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SHORT REPORT

Multispectral imaging for highly accurate analysis of tumour-infiltrating lymphocytes in primary melanoma

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Aims: The quality and quantity of the infiltration of immune cells into tumour tissues have substantial impacts on patients' clinical outcomes, and are associated with response to immunotherapy. Therefore, the precise analysis of tumour-infiltrating lymphocytes (TILs) is becoming an important additional pathological biomarker. Analysis of TILs is usually performed semiquantitatively by pathologists on haematoxylin and eosin-stained or immunostained tissue sections. However, automated quantification outperforms semiquantitative approaches, and is becoming the standard. Owing to the presence of melanin pigment, this approach is seriously hampered in melanoma, because the spectrum of melanin lies close to that of commonly used immunohistochemical stains. Aim of this study is to overcome the technical issues due to the presence of melanin for an automated and accurate quantification of TILs in melanoma.

Methods and results: Here, we successfully applied a novel multispectral imaging (MSI) technique to

enumerate T cells in human primary melanomas. This microscopy technique combines imaging with spectroscopy to obtain both quantitative expression data and the tissue distributions of different cellular markers. We demonstrate that MSI allows complete and accurate analysis of TILs, successfully avoiding the blurring of images by melanin pigments, in whole tissue slide primary melanoma lesions, which could otherwise not be accurately detected by conventional digital image methodologies.

Conclusions: Our study highlights the potential of MSI for accurate assessment of immune cell infiltrates, including those in notoriously difficult tissues, such as pigmented melanomas. Quantification of tumour infiltration by different immune cell types is crucial in the search for new biomarkers to predict patient responses to immunotherapies. Our findings show that this innovative microscopy technique is an important extension of the armamentarium of pathologists.

Keywords: digital pathology, melanin unmixing, melanoma, multispectral imaging, T-cell infiltration

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Introduction

Melanoma is a potentially lethal skin cancer generated from the malignant transformation of melanocytes, the melanin-producing cells of the skin.^{1,2} Although the

development of successful strategies remains a challenge, in the past decade there have been encouraging advances in melanoma management.^{3,4} Perhaps the greatest improvement in melanoma therapy has come from immunotherapeutic approaches, in particular immunomodulatory monoclonal antibodies, for which durable clinical responses and increased overall survival have recently been reported.^{5–8} Nonetheless, the costs and toxicity associated with these novel immunotherapies have created a need for the development of biomarkers to predict treatment efficacy and disease outcome.⁹

The tumour microenvironment influences therapeutic response and clinical outcome.^{10,11} Immunohistochemical (IHC) analysis of the type, location and density of tumour-infiltrating immune cells has identified immune cell types that can be either beneficial or harmful to patients.^{12–15} For example, tumour core infiltration by CD8+ tumour-infiltrating lymphocytes (TILs) positively correlates with survival for multiple cancer types.^{16–19}

So far, the density and location of TILs have been estimated by tissue IHC analysis of limited areas of the sample, by independent histopathological reviews. The total number of TILs is generally calculated by multiplying the number of cells counted in one region by the entire surface of the tumour.¹⁴ Others use a semi-automated approach, based on mathematical algorithms that can recognize and quantify cells of interest within the tumour area. Although this methodology is relatively accurate, it is extremely time-consuming. Moreover, in the case of melanoma, it falls short because of intense melanin pigments creating high backgrounds in bright-field IHC stains.^{20–22} Recent advances in the field of tissue imaging have resulted in the development of multispectral imaging (MSI) for application to whole-slide tissue sections.²² Here, we exploit MSI for linear spectral unmixing of melanin pigmentation in human primary melanoma biopsies, and demonstrate that melanin unmixing allows for highly accurate quantification of TILs in highly pigmented tumours.

Materials and methods

SPECTRAL LIBRARY GENERATION FOR MELANIN UNMIXING

Images of single-stained and unstained tissue were used to extract the spectrum of each chromogen (haematoxylin for nuclei; Vector Nova red for CD3+ T cells) and melanin pigments, respectively, with the Nuance Multispectral Imaging System (PerkinElmer, Waltham, MA). The extracted spectra were used to build a spectral library (Figure 1) that enables quantitative separation (unmixing) of each marker into its own channel/image, thus removing crosstalk between chromogens and tissue pigmentation.

