

Invited Review

Spectral imaging for quantitative histology and cytogenetics

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Summary. Evaluation of cell morphology by bright field microscopy is the pillar of histopathological diagnosis. The need for quantitative and objective parameters for diagnosis gave rise to the development of morphometric methods. Morphometry combined with spectral imaging provides multi-pixel information from a specimen, which can be used for further image processing and quantitative analysis. The spectroscopic analysis is based on the ability of a stained histological specimen to absorb, reflect, or emit photons in ways characteristic to its interactions with specific dyes. Spectral information obtained from a histological specimen is stored in a cube whose appellate signifies the two spatial dimensions of a flat sample (x and y) and the third dimension, the spectrum, representing the light intensity for every wavelength. By mathematical analysis of the cube database, it is possible to perform the function of spectral-similarity mapping (SSM) which enables the demarcation of areas occupied by the same type of material. Spectral similarity mapping constructs new images of the specimen, revealing areas with similar stain-macromolecule characteristics and enhancing subcellular features. Spectral imaging combined with SSM reveals nuclear organization and identifies specifically the nucleoli domains. Therefore, differentiation stages as well as apoptotic and necrotic conditions are easily quantified. The commercial SpectraCubeTM system was developed for the application of spectral imaging in biology, recording both transmitted light and fluorescence. The SKYTM technique utilizes the advantages of the SpectraCubeTM for multi probe FISH and chromosome karyotyping, identifying marker chromosomes, detecting subtle chromosome translocations and clarifying complex karyotypes.

Key words: Spectral-imaging, Fourier spectroscopy, Spectral similarity mapping, Optical density, SKY

1. Principles of spectral imaging

The histopathological examination experienced an important technical improvement with the development of image analysis techniques. Histopathological classification and malignancy grading of tumors have traditionally been based on subjective qualitative evaluation of morphology seen in two-dimensional tissue sections (Sorensen, 1992). However, as Mather (1953) has pointed out: "no science could reach its flowering until it becomes quantitative in both its observation and its theory". Thus, morphometrical studies have been recommended in order to achieve an objective diagnosis (Weibel and Elias, 1967). The development of spectroscopic analysis for histological and cytological specimens provides an additional tool for objective and quantitative diagnosis.

Spectroscopic analysis is based on the ability of histological and cytological specimens to absorb, reflect, or emit photons in ways characteristic to their interactions with specific stains. A high-resolution trace of the intensity of light radiation versus wavelength forms a graphical record unique to a given material. These characteristic absorption and emission bands occur in narrow wavelength ranges, 10 nm or less, and unless the measuring instrument has that resolution, these details cannot be detected. Spectral imaging, as the terminology suggests, combines spectroscopy and imaging. In dramatic contrast to conventional microscopy in which fluorochrome discrimination is based on the measurement of a single intensity through a specific optical filter, spectral imaging allows one to measure and analyze the full spectrum of light at all pixels of an image. This technology is highly data intensive, requiring the collection of hundreds of spectral data points for every pixel of an image. Other methods utilize limited spectral data obtained from single points or lines along the cell, so that image reconstruction on the basis of the measured spectra is necessary. Spectral image analysis creates a unique data-base which enables demarcation of features and evaluation of quantities from multi-point spectral information of an histological specimen. The correlation between spectrum and composition is easy in the study of known molecules; in

this case the spectra are well known and their interpretation is straightforward. In a composite biological material such as a stained histological section, the interpretation of the spectra is complicated. Any single position in space defined by a pixel of the image is actually a volume which is very large compared to molecular size, so that the spectral signal coming from that pixel contains contributions from a number of different types of molecules, each with its own complex spectrum and modified by the interactions between them.

2. The SpectraCube™: a multi-pixel Fourier spectroscopy and imaging system

The SpectraCube™ SD 200 is a spectral imaging system based on Fourier spectroscopy (Bell, 1972). The SpectraCube™ has a high optical throughput, high spectral resolution, a broad spectral bandwidth and it is independent of polarization. The 12 bit cooled CCD camera used in the SD 200 system is of a high performance, collecting 512x512 pixels (Garini et al., 1996a).

A Spectral Imager is composed in principle of an optical system containing a light source illuminating the specimen. The light emitted from the specimen is collected and collimated by an optical objective of the microscope; it is spectrally dispersed, and then imaged onto a CCD by a focusing lens. While the dispersion element is scanned, CCD frames are acquired in synchronization with it, and memorized in the computer. As the interferometer is scanned and the CCD frames collected, an interferogram for each of the pixels of the image is built simultaneously, so that on the order of >100,000 interferograms are acquired in a time between a few seconds to a minute, depending on the strength of the signal. All the interferograms are then Fourier transformed to obtain >100,000 spectra which are stored in the so-called spectral cube. The "cube" signifies the two spatial dimensions of a flat sample (x and y) and the third dimension, the spectrum, representing the light intensity for every wavelength. The SpectraCube™ design is shown in Figure 1.

The SpectraCube™ technique has several advantages over systems already used for image analysis. Fourier spectroscopy is widely used for high resolution spectral measurements in a variety of applications, mainly in the infra-red spectral region (Bell, 1972; Chamberlain, 1978; Jain, 1989). Interferometric Fourier spectroscopy exhibits several important advantages as a dispersion method, especially when combined with fluorescence imaging microscopy: (i) high optical accuracy; (ii) high and variable spectral resolution; (iii) wide spectral range and (iv) mechanical/temperature stability (Chamberlain, 1978; Vane et al., 1988). In cases where the noise is independent of the signal, or when the spectrum has narrow regions with the signal higher than average and the noise proportional to the square root of the signal, Fourier spectroscopy has a sensitivity advantage (the Fellgett advantage, see for

example Chamberlain, above) over any other filter, grating, AOTF, and LCTF based dispersion technique.

The interferometer used in the SpectraCube™ system is of a "common path" type, with specific advantages of compactness and ruggedness which allow working with the relatively short wavelength of visible light. The spectral range of a transmission or fluorescence measurement is a function of: the illuminating source, the spectral transmission of the optics and the spectral sensitivity of the CCD. The optical elements are designed for maximum transmission to ensure flexibility. Typical ranges are 420 to 750 nm for UV enhanced sources and 550 to 1000 nm for transmission or reflection of a standard halogen source. In fluorescence, the emissions are usually narrower, but in the multicolor fluorescence applications where the instrument has special advantages, the total range is again wide, and is typically covered between 520 and 800 nm in the same measurement. The spectral resolution of the instrument is tested by measuring the WHM (width at half maximum) of a laser line. The spectral resolution (FWHM, full width at half maximum) is 5 nm at 400 nm and 12 nm at 600 nm (Garini et al., 1996b).

In spectral imaging of histological and cytological specimens, >10⁴ pixel-spectra (400-850 nm) are obtained for an image, providing more information than conventional gray scale image analysis (Stenkvist et al., 1978), even when the latter is used in combination with color filters (Wells et al., 1992). The transformation from a fully colored image to a gray scale image, in morphometric methods, introduces some inaccuracy that is totally eliminated by spectral imaging. A colored image displaying closely related wavelengths with the same intensity might appear similar in a gray scale image. However, spectral imaging differentiates between closely related colors even when the total intensity is similar and is thus more sensitive and specific.

