Mapping of the three-dimensional lymphatic microvasculature in bladder tumours using light-sheet microscopy

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BACKGROUND: Cancers are heterogeneous and contain various types of irregular structures that can go undetected when examining them with standard two-dimensional microscopes. Studies of intricate networks of vasculature systems, e.g., the tumour lymphatic microvessels, benefit largely from three-dimensional imaging data analysis.

METHODS: The new DIPCO (Diagnosing Immunolabeled Paraffin-Embedded Cleared Organs) imaging platform uses three-dimensional light-sheet microscopy and whole-mount immunolabelling of cleared samples to study proteins and micro-anatomies deep inside of tumours.

RESULTS: Here, we uncovered the whole three-dimensional lymphatic microvasculature of formalin-fixed paraffin-embedded (FFPE) tumours from a cohort of 30 patients with bladder cancer. Our results revealed more heterogeneous spatial deviations in more advanced bladder tumours. We also showed that three-dimensional imaging could determine tumour stage and identify vascular or lymphatic system invasion with higher accuracy than standard two-dimensional histological diagnostic methods. There was no association between sample storage times and outcomes, demonstrating that the DIPCO pipeline could be successfully applied on old FFPE samples.

CONCLUSIONS: Studying tumour samples with three-dimensional imaging could help us understand the pathological nature of cancers and provide essential information that might improve the accuracy of cancer staging.

INTRODUCTION
Cancers are heterogeneous, and there are various types of irregular structures that exist in three-dimensions (3D). To date, the lack of techniques and methods has limited researchers’ and physicians’ ability to spatially elucidate the entire cancer landscape. Studying solid tumours with traditional two-dimensional (2D) light microscopy restrict our findings to surface pictures. However, advances in tissue clearing techniques and light-sheet microscopy have enabled high-end 3D visualisation deep inside samples. Additionally, we recently optimised the use of formalin-fixed paraffin-embedded (FFPE) samples for whole-mount immunolabelling, clearing, and imaging with light-sheet microscopy, naming the approach DIPCO (Diagnosing Immunolabeled Paraffin-Embedded Cleared Organs, Fig. 1a). The time is ready for a new imaging platform to characterise cancers, which will fill the information gap in studying 3D objects, such as cancerous tumours, with 2D microscopy.

Studies of vasculature systems, e.g., the tumour lymphatic microvessels, benefit largely from using 3D imaging data analysis. Lymphatic dissemination is the major pathway for systemic tumour spread in patients with urinary bladder cancer. However, little knowledge exists about the spatial distribution of lymphatic microvessels within intact human bladder tumours. Herein, we applied the DIPCO pipeline to answer this question. Further, we demonstrated that cancer staging by 3D imaging data analysis provide more accuracy than standard 2D histological diagnostic methods.

MATERIALS AND METHODS
Sample collection
Thirty human FFPE samples from bladder cancers were included; namely, 2 from the Karolinska University Hospital in Sweden and 28 from the Medical University of Lublin in Poland. The tissues were fixed after surgery using formaldehyde and were then embedded in paraffin. One tissue block was randomly picked from each patient for further experiments. All tumours were histologically confirmed to be urothelial carcinomas. The tumours were staged according to the 2002 TNM staging system and graded according to the 2004 WHO classifications. All experiments were performed in accordance with theDeclaration of Helsinki.

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Immunohistochemistry of paraffin-embedded sections (IHC-P) FFPE sections (4–6 μm) were deparaffinised and rehydrated. Then, the antigen was removed, and endogenous peroxidase was quenched. After blocking, the sections were incubated overnight with the appropriate species-specific primary antibody for LYVE-1 (1:100, # ab33682, Abcam) with the appropriate species-specific secondary antibody. The specificity of the LYVE-1 immunosignal for detecting tumour lymphatic vessels was tested and confirmed using an alternative lymphatic marker Podoplanin (1:100, # ab10288, Abcam). Images were acquired with a fluorescence microscope (Cell Observer, Carl Zeiss, Jena, Germany).

Preparation and image processing for 3D analysis
Preparation and 3D imaging data processing of samples are described elsewhere.\textsuperscript{5} The lymphatic endothelial hyaluronan receptor LYVE-1 was targeted to label lymphatics within tumours.\textsuperscript{11,12} The primary and secondary antibody used was anti-LYVE-1 (1:100, # ab33682, Abcam) and Alexa 647-conjugated affinