Article

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The principles of scale space applied to structure and colour in light microscopy

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Abstract

This article shows that scale space theory and spatial colour models can be quantitatively applied to light microscopy images through the use of computer software algorithms. The use of scale space is particularly helpful in obtaining information about an image in which the conditions were less than optimal, thus reducing the pressure and time during experimental setup and data acquisition. In particular, this article considers two dimensional brightfield and fluorescence microscopy. However, the principles described can be applied to other forms of microscopy, including three dimensional images and time series images, thus introducing a number of avenues for assisting in future research.

Introduction

Both structural features and colour are powerful cues in human vision to distinguish objects like cells or tissue regions in light microscopy. Taking these structural features or geometry and colour into consideration in image processing often enables a more robust segmentation than based on traditional thresholding. However, spatial structures are not easily captured in an algorithm. Even worse, a common language for explaining the variety of shapes in biological preparations to an image processing system is presently not available to the biologist.

Closest to what is manageable for an image processing system nowadays, and easily grasped by a human expert, is the decomposition of shapes into local primary details, like 'bended', 'elongated', 'circular', convex here and concave there, and so on. Such local shape directives are mathematically described by differential geometry, which provides one of the tools for describing shapes of objects. However contrary to the ideal world of mathematics, in the real world under the microscope, the apertures with which we observe the world are not infinitesimally small, but have a certain size and spatial extent and are influenced by small perturbations, deformation and noise.

Linear scale space provides us the framework of measuring, or better estimating, those mathematical quantities, as discussed in Koenderink (1984), Lindeberg (1990), Lindeberg (1994), ter Haar Romeny (1994). The scale space framework tells us how differential geometry can be applied to image processing. The mathematical derivative operator, the basic tool for differential geometry, is replaced by a convolution of the image with the derivatives of a Gaussian kernel. Analysis of objects of a particular size is done by studying the structure at the appropriate scale, given by the standard deviation of the kernel. By using the Gaussian ker-

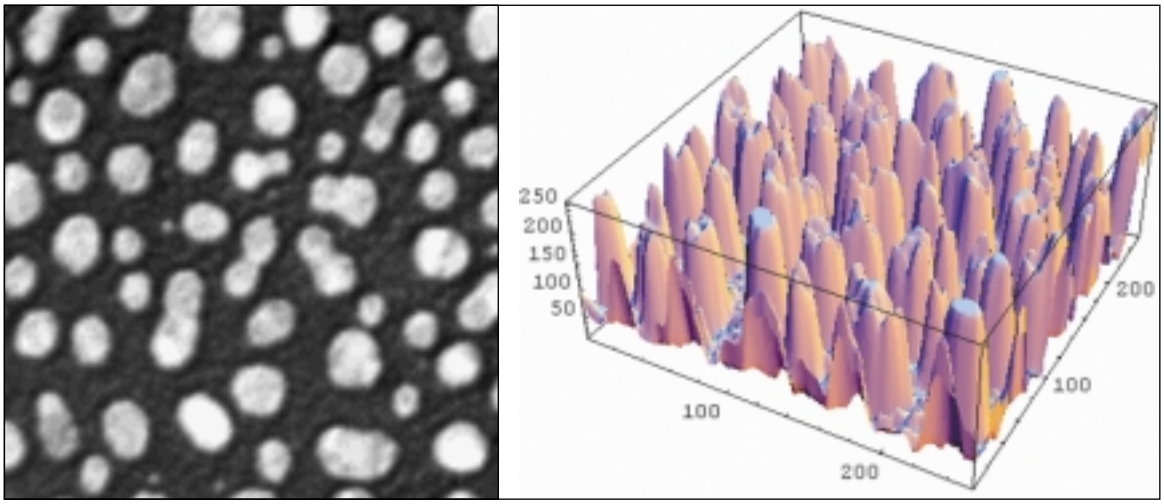

Fig. 1. The luminance image on the left is presented on the right as a 3D landscape with hills and valleys.

nel with a certain scale σ, it is possible to select cells, nuclei and tissue regions of certain size and shape (Florack *et al.*, 1992). In this way the scale space framework enables the robust extraction of local shape features from light microscopical images. The recent development of the scale space framework to combine local shape, hence spatial detail, with colour, thus spectral information, extends its applicability from single stained preparations to full colour preparations (Koenderink & Kappers, 1998; Geusebroek, 2000; Geusebroek *et al.*, 2001). In this paper we advocate the use of the scale space framework, and show a few successful biological image processing applications.

Before we continue we will first give an overview of the symbols used in this article and which might help to explore the literature on scale space. The image itself is described as L (from Luminance), while L_x and L_y are the first order Gaussian derivatives in the X and Y direction of the 2D image. L_x and L_y may be regarded as the edges in the image for the X and Y direction respectively. L_{xx}, L_{yy}, etc. are the second and higher order derivatives. For the Gaussian colour model, E (from Emissivity) is the zero order Gaussian derivative to wavelength λ, E_λ and $E_{\lambda\lambda}$ are the first and second order derivative to λ. Note that E in the Gaussian colour model is just another notation for L in luminance (grey) scale space. Hence, a colour scale space is an extension of the echromatic luminance scale space by two chromatic entities E_λ and $E_{\lambda\lambda}$. The chromaticity E_λ represents the opponent colours yellow and blue, whereas $E_{\lambda\lambda}$ represents the opponent colours red and green.

In principle we can look at an image as to a landscape in which we describe mathematically the shapes we see (Fig. 1). In the case of bright objects on a dark background (or vice versa) the problem can in most cases be considered to be the detection of elliptic patches and ridges, depending on their shape, either round or elongated (Koenderink, 1984; Florack *et al.*, 1993).

Applying scale space to intensity microscopy

Microscopy images acquired with a grey-level (B/W) camera contain structural features for which grey-level scale space provides a robust detection framework. The work of J.J. Koenderink provides us with the theoretical basis for this approach. From his and other research we know that the linear scale space framework offers robust structural feature selection for image distortion, noise and intensity changes. Feature detectors can be constructed based on differential invariants which can be designed such that robustness against changes in illumination condition and signal-to-noise ratio is obtained. This is an important feature in light microscopy.

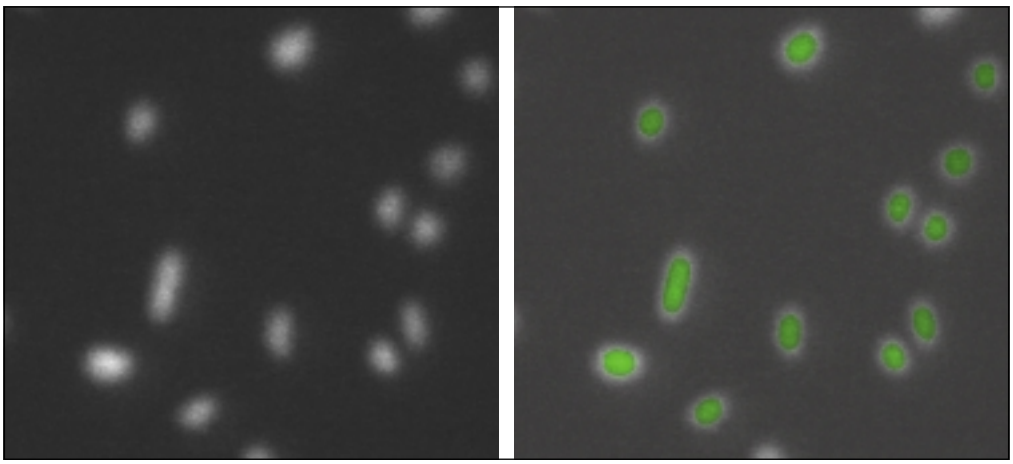

Fig. 2. Cell nuclei acquired with a Photonic Science® ISIS-3™ intensified camera at 40× magnification on a Carl Zeiss® Axiovert 135™ inverted microscope. Hoechst 33342™ stained cell nuclei shown on the left and the result of elliptic patch detection shown on the right (green overlay). Field of view approximately 500μm.

As a general example of the use of scale space to microscopy, we can consider two problems. In the first example we will apply the principles of scale space to the detection of nuclei in fluorescence microscopy. The second example shows us the detection of neurites in brightfield microscopy.