The spectral data-base of a histological image enables further processing by an algorithm called "Spectral Similarity Mapping" (SSM). The ideas behind SSM are that: i) the same type of material present in different pixels will give the same spectrum; ii) a selected feature (e.g. organelles, chromatin, etc) will be highlighted by means of a gray-level map based on spectral comparison between a chosen reference spectrum and other pixels of the image. The comparison algorithm for SSM is the following: $I_{x,y}(\lambda) - I_0(\lambda)$, $I_{x,y}(\lambda)$ is the spectrum of the pixel of coordinates x and y on the image as a function of λ , and $I_0(\lambda)$ is the spectrum of a reference pixel chosen in a region where the feature of interest is present. The function $f_{x,y}$ is defined for every pixel of the image as follows:

$$f_{x,y} = \left(\int_{\lambda} [I_{x,y}(\lambda) - I_0(\lambda)]^2 d\lambda \right)^{1/2}$$

where the integral over λ stands for an integral over a predetermined spectral range $\lambda_1 - \lambda_2$. A black and white image is displayed on the screen, where the gray level of each pixel is defined as brighter for lower $f_{x,y}$,

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and as darker for higher $f_{x,y}$, so that the pixels with spectra more similar to I_0 are highlighted (Fig. 1A, B).

Choosing specific pixel spectra from a given specimen forms a spectral library. The library serves to scan the samples, cells or chromosomes, assigning an

arbitrary color to each spectrally similar region as calculated by minimal square error (Fig. 1C-D, Fig. 1E-F, respectively). The reconstructed image is a classified map representing spectrally similar pixels in the specimen and enhancing the spectral differences to the

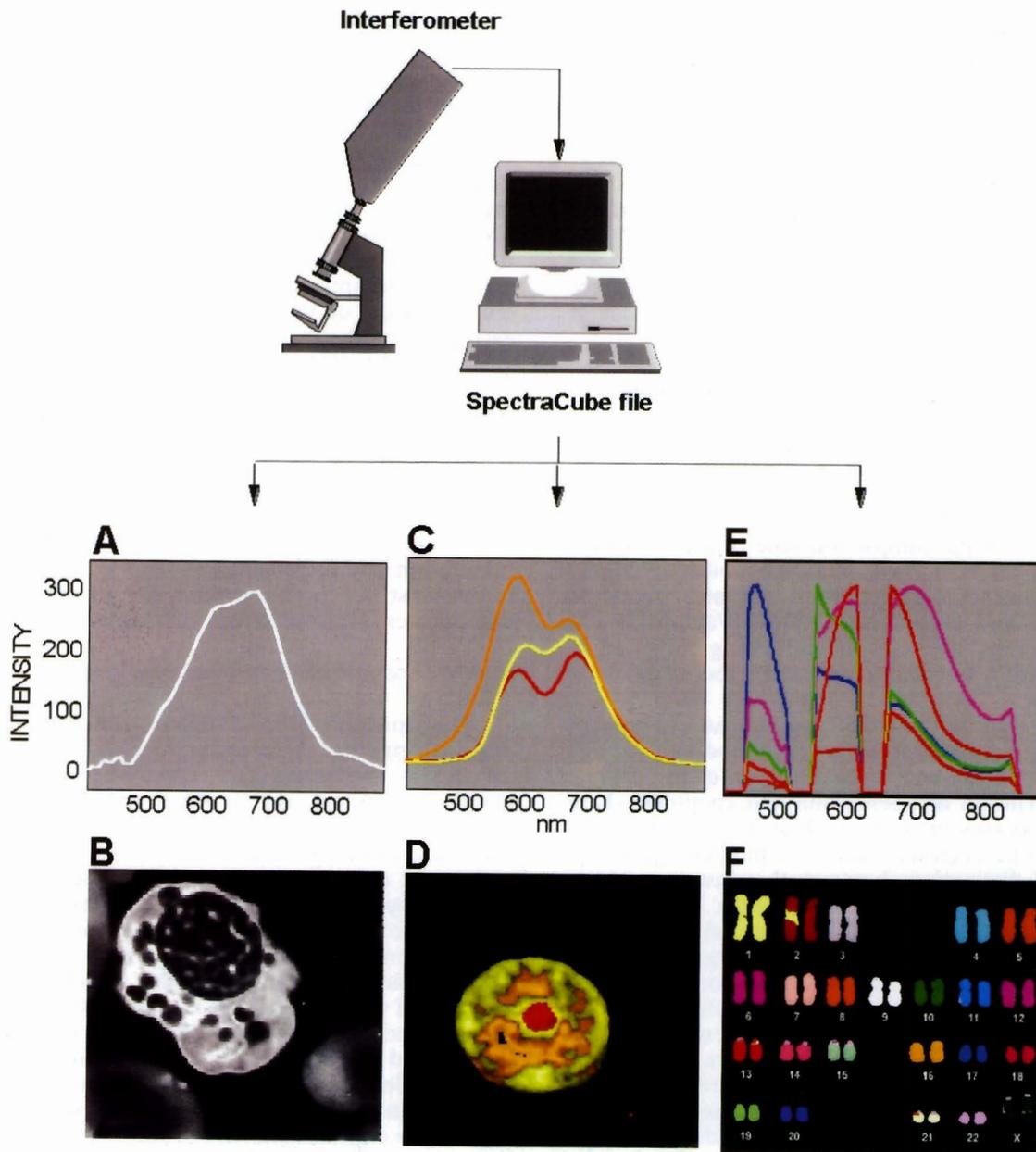


Fig. 1. The SpectraCube™ system and its applications. Acquisition of a multi-pixel spectral-cube of an histological specimen is performed by a Sangac interferometer attached to a cooled CCD camera (PI, Princeton Instruments) and mounted on top of a bright field or epifluorescence microscope. The light emitted from the specimen is collected by the objective lens, dispersed in the interferometer and imaged onto the CCD camera. Light intensity at any wavelength (450-850 nm) over $>10^4$ pixels is measured by the SpectraCube™. Spectral similarity mapping using a single reference spectrum (A) produces a gray-level image where regions with similar spectra are highlighted (B). SSM using a spectral library with spectra depicted from different subcellular regions such as the heterochromatin, euchromatin and nucleoli (C) will assign arbitrary colors to each spectrally similar domain (D). Spectral karyotyping of chromosomes is performed using the SKY™ method. The chromosomes are hybridized with whole chromosome probes, each one emitting a different fluorescence spectrum. A spectral library composed of known spectra (E) is used to scan the chromosomes. Thus, individual chromosomes are identified and displayed in enhanced pseudo-colors (F).

observer.

3. Applications of spectral imaging in cytology, histology and cytogenetics

The SpectraCube™ has already been used in a large number of biological fields. The subcellular localization of porphyrins in treated cancer cells was determined using the known fluorescence spectra of the porphyrines as a marker for tracing (Malik et al., 1996b, 1997). In addition, the localization of protoporphyrin auto-fluorescence was determined in treated human patients with skin cancers (Malik et al., 1996a). Nuclear organization in normal erythropoiesis and apoptosis was studied by the transmitted light spectra of stained cytological specimens (Rothmann et al., 1997). In addition, a novel karyotyping method was developed using the SKY™ technique in order to detect chromosomal abnormalities in solid tumors (Schrock et al., 1996) and leukemias (Veldman et al., 1997).

Subcellular morphology resolved by spectral imaging

Nuclear chromatin structure is modulated by histone-histone, histone-DNA interactions, histone modifications and by the presence of non-histone proteins. The accessibility of a specific DNA sequence is a consequence of the compaction-state of the chromatin in which it is located. Thus, the accessibility of highly condensed chromatin is likely to be extremely restricted (Woodcock and Horowitz, 1995). Variability in chromatin organization in differentiating cells and in abnormal cells is regarded as a common tool in cytology and histology.

Nuclear compartmentalization and chromatin organization was studied in the red blood cell lineage using the SpectraCube™ (Rothmann et al., 1997). Terminal erythroid differentiation and apoptosis share common processes of nuclear chromatin condensation and cell-volume decrease. However, morphological and biochemical distinctions between these two processes have been described (Kelley et al., 1993). Rothmann et al. (1997) have demonstrated with spectral imaging the chromatin condensation and nuclear organization of May-Grunwald Giemsa (MGG) stained normal erythropoietic bone marrow cells and of apoptotic red cell precursors obtained from an agnogenic myeloid metaplasia patient. Two groups of spectra, of low and high intensity transmitted light, were revealed in the nuclei of each cell. The localization of these two spectral components in the nuclei was resolved by spectral similarity mapping. High-symmetry chromatin structures with a windmill-like organization were detected in the nuclei of normal basophilic, polychromatic and orthochromatic normoblast cells. Matching structures were detected in apoptotic normoblasts of the agnogenic myeloid metaplasia patient. The process of apoptosis was associated with a gradual breakdown of the ordered arrays in the nucleus. It was proposed that DNA cleavage might lead to a process of fragmentation to its

basic nuclear domains of the symmetrical windmill-like superstructure. The results showed that spectral similarity mapping reveals nuclear structural features undetectable by conventional methods.

Absorbance images

The characteristic absorbance of molecules is the basis for another approach to the determination of subcellular compartments. Application of the Beer-Lambert equation on a spectral data-base enables the formation of new spectral images depicting the absorbance characteristics of a stained cell. The Beer-Lambert equation requires two variables: the intensity of the incident light and of the transmitted light. The division of the intensity of the transmitted light by the intensity of the incident light on a logarithmic scale produces the optical density of a measured pixel. The optical density of MGG stained cells was calculated for each pixel of the image on the basis of the transmitted spectra "cube". The newly calculated optical-density "cube" served for the construction of optical density images reflecting the absorbance properties of each compartment in the examined cell. The optical density images produced a high-resolution picture emphasizing the difference in absorbance between the nucleus and the cytoplasm. The absorbance of the cytoplasm of an MGG-stained proerythroblast decreased with its maturation and differentiation into an orthochromatic normoblast. At the same time, the absorbance of the nucleus increased due to the condensation of chromatin.

Spectral analysis of histological specimens

Histopathological examination uses different staining protocols in order to emphasize cellular structures of tissues and cells. The most common staining procedure used is H&E; other procedures are the PAS stain for the demarcation of hydrocarbon moieties, Masson's trichrome stain for extracellular staining of collagen and the Romanowsky-Giemsa stain in hematopathology. The spectroscopic information obtained from histopathological specimens generates a new dimension of quantitative information to the standard morphometric evaluation. Spectral analysis is considered to be an objective tool for characterization and comparison between different cells, answering to an urgent need for objectivity in histopathology.

In order to demonstrate the power of spectral imaging in the analysis of stained histological specimens, we examined the contribution of distinct staining methods to the spectral information. Rat hepatocytes stained by Hematoxylin and Eosin (H&E), Romanowsky-Giemsa, Periodic Acid Schiff (PAS) and Masson's trichrome were assessed. The SpectraCube™ recorded light intensity from 10^4 pixels in the range of 450-850 nm from the nuclear domains of each stained specimen. The spectra of the heterochromatin, euchromatin and nucleolus were determined for each staining method and stored in a spectral library. The

areas of spectrally similar regions were classified using the SSM approach. The spectral fingerprints of Masson's trichrome stain distinguished the nucleolus from the rest of the nuclear chromatin, enabling the demarcation and calculation of the nucleolar area. Preliminary studies were performed using human histological sections. Spectral imaging of human hepatocytes stained by Masson's trichrome followed by morphometric measurements revealed marked differences between the nucleolar area in normal human hepatocytes compared to hepatocellular carcinoma. In addition, spectral imaging of normal human keratinocytes and of breast carcinoma tissue sections using the Masson's trichrome classification library also distinguished the nucleolus from the rest of the nuclear chromatin, proving that this technique is not tissue specific. One of the most attractive features of the SpectraCube™ is that all spectral morphometric analyses can easily be automated. Spectrally resolved imaging of various combinations and proportions of dyes may reveal the ideal mixture of dyes for any desired purpose.

