before it is compared (with previous background reduction, contour definition, extraction).
2.2 Color composites

Initially, we take monochrome images in appropriate (arbitrary) wavelengths and generate their fusions into functional coloring, or, rather make color galleries of different mixes. Instead of original monochromes, we can preprocess collections of monochromes towards more differing inputs, i.e. in order to obtain better spanning base vectors (a sort of orthogonalisation process). Before mix the vectors should be properly aligned.

Our desire was to produce an efficient and comfortable work environment that would provide for “false color fusions” of all astronomic sources, with ease and necessary input preprocessing. Existing software on the market did not support all needs average researcher would demand, which was sufficient motivation. The method and software provide for fusions of data obtained with visual, IR, all radio, X and gamma recordings prepared as standard monochrome image inputs. Here described software is still under development, expected to expand the number of features and to incorporate in future other specifics, especially virtual optical components – spectroscopic lensing, based on our method of image spectroscopy, applied in the corrections of defects present in the microscopy at high magnifications.

Similar approach to ours must have been applied in the Hubble Space Telescope image processing, report of which was published in the August, 2002, issue of Sky and Telescope magazine, though all interesting details on the software were not shown, nor that software is publicly available.

Shortly the method comprises the following. Suppose the recordings (of e.g. astronomic or microscopic data), originating in all perceptible windows, are prepared in the form of monochrome (e.g. .bmp) inputs, i.e. made available in some sort of standard visual form. Designate the mentioned windows as

$$W = \{ W_{s1,i1} \ldots W_{s1,ni1}; W_{s2,i2} \ldots W_{s2,ni2}; \ldots W_{sk,i} \ldots W_{sk,ni} \},$$

with source type domains \{\(s_1, s_2, \ldots, s_k\}\}. Allow the preprocessing operations on the separate domains that would provide for filtering, noise reduction, sharpening, some feature enhancing, centering consisting of the reshaping of involved coordinate systems (including translations, rotations, and magnification tuning if necessary), so that the contained objects are repositioned to the same coordinates, aiming finally to the linear combinations that will integrate an output for each source type

$$o_i = \sum_j f * \lambda_j * W_{adj} \quad \text{for} \quad i \in \{ 1, 2, \ldots, k \}, \; f \; \text{a correction factor (usually 1)}. \quad (0)$$

So obtained type representatives are further individually and combined processed and aligned-centered before they are entered into final pre color monochrome fusion:

$$m_i = P(o_{i1}, \ldots, o_{il}) \quad i \in \{ 1, 2, \ldots, l \}$$

Both initial and final centering consists of combined translations, rotations and zooming. The coordinate system transformations are not fully integrated yet but are experimented with, to provide for unification of diverse prevailing standards. In such a way, efficiently, a sequence (gallery) of color composites

$$c_{cj} = (k_{cj} m_{i1} l_{j1}, m_{i2} l_{j2}, \ldots, m_{in} l_{jn}), \; j \in \{ 1, 2, \ldots, n \}; \; i_1, i_2, i_3 \in \{ 1, 2, \ldots, n \}; \; k_{cj}, l_{j1}, n_j \; \text{are nonnegative scalars},$$

is generated in real time, supplying researchers with potentially reach insight into the investigated phenomena and physics, color galleries being 4D objects - predicates, potentially expressing more complex properties than present in the original images. The Figs 3 - 5 demonstrate the simple use of the method of color composites, a generalization of earlier used term false coloring.

Further functionality and applicability of the method is shown in [1].

For the inverse, the method of colour fragmentation towards the elimination or extraction of certain features, the procedure is the opposite to the one described above.

Starting with different color images, we extract their color components to obtain a collection of coordinate vectors-images, the initial vector base. Then proceeding in the similar fashion as in the case of color composites, with initial preprocessing of coordinate vectors – a selection of monochrome images, the improved vector base is obtained by the broadening of the initial base span. This is done in order to enable larger manipulation capacity in further color recompositions.

The method of color fragmentation and recomposition has a broader applicability and, as alternative to the prevailing techniques represents alternative and complementary solution in a variety of rather distant problem areas. One simple application is shown in Fig. 6.
Here we have 3 FISH inputs in 3 (standard) different wavelengths; the light dots correspond to FISH signals, which are surrounded by less or worse visible nuclei contours; the third input is especially poor with information. The three inputs are preprocessed for alignment and towards better vector spanning and finally fused into color composites in the next figure.