MULTISPECTRAL ANALYSIS

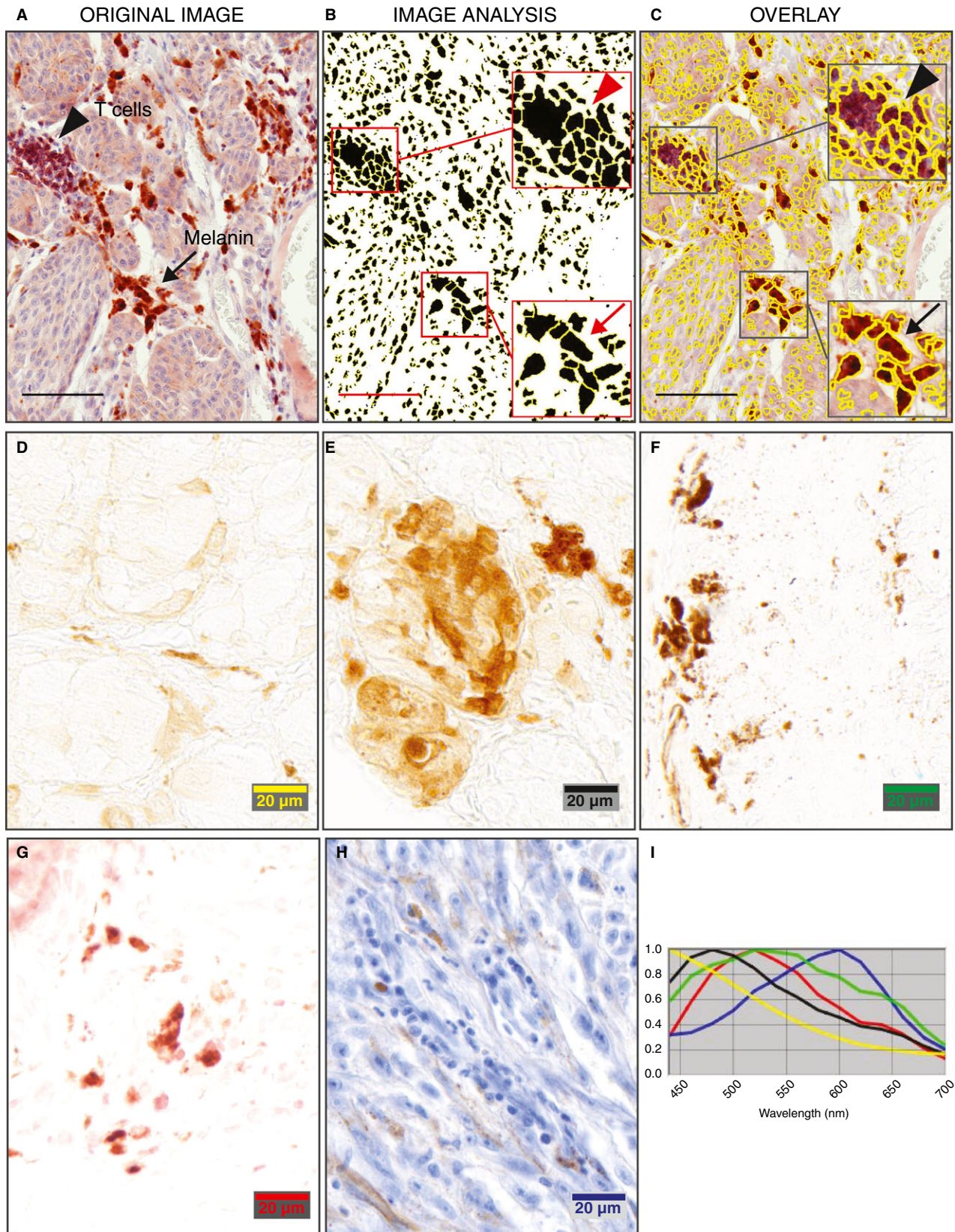
Whole tissue slides were imaged with the Vectra Intelligent Slide Analysis System (PerkinElmer). Ten to 15 representative multispectral images were loaded into advanced user-trainable image analysis software (INFORM Version 2.1; PerkinElmer), which allows the automated recognition of tissue regions and individual cells, mainly on the basis of morphological features (Figure S2). Next, using multispectral data, the software calculates per-cell and per-cellular compartment (i.e. nuclear, cytoplasmic, and membrane) optical intensity values to score cell positivity for each specific marker.^{23,24}

All the settings for cell segmentation and positivity score applied to training images were saved within an algorithm used to run batch analyses of multiple multispectral images belonging to different patients.

STATISTICS

Statistical significance ($P < 0.05$) was assessed with the *t*-test in PRISM v.5.03 (GraphPad Prism, La Jolla, CA).

Figure 1. Semi-automated image analysis limitations and melanin spectral unmixing in pigmented melanomas. (A–C), Assessment of the presence of CD3+ T lymphocytes in highly pigmented melanoma tumour by the use of semi-automated image analysis. (A), Original immunohistochemical (IHC) image of human melanoma, containing clusters of infiltrating CD3+ T cells (Nova red, arrowhead) and brown melanin pigments (arrow). (B), Output image of the semi-automated algorithm for image analysis and cell count. (C), Overlay of output and original multispectral image. (B, C), The image processing has some limitations (see zoom-in): T cells in clusters cannot be efficiently separated (top, arrowhead), and melanin pigments cause false-positive results (bottom, arrow). Scale bars: 50 μ m. (D–I), Representative IHC images of cellular components and melanin pigments used to build a spectral library for image analysis of tumour-infiltrating lymphocytes in melanomas. (D–F), Three differently pigmented melanin samples (D–F), CD3-Nova red-positive T lymphocytes (G), and haematoxylin staining of nuclei (H). (I), A spectral library of Nova red (red) and haematoxylin (blue) and three different melanin pigments (green, black, and yellow) was built in NUANCE software by the use of single-stained or unstained human melanoma tissues. Scale bar: 20 μ m.



Results

MELANIN PIGMENTATION AFFECTS ANALYSIS OF TILS IN MELANOMA

Melanoma tissue sections stained for IHC evaluation of CD3+ T cells were analysed with a four-step, semi-automated algorithm: (i) colour threshold; (ii) cell segmentation; (iii) cell counting; and (iv) overlaying of the resulting mask with the original image (Data S1, Figure S1). However, this approach has limitations that affect the outcome of the analysis. In particular, the presence of melanin pigments alters IHC quantification, owing to its high background signal, which is often indistinguishable from brown or red chromogens. In addition, the semi-automated algorithm fails to efficiently split cell clusters, which are then erroneously reported as a single cell (independently of the size of the cluster) (Figure 1A–C).

SPECTRAL LIBRARY GENERATION

To overcome the above-mentioned limitations, we used an MSI system that combines imaging with spectroscopy, thereby allowing quantification and determination of the tissue distribution of T cells in the whole tissue section. In our cohort of patients, we observed that different tumours synthesize melanin with varying intensities and distinct spectral characteristics (Figure 1D–F), and we identified three different melanin spectra. One of these showed strong overlap with the spectrum of the Nova red chromogen spectrum (Figure 1F), and was therefore responsible for the failure of the semi-automated algorithm to accurately quantify positive cells, especially within the tumour area. We combined the spectra of melanin, haematoxylin and Nova red in a spectral library (Figure 1), which was then applied to all of the acquired images. This resulted in new images (Figure 2B–D), in which the brown melanin signal became distinguishable from the Nova red signal, and was therefore not quantified as positive in the T-cell score.

T-CELL DENSITY ASSESSMENT: SEMI-AUTOMATED VERSUS MULTISPECTRAL ANALYSIS

The density of CD3+ T lymphocytes was quantified in intratumoral and peritumoral regions of melanomas, which were defined by trainable tissue segmentation, on the basis of morphological features (Figure S2). Furthermore, nuclear staining allowed for efficient segmentation of cell clusters. We observed variable levels of melanin pigmentation (intensity and

content), and scored 14 tumours as highly pigmented (>50% pigmented tumour cells) and nine as low pigmented (5–20% pigmented tumour cells). T-cell density was assessed with both our semi-automated algorithm and MSI (Data S1, Table S1). Our results show that the T-cell density in highly pigmented tumours was significantly higher with conventional analysis (owing to false-positive cells) than with the MSI approach (Figure 2K–M). This difference, was not significant in low pigmented melanomas (Figure 2L), supporting the notion that linear unmixing of melanin and chromogens with spectra close to one of the melanin spectra [such as Nova red and 3,3'-diaminobenzidine (DAB)] is particularly relevant in highly pigmented melanomas for accurate estimation of T-cell infiltrates. In some tumours, the T-cell density was slightly higher when quantified by the automated software (Figure 2H–J; Data S1, Table S1), but this was mainly attributable to more accurate cell segmentation, whereby all of the cells are detected and clusters are correctly split.