Applications of the SpectraCube™ in cytogenetics

Chromosome analysis is a most important tool in cytogenetics, both for research and for diagnostics in a clinical setting. Since the discovery that chromosomal abnormalities are a leading cause of genetic diseases, their detection has been used extensively over the years for improved diagnosis and therapy. Chromosomal structure rearrangements have also gradually begun to be correlated with tumor aggressiveness, morphology and staging and therefore can be of prognostic value. In addition, the identification of abnormal chromosome regions as suggested by cytogenetic analysis has led to the isolation of new genes that are involved in specific diseases (Solomon et al., 1991; Heim and Mitelman, 1995).

The most common method used today for cytogenetic analysis is the G-banding technique. This method is used in routine screening of cells for the detection of numerical as well as structural aberrations associated with specific genetic syndromes or with certain types of cancers. However, G-banding is sometimes insufficient because of difficulties encountered in analyzing complex karyotypes or subtle chromosomal rearrangements. Fluorescence in Situ Hybridization (FISH) makes use of DNA probes covalently bonded to fluorescent dyes to permit rapid characterization of genetic aberrations at the cellular level (Lichter and Ward, 1990; Tkachuk et al., 1991; Ledbetter, 1992).

Until now, the use of FISH has been restricted to cases in which only one or a few specific abnormalities are investigated in one sample, and there is prior knowledge of which chromosomes are involved. The analysis has therefore been directed at finding evidence of specific changes that are suspected to be present. At the same time, the cytogeneticist's task has been difficult and prone to mistakes due to the limited number of available fluorescence probes and the fact that their

spectra overlap significantly. Simultaneous screening would require the hybridization of the same sample with a large number of probes for different genes, each labeled with different fluorophores.

Spectral Karyotyping™ (SKY™), developed by Applied Spectral Imaging (ASI), combines the resolution and power of FISH with the advantages of conventional cytogenetic methods (Schrock et al., 1996). SKY™ uses the SpectraCube™ technology, integrated with a modified fluorescence microscope (Simon-Blecher et al., 1996; Katz et al., 1997). Once chromosomes extracted from a metaphase nucleus are hybridized with 24 whole chromosome probes, each one labeled and emitting a different fluorescence spectrum, the SKY™ system with its high spectral resolution, can identify and display all the pixels of the image in enhanced pseudo-colors of each chromosome (Fig. 1E,F). In this manner, a color karyotype can be created, enabling easy screening of cells for the detection of numerical and structural aberrations (Garini et al., 1996a; Liyanage et al., 1996; Schrock et al., 1996; Veldman et al., 1997). The advantage of SKY™ was also shown recently by the groups of Ried and Rowley (Veldman et al., 1997). The two groups analyzed hematological malignancies to detect hidden chromosomal abnormalities. Fifteen cases were analyzed and in all instances SKY™ provided additional cytogenetic information that could not have been detected using the conventional banding technique, including the identification of marker chromosomes, the detection of subtle chromosome translocations and the clarification of complex karyotyping. The conclusions from this work are that "SKY has the potential to become an important cytogenetic technique for the identification of subtle translocations, marker chromosomes and the delineation of complex chromosomal aberrations without requiring any preconceived notion of the abnormalities involved" (Veldman et al., 1997).

SKY™ can also be very useful in the analysis of solid tumor material. Very little is known about specific rearrangements in solid tumors (carcinomas, sarcomas and melanoma) despite the fact that these cancers contribute significantly more to morbidity and mortality than the hematological neoplasms. This is mainly due to technical difficulties in analyzing solid tumor material with their complicated karyotypes and the often suboptimal morphology of the chromosomes. SKY™ can help overcome these limitations and can contribute to the correct identification of specific chromosomal alterations, which may be of diagnostic and prognostic value.

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