The FISH monochrome inputs shown in Fig. 3, after preprocessing are used as generators for the above false color sequence. The structures exhibited in the gallery are better seen and resolved in the left column. The nucleus left of the center, clearly shows presence of trisomy, which was not as certain in the original image inputs.

In the top row we have 3 FISH monochromes in 3 different (standard) wavelengths, with visible FISH signals in rather fuzzy chromosome contours. In the lower row we have 3 color composites obtained as linear combinations applied to the top 3 monochromes, after their alignment. Beside two central bright signals, one can notice immediately below them, the three spots
(bluish in the middle image, reddish to the right), with max at different wavelength, which provides means for multiple FISH signal detection/resolution.

Fig. 6  The inverse method of color fragmentation and recomposition, again using a gallery of recombined coordinate vectors – monochromes in the original color images, can be used to remove artifacts (texts over fingerprints here), or for spectroscopic structural separation.

2.3 Small object recognition

In this section we will shortly overview an alternative method for the efficient recognition of smaller, dot-like objects, with diameter < 10 pixels. Method applies to both matrices and vectors, thus to both images and signals. Spectral features which are stable and narrow in frequency might be examples of such sort of vectors. Previously, we have developed procedures for small object recognition and filtering by size based on the intensity discrimination (intensity of considered pixels). The method we present here is an improved Tomasi, Shi, Kanade procedure (see e.g. [24-26]) for the extraction of characteristic features from a bitmap. It is robust and proved efficient, possessing all highly desirable properties, as illustrated in subsequent figures. As an input we have a simple monochrome (0 = white, 255 = black) bitmap (matrix) $A$ of a fixed format, (here presented with $400 \times 400$ pixel resolution). The components of $A$ signal amplitude values, or e.g. spectrogram intensities will be denoted by $A(x, y)$, where $x$ indicates the corresponding row and $y$ indicates the corresponding column. Spatial $x$-wise and $y$-wise differences $I_x$ and $I_y$ are defined as follows:

$$ I_x = \frac{\partial A(x, y)}{\partial x}, \quad I_y = \frac{\partial A(x, y)}{\partial y} $$

(1)

The matrix $G$ of sums of spatial square differences is defined by

$$ G = \sum_{x=p_x-\omega_x}^{p_x+\omega_x} \sum_{y=p_y-\omega_y}^{p_y+\omega_y} \begin{bmatrix} I_x^2 & I_x I_y \\ I_x I_y & I_y^2 \end{bmatrix}. $$

(2)

where $\omega_x = \omega_y$ is the width of integration window (best results are obtained with values between 2 and 4), while $p_x$ and $p_y$ are the indices corresponding to the indices $x$ and $y$ such that the formula (2) is defined. That is, all inner pixels (i.e. pixels for which $I_x$ and $I_y$ can be defined) are included in the computation. We rewrite $G$ in the more compact form as

$$ G = \begin{bmatrix} a & b \\ c & d \end{bmatrix}. $$

(3)

Using the above compact form (3) of $G$ we can compute its eigenvalues by

$$ \lambda_{1,2} = \frac{a + d}{2} \pm \sqrt{\left(\frac{a - d}{2}\right)^2 + 4bc}. $$

(4)

Furthermore, for each inner pixel with coordinates $(x, y)$ we define $\lambda(x, y)$ by

$$ \lambda(x, y) = \min(\lambda_1(x, y), \lambda_2(x, y)). $$

(5)

Finally, for the given lower threshold $T_{\text{min}}$ and parameter $A_{\text{max}}$ (here equal to 255) set the value

$$ \lambda_{\text{max}} = \max(\lambda(x, y)) \text{ at } (x, y) \text{ is an inner pixel}. $$

(6)
Define the extraction matrix by
\[ E(x, y) = \begin{cases} \frac{\lambda_{\text{max}}}{\lambda_{\text{max}}}, & \lambda(x, y) \geq T_{\text{min}} \\ 0, & \lambda(x, y) < T_{\text{min}} \end{cases} \] (7)

When two images or spectrograms are available (two consecutive shots or two significantly linearly independent channels) we obtain a solution in even harder case for automatic extraction. Let \(B\) and \(C\) be two images where every pixel is contaminated with noise which has a normal Gaussian distribution, in which stationary signal is injected, objects at coordinates \((x_1, y_1), \ldots, (x_{10}, y_{10})\), all with intensity \(m\) (within \([0, 255]\) interval) and fluctuation parameter \(p\); we generate the new binary image \(A\) in two steps:

\[ A(x, y) = \text{abs}(B(x, y) - C(x, y)) \]

If \(A(x, y) < p\) then \(A(x, y) = 255\) else \(A(x, y) = 0\); (8)

The above simple discrimination reduces random noise significantly and exhibits signals together with residual noise. Performing procedure defined by equations (1) thru (7), we obtain the filtered image with extracted signals. The method is adaptable, using two parameter optimization (minimax): minimal integral surface of detected objects, then maximization of the number of small objects.