Cells were cultivated in COSTAR™ clear-bottom 96 well plates. Nuclei were stained with Hoechst

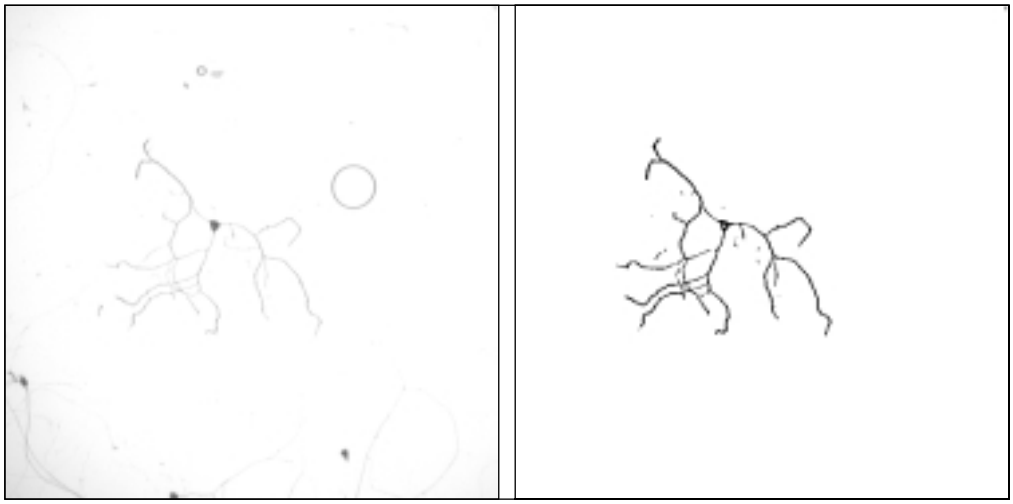

Fig. 3. An example of brightfield microscopy of a neural cell and its neurites on the left and the result of applying a filter for dark ridges shown on the right. Despite the uneven illumination, the proposed method is clearly capable of segmenting the neurites. Neurites are typically 50μm across.

33342™. Images were acquired on a Carl Zeiss® Axiovert 135™ inverted microscope at 40× magnification with a Photonic Science® ISIS-3™ intensified camera. By using an intensified camera we are able to detect extremely faint fluorescence signals without the need for temporal integration of the signal, thereby minimising the time needed to acquire an image. The use of an intensified camera results in a considerable reduction in signal-to-noise ratio (SNR). The acquired images were analysed on a Silicon Graphics® O2™ workstation with software developed in SCIL Image 1.4.1™ (van Balen *et al.*, 1994).

Figure 2 shows the result of applying the feature detector for bright elliptic patches on a noisy fluorescence image. At the appropriate scale (σ) of 9.0 we can easily detect the cell nuclei amidst the noisy background. The scale correlates to the size of the objects in the image we want to select and is a measure for the spatial integration by the Gaussian kernel. The scale space equation solved is given by:

$$L_{xx} < 0 \text{ and } L_{xx}L_{yy} - L_{xy}^2 > 0$$

Linear scale space allows us to detect the bright spots, even in very noisy images like Fig. 2 (Lindeberg, 1993). Instead of temporal integration to improve the SNR, the scale space framework uses the Gaussian filter to integrate the spatial extent, hence improves the SNR.

Another example of a feature detector is shown in Fig. 3. Detection of dark ridges in brightfield microscopy of neurites, the scale of the Gaussian kernel σ=2.0 and the threshold t=1.0. In this example the filter looks at the local difference in intensity gradients (Lpp) and the response of the filter is a measure for the difference in the intensity gradient in orthogonal directions (Steger, 1998). The applied threshold is a measure for the minimum level of linearity for a pixel to be considered being part of a ridge or line.

$$Lpp > 4.0 * t / \sigma^2$$

The expression is invariant to a change in illumination, or a non-uniform illumination (Fig. 3). The same princi-

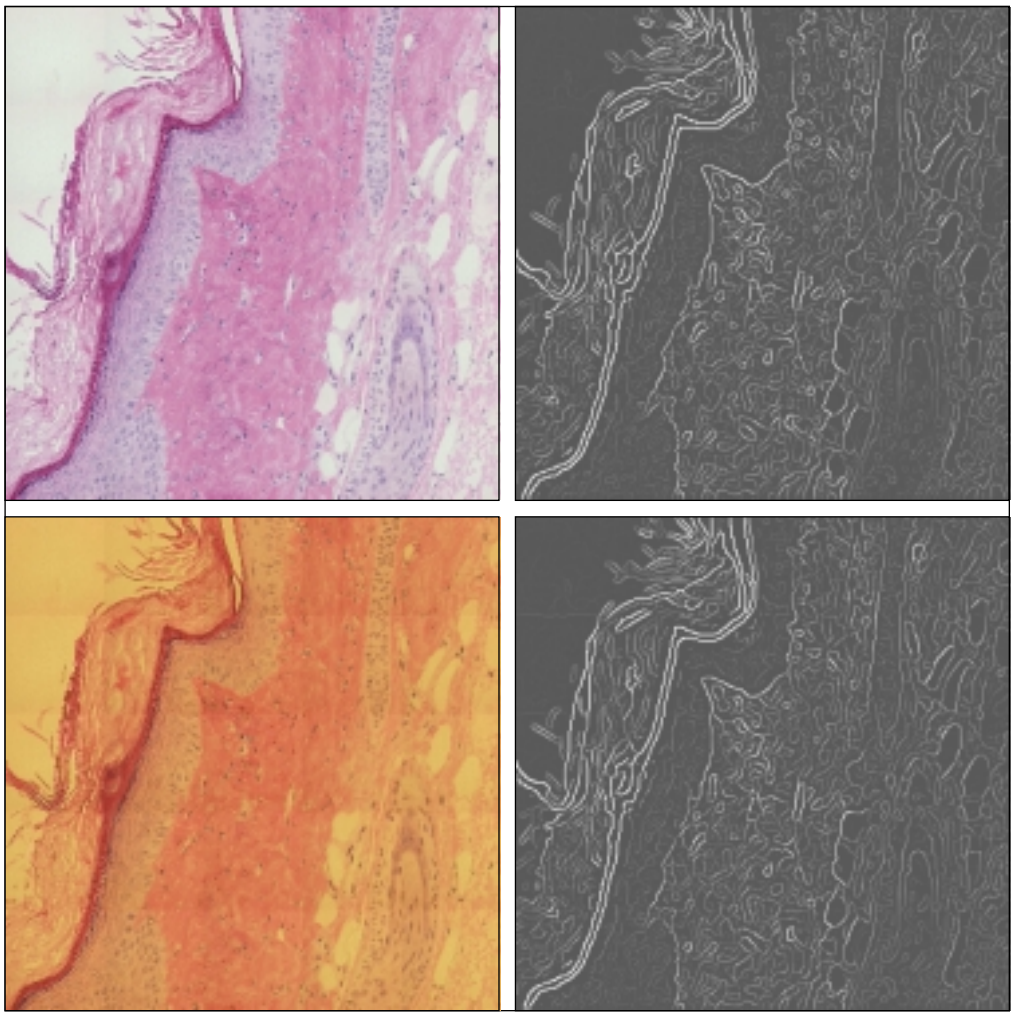

Fig. 4. Colour ridge detection (on the right) of a skin tissue section illuminated by a halogen bulb at 4000K (top) and 2600K (bottom) colour temperature. Image taken with a Carl Zeiss Axioskop. Field of view approximately 1.5mm.

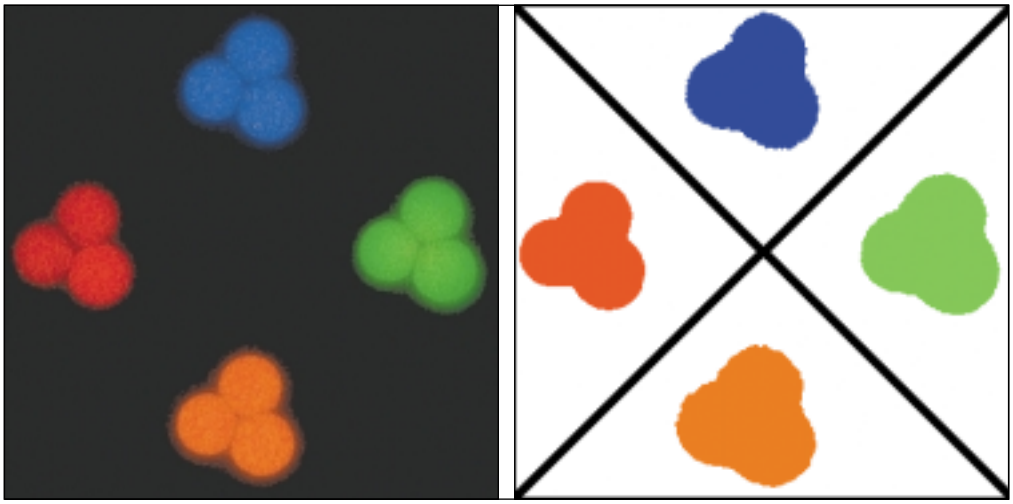

Fig. 5. TetraSpeck™ 4.0 μm beads photographed using optical filter sets appropriate for DAPI, fluorescein, rhodamin and Texas Red dye (left) and the result of colour detection with the spatial colour model (right). The field of view is about 25 μm. Courtesy of Molecular Probes, Inc.

ple can be applied to other images in which elongated structures need to be detected. See Geusebroek *et al.*, 2001a for the application of this technique to heart tissue segmentation. Other examples include Spirochetes stained with a Warthin-Starry silver stain or myelin sheaths stained black with Toluidin blue in a peripheral nerve preparation, in which case the circular black rings are considered to be dark ridges.

Another application of scale space in microscopy is in robust real-time autofocussing in automated microscopy. The first order Gaussian derivative can be used as a measure for calculating a focus score. By increasing the scale of the derivative, even images with an extremely low SNR can be reliably focused. A patented application of this principle is already in use for high-volume automated microscopy (Geusebroek *et al.*, 2000a).

Applying the spatial colour model

For colour light microscopy the spatial colour model as proposed by J.J. Koenderink and J.M. Geusebroek to select different coloured regions and objects allows for elegant solutions in light microscopy (Koenderink & Kappers, 1998; Geusebroek, 2000; Geusebroek *et al.*, 2001). The methodology applies to both brightfield microscopy as well as fluorescence microscopy. In the spatial colour model the visible spectrum is probed with a Gaussian kernel (E) and its first (E_λ) and second order derivative ($E_{\lambda\lambda}$) relative to wavelength (λ), all centred on 520 nm and with a scale sigma (σ) of 55.0 nm. These theoretical values are derived from the human visual system and are well approximated by RGB cameras. A 3CCD colour camera 'probes' the visible spectrum more or less equivalent to the human visual system. After a linear transformation of the RGB values, equivalent to the Gaussian colour model, differential invariants can be constructed which are insensitive to changes in illumination colour temperature and illumination intensity.

Figure 4 shows the robust detection of colour ridges in varying conditions of illumination. The colour temperature of the halogen bulb was changed from 4000K (top) to 2600K (bottom) and the result of this change is shown. The ridge detection is only determined by the colour transitions in the underlying sample and is insensitive to the change in illumination conditions. Certainly in long-term experiments in which large numbers of samples need to be compared or in the day to day changes in illumination conditions, this feature is a serious advantage in quantitative microscopy.