Discussion

Analysis of the location, type and density of immune infiltrates and their correlation with clinical outcome is of crucial importance in cancer research.^{11,13,14,25} Assessment of T-cell density in solid tumours is usually investigated with traditional IHC analysis and bright-field microscopy.¹⁴ Here, we compare two different digital imaging approaches to estimate the number of TILs in tumoral and peritumoral areas of melanoma.

When developing and testing a semi-automated digital image approach, we noted that, especially in melanoma samples containing dark brown melanin pigments, a large proportion of the immune cells were obscured, thereby hampering IHC measurements.²⁶ This is because different colours in bright-field images are perceived as a mixture of the red, green and blue channels, and routinely used imaging software solutions are not able to discriminate between single colour components. Consequently, melanin was detected in all three channels, at different intensities.^{20,27} An established technique for studying immune infiltrates is melanin bleaching with strong oxidants.^{28,29} Although this technique can give satisfactory results in many cases, certain antigens (such as CD3 and CD45RO) and the tissue itself can be partially destroyed. Furthermore, conventional brown chromogens used for antibody detection (e.g. DAB) are also affected by the intense oxidative procedure, thus making the analyses less accurate.^{30,31} The use of red

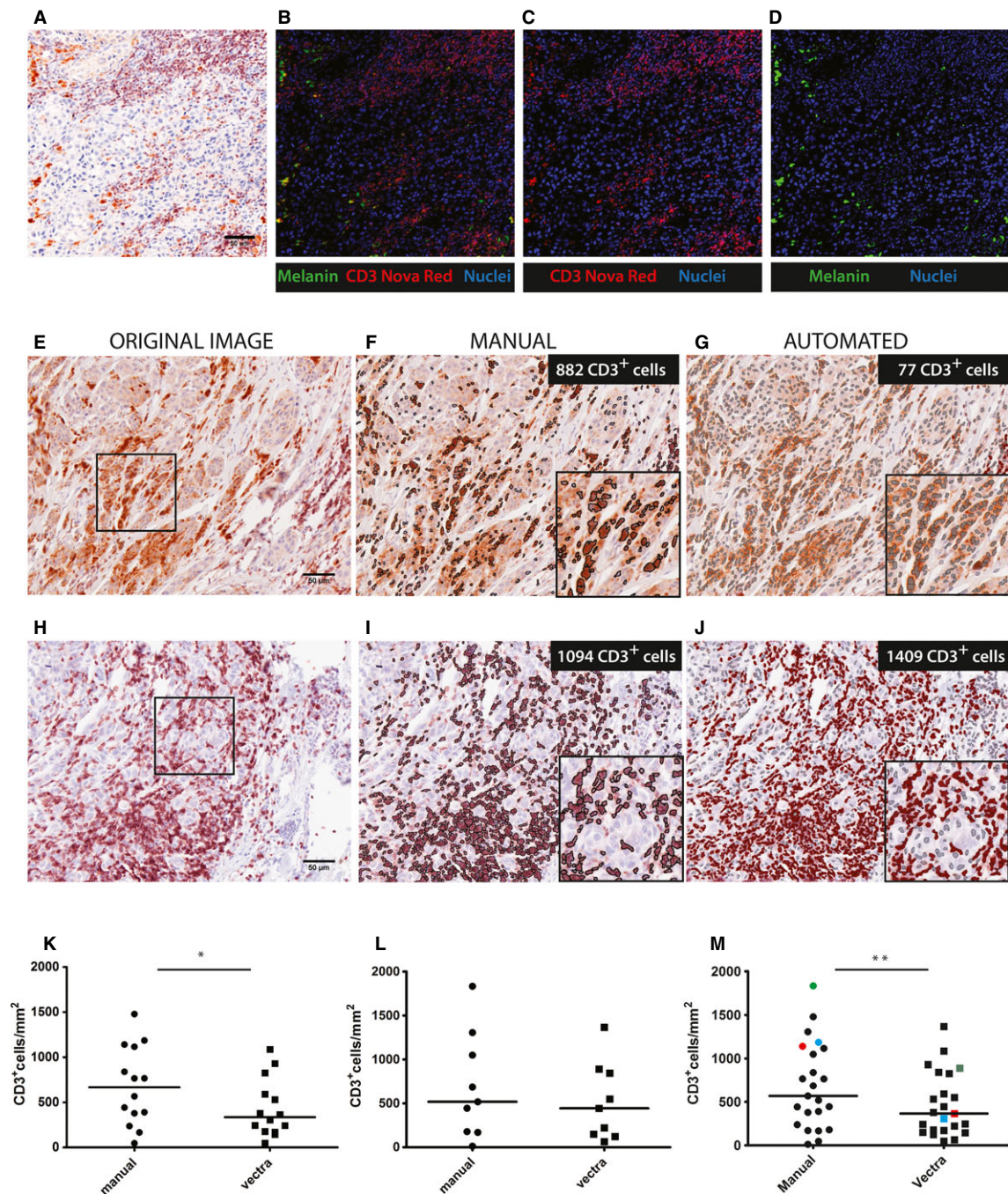


Figure 2. Comparison of manual and multispectral automated cell counts in pigmented and non-pigmented melanoma. Melanin unmixing allows visualization of infiltrating T lymphocytes in melanoma tumour. (A), Original coloured image of human melanoma. (B), Composite pseudo-fluorescent image containing both melanin (in green) and CD3+Nova red T cells (in red). (C, D), Pseudo-fluorescent images of (C) unmixed CD3+Nova red T cells and (D) melanin. Nuclei are shown in blue (haematoxylin staining). Scale bar: 50 μ m. (E–J), Comparison of the quantitative assessment of CD3⁺ T lymphocytes in human primary melanomas performed with the manual image software *fiiji* (F, I) or the fully automated *VECTRA/INFORM* platform (G, J). For tumour with >50% of cells pigmented (E–G), the absolute CD3⁺ T cell counts are significantly higher with the manual (F) than with the automated (G) analysis, because of false positive results caused by melanin pigmentation. In tumours with no or low pigmentation (H–J), the CD3⁺ T cell counts are comparable in manual (I) and automated (J) analysis. (K–M), Dot plots of CD3⁺ T-lymphocyte enumeration for pigmented (K) versus non-pigmented (L) melanomas and the combination of the two groups (M). Matched colour symbols indicate differences in T-cell counts in the same tumour, obtained with manual or automated analysis (M). Scale bars: 50 μ m.

chromogens in the immunostaining of melanocytic lesions, if limited to tumour markers, can partially overcome this problem; however it remains suboptimal for immune cell detection.³²

On the basis of these findings, we applied MSI to discriminate between melanin and red chromogens. We found that linear spectral melanin unmixing could effectively overcome the limits of the conventional semi-automated algorithm.^{22,26} A major advantage of MSI over conventional imaging is the possibility of separating the emission spectra of the different chromogens in the tissue section. Likewise, the spectrum of melanin, which otherwise distorts the Nova red signal, can now be unmixed from the original multispectral image. Our findings demonstrate major differences in T-cell densities, depending on the two methods described. Conventional analysis leads to overestimation of T-cell infiltration, and therefore to possible misinterpretation of the data. This is of especial importance different groups of melanoma patients showing dissimilar degrees of tissue pigmentation are compared.

In conclusion, our study highlights the potential of MSI for accurate assessment of immune cell infiltrates, including those in notoriously difficult tissues, such as highly pigmented melanomas. By using this innovative microscopy technique, we recently demonstrated that the density of pre-existing lymphocytes inside the tumour and at the tumour margin is an accurate predictor of survival for metastatic melanoma patients receiving dendritic cell vaccination.³³ We strongly believe that this approach is an important addition to the armamentarium of pathologists and in melanoma research, where the assessment of tumour infiltration is crucial in the search for new biomarkers to predict patient responses to immunotherapies.

Furthermore, IHC interpretation may be standardized by the use of MSI digital imaging technology, avoiding the use of subjective scoring methods for the assessment of TILs in different cancer types.

Furthermore, MSI enables simultaneous measurement of multiple and often overlapping chromogens or fluorophores in multicolour IHC analysis, on a per-cell basis, in the context of the original anatomy. This, in turn, enables a better understanding of the mechanism of diseases, potentially leading to better avenues for therapeutic intervention.

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Conflict of interest

The authors disclose no potential conflicts of interest.

Author contributions

A. Vasaturo and D. Verweij performed the experiments and analysed the data. A. Vasaturo and C. G. Figdor designed the research study. A. Vasaturo and S. Di Blasio wrote the paper. Willeke A. M. Blokx assisted with image interpretation. J. Han van Krieken, I. Jolanda M. de Vries and Carl G. Figdor read and revised the paper, and agreed with the final version of the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Table S1. Comparison of tumour T-cell density by manual versus automated analysis.

Figure S1. Overview of the semi-automated image analysis process for the assessment of T-cell density in primary human melanoma.

Figure S2. Fully automated digital image analysis of primary melanoma on the VECTRA/INFORM platform.