In the following example we have introduced several dots (useful signals) with the amplitude \(a = 120\) and we have contaminated the image with random and the cloudlike noise. The lefthand side image in Fig. 7 shows bitmap with the random contamination of signal – dots. The righthand side image of the same figure shows the resulting bitmap after the application of the procedure for the noise reduction. After the initial setting \(A_{\text{max}} = 255\) and \(T_{\text{min}} = 124\), the extraction procedure yields image shown below in Fig. 7 right. Somewhat different situation we have in Fig. 8.

**Fig. 7** Dot like structure is embedded in the noise (left), which is properly reduced (right).

**Fig. 8** The left hand side image shows similar example of a signal – dots as in Fig. 7 contaminated with cloudy noise containing granular elements similar in size and intensity to the signal. The right hand side image shows results of the reduction of noise: some new dots belonging to noise cannot be distinguished from the signal – top and low right. Note that the amplitude of the target signal is lower than the chosen lower threshold.

We illustrate the application of the method of small feature extraction with the two independent sources, shown on Fig. 9, with signals embedded in the noise and the process of signal extraction.
2.4 Small object recognition using Kalman filter banks

Alternative method for the detection/extraction of small features is based on a bank of Kalman filters. After the construction of the initial sequence of images $Z_k$, the bank of one-dimensional simplified Kalman filters (see e.g. [27]) is defined using the iterative procedure as follows:

$$K_k(x, y) = \frac{P_k(x, y) + Q}{P_k(x, y) + Q + R}$$  \hspace{1cm} (9)

$$\tilde{X}_k(x, y) = \tilde{X}_{k-1}(x, y) + K_k(x, y) \cdot (Z_k(x, y) - \tilde{X}_{k-1}(x, y)) ;$$  \hspace{1cm} (10)

$$P_k(x, y) = (1 - K_k(x, y)) \cdot (P_{k-1}(x, y) + Q).$$

Initially

$$P_0(x, y) = \tilde{X}_0(x, y) = 0, Q = 1, R = 100,$$

where $Q$ is the covariance of the noise in the target signal, $R$ is the covariance of noise of the measurement. We put (depending on the dynamics of the problem): the output filtered image in $k^{th}$ iteration is the matrix $\tilde{X}_k$, the last of which is input in the procedure described by equations (1) to (7), finally generating the image with extracted objects.

This method shows that it is not necessary to know about the signal level if we approximately know statistical parameters of noise and statistics of measured signal to some extent. In our basic case we know that its mean is somewhere between 0 and 255 and that it is contaminated with noise with unknown sigma.

Application of Kalman filters in small object extraction. In the experiment shown, the initial sequence of images $Z_k$ of the size $200 \times 200$ pixels is generated as follows. First, in each image we have introduced the noise by

$$Z_k(x, y) = \text{randn}(0, 90);$$

where "randn" generates random numbers in the interval $[0, 255]$ using Gaussian distribution with $\mu = 0$ and $\sigma = 90$.

Then, in each image we injected 10 objects (useful signal) at the same positions, each of them of the size around 10 pixels, with random (Gaussian) fluctuation in intensity around mean value (here 120). After the construction of the initial sequence of images, the bank of $200 \times 200 = 40000$ one-dimensional simplified Kalman filters is defined using the iterative procedure as above.

The resulting process of noise elimination and consequent pattern-feature extraction is shown in Fig. 10. We can notice that the minor small objects reshaping is present in the result, with the whole pattern preservation, but further improvements/corrections are possible.
The method of small object recognition originally developed for marine radar object tracking, works with vectors equally well. It is applicable for automatic extraction of signals which are embedded in noise and are imperceptible (also in spectra) in case when we can provide at least two sources which are sufficiently linearly independent (their linear dependence on signal components is essential for object filtering – extraction), or when the conditions for application of Kalman filters are met.