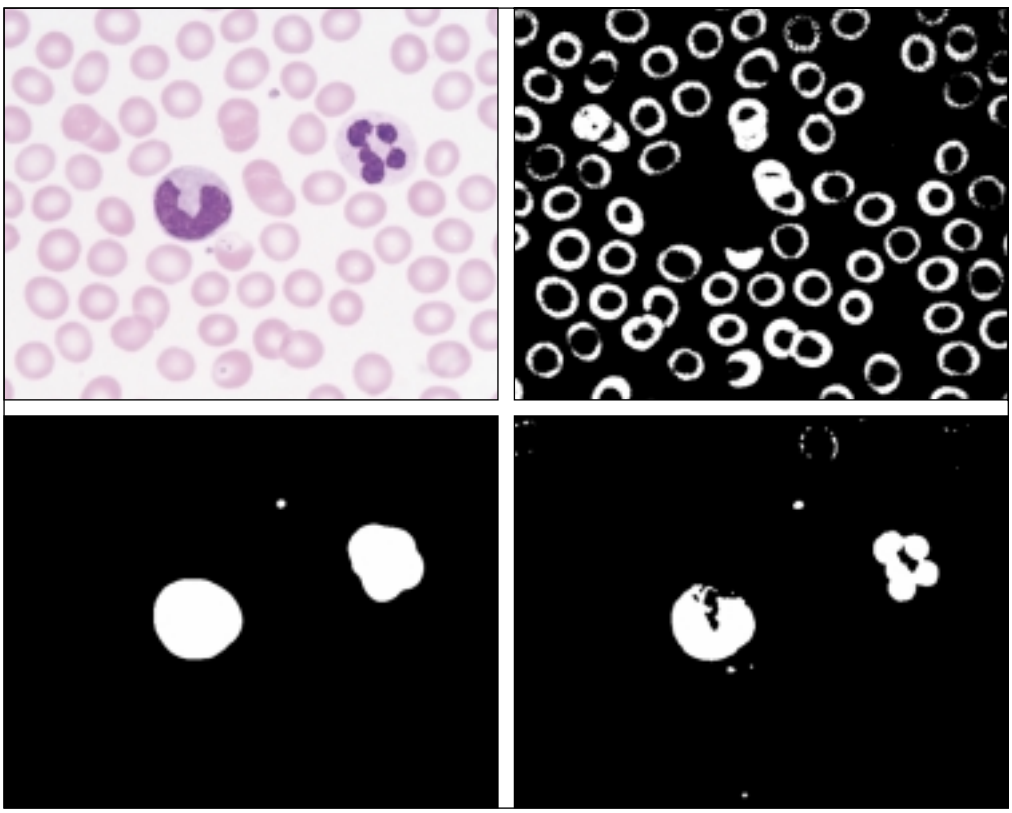

Fig. 6. Blood smear, Giemsa stain. Courtesy of University of Western Australia, Department of Anatomy and Human Biology. Blood cells are typically 5μm in diameter.

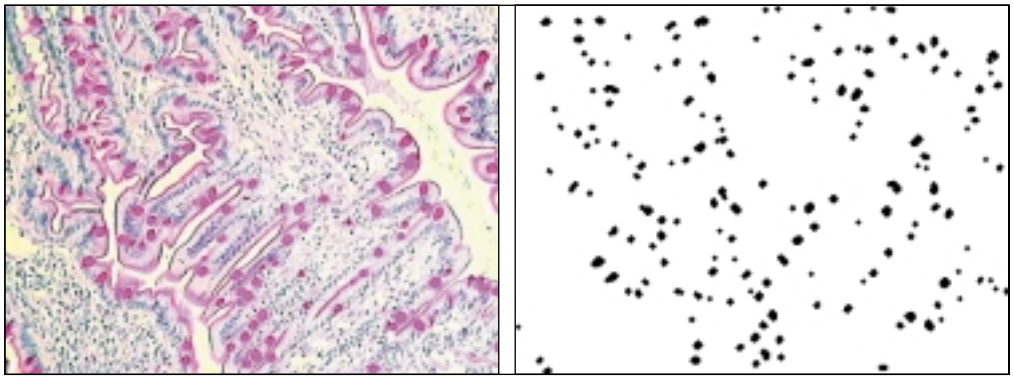

Fig. 7. P.A.S. stain for carbohydrates (goblet cells, gut) stain magenta - elliptic patches. Field of view approximately 0.5mm. Courtesy of Department of Pathology & Microbiology, University of Bristol, UK.

Not only does the spatial colour model allow us to detect colour transitions, but also the detection of different colours as such. Figure 5 shows one possible application for the spatial colour model in fluorescence microscopy. The different colours are easily selected by applying a combination of spatial colour derivatives. Combining several derivatives allows us to fine tune the spectral range we want to select. The spatial integration by the Gaussian kernel (σ=2.0) allows for improving the SNR, which is an important feature in fluorescence microscopy. The different colours are selected by:

Red:	$E_\lambda>0$, $E_{\lambda\lambda}>0$, $E_\lambda-E_{\lambda\lambda}<0$
Green:	$E_\lambda>0$, $E_{\lambda\lambda}<0$
Blue:	$E_\lambda<0$, $E_{\lambda\lambda}-E_\lambda>0$
Orange:	$E_\lambda>0$, $E_{\lambda\lambda}>0$, $E_{\lambda\lambda}-E_\lambda>0$

In Figure 6 an example is shown of the application of the spatial colour model to a blood smear and the discrimination between the erythrocytes and leukocytes. In a second step the cell nuclei of the leucocytes are discriminated from the cytoplasm based on their colour. Notice the use of the scale to include the spatial extent of the coloured cells.