2.5 Image spectroscopy

We can define a locally periodic function as a roughly periodic function on a finite interval. The concept is applicable in microscopic image analysis. If we have two adjacent matrix columns or rows of an image (with reduced random noise), those will exhibit essential similarity, which would include all or some of tiny details. Concatenating adjacent cuts into a signal (see Fig. 11), it will become more or less locally periodic – depending on the features present in images. Here
we present the potential of the idea in image processing by essentially introducing new nonstandard mathematical-optical components in the optical path corresponding to optical membranes – correctors, nonhomogeneous in thickness or density, whose simpler forms are often used in astronomy as corrector lenses. Thus, initial image $F$, cut into $m$ rows and $n$ columns respectively, concatenated into corresponding locally periodic signals $A_1$ and $A_2$ are shown in detail in Fig. 11, with the respective Fourier spectra shown in Fig. 12 (taking for sample rates length of a row and column respectively). On the consecutive spectra, the following filtering operations are performed: intervals of the form $(i+\varepsilon, i+1-\varepsilon)$, for $\varepsilon > 0$, $i > 1$, are annihilated and inverse FFT of the filtered spectra are taken which are restored to images in the same fashion the originals were cut/concatenated into signals $A_1$ and $A_2$.

![Fig. 12](image_url) The previously generated sequences $A_1$ and $A_2$ from Fig. 11 with their spectra and spectral fragments in the lower and higher “frequency” domains.

Taking linear combinations or mux of these transforms, starting with the original image $F$, the filtered image $F^T$ is obtained

$$F^T = k_1 \cdot f^R + k_2 \cdot f^C$$

(11)

where $f^R$ is a both rowwise and $f^C$ both columnwise composition of transformations, as explained above, $k_1$ and $k_2$ are scalars.

In this way we obtain the decomposition of the original image $F$ into a pair consisting of the filtered or transformed component $F^T$ and the residual component – image $F^C$

$$F = F^T + F^C$$

(12)

The residual component $F^C$ is obtained after filtering the original image $F$ and we can consider it as the complementary element inserted into the optical path, or as a complex corrector lens.

Examples of contaminated original images, followed by filtered images and the residuum images – corresponding to the new inserted mathematical-optical component like a complex meniscus correcting lens, as used in astronomy for comma correction, are presented in the Figs 13 – 15.
Fig. 13 Three examples of contaminated original images, followed by filtered images and the residuums – corrector membrane lens. The chromosome on the left has a stroke-like artifact, which is filtered in the middle image, residuum on the bottom left; chromosomes on the right side have artifacts induced by bubbles around impurity particles present in the immersion oil between objective and preparation, visible in both examples closer to the left edge. Filtered images are with highly reduced artifacts, thus providing images as if they would be with no bubbles initially.

Fig. 14 to the left: original image with contamination – bubbles of the same origin as in Fig. 13. In the middle, we have the artefacts removed applying the above described method, resulting in the filtered image – in the middle; to the right: the residual image with a cluster of diffraction structures is shown with tiny details, representing a new complex mathematical-optical component in the optical path.

This technique provides some important mathematical invariants of objects in images. The application of photo morphology of section 2.1 might provide here some more details in the structures obtained with this technique, potentially offering optimised selection of parameters used in the process of filtering and the subsequent linear combinations. Combined in real time the presented techniques offer an expanded optical microscope securing the insights not available as hardware devices.
Fig. 15 A photomorphology showing the fragment of the original image in Fig. 14, top left, with the structurality cleared of the artifacts, top right and subtle details in the structure of the corrected element (bottom image).

2.6 Linear combination

All the methods for small object extraction/ recognition can be used for the localisation of FISH signals in chromosomes; together with photomorphology, chromosome normalisation and comparison of chromosomes this provides a method for highly precise addressing of positions of genes in chromosomes. With cheap high resolution CCD chips and with reduced pixel size, we can easily obtain a few thousand pixels per chromosomal length, thus providing new ways of investigation of chromosomal structures in technologies complementing more sophisticated microscopic techniques with features specific for optical microscopy.

Fig. 16 a) FISH signal on a chromosomes; b) normalized – „rectified“ chromosomes with signals, well aligned, with the FISH signal localization within 2 pixel error of longitudinal sections, exhibiting some of absorption structure.

3. Conclusion - comments

The methods presented here have been corroborated in extending the detecting capacity in optical and UV microscopy, uncovering hidden or hardly perceptible objects, their complex relations, increasing resolution and in the increased measurement precision; they have been extended and expanded with more sophisticated ideas, involving more complex


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