Erythrocytes:	$E_\lambda>0$, $E_\lambda+E_{\lambda\lambda}>0$; σ=0.5
Leucocytes:	$E_\lambda<0$; σ=12
Leucocyte nuclei:	$E_\lambda<0$, $E_{\lambda\lambda}>0$; σ=3

Combining scale space and the spatial colour model

Scale space not only allows for feature detection on its own in grey value images, but can also be combined to detect objects of a certain colour and shape, such as shown in Fig. 7. In this case the magenta stained carbohydrates are selected from their background by applying a feature detector for elliptic patches in combination with a spatial colour model detector for magenta, scale σ=2.0. Using the detector for magenta on its own would also select the magenta stained brush border lining the gut lumen. The magenta elliptic patches detector is given by:

$L_{xx}>0$, $L_{xx}L_{yy}-L_{xy}^2>0$ and $E_{\lambda\lambda}-E_\lambda>0$

Anisotropic scale space

Besides the use of linear scale space as shown in the previous examples, the Gaussian kernel can also be modified to result in a more directionally oriented detection (Geusebroek *et al.*, 2002; Seinstra *et al.*, 2001; Weickert, 2001). An anisotropic filter allows us to align the filter with an elongated structure, such as a neurite or an image of the nematode *C. elegans* to name a few (figure 8). In this example, *C. elegans* nematodes were dispensed into a COSTAR™ clear-bottom 384 well plate with a COPAS™ BIOSORT (Union Biometrica, Inc.) and grey value images were acquired on a Carl Zeiss® Axiovert 135™ at 5× magnification. This resulted in images with an uneven and varying background and containing touching and crossing structures. The directional response of the filter gives it a clear advantage over the isotropic filter, as it allows an even more robust selection of elongated objects with a high sensitivity.

Discussion and conclusion

In this article we have shown that scale space and the spatial colour model can be applied to light microscopy. Structural features and colour can be detected separately or they can be combined to detect objects for quantitative microscopy. Scale space combined with the spatial colour model provides the scientist interested in quantitative microscopy with a theoretical framework from which several algorithms can be developed. These

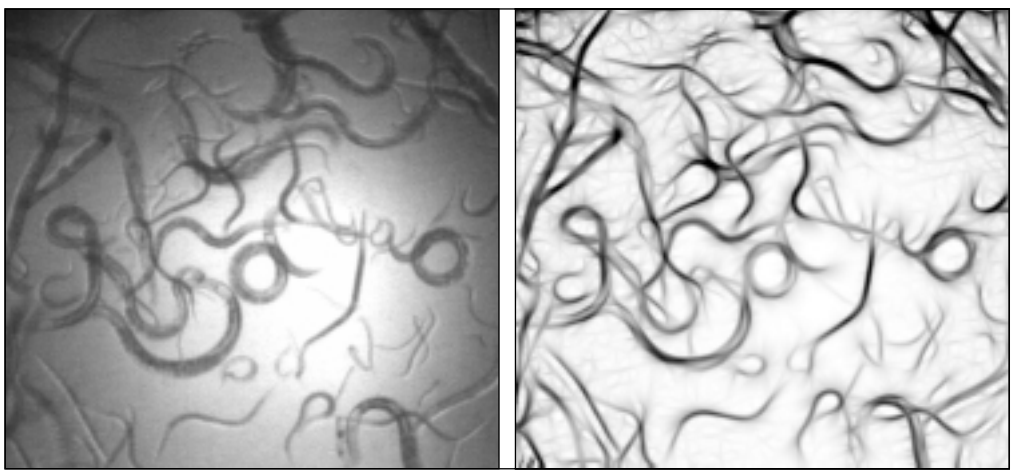

Fig. 8. An image from *C. elegans* taken at 5× magnification in a 384 multiwell plate, showing considerable illumination inequality (left). The result of applying an anisotropic filter for dark ridge detection is shown on the right. Image taken with an Adinuec MX5™ camera. Field of view approximately 500μm.

algorithms allow us to meet the needs of the cell biologist, tissue morphologist or model organism researcher, to name a few.

The robustness of the feature detectors is very useful in developing robust algorithms for the detection of objects under varying or less than optimal conditions. By using scale space, experimental conditions may be relaxed, thereby reducing the pressure on the researcher to optimize the experimental setup, and relaxing the burden to maintain the quality over time. This optimisation is often a very time consuming process, which has an impact on the resources needed to do the experiments. In highly automated high-volume industrial research in biotechnology and pharmaceutical industry, day-to-day variations are unavoidable.

The theoretical basis of scale space allows us to reduce the time needed to develop an algorithm, while at the same time retaining the flexibility to use the developed algorithm under varying conditions. This combination can be of significant importance in an increasingly competitive environment, to allow for complex and still reliable analysis of images in light microscopy.

Although we limited ourselves in this article to 2D brightfield and fluorescence microscopy, the principles of scale space can be applied to other forms of microscopy, 3D and time series. Scale space has also been applied to 3D MRI and CT imaging as mentioned in some of the references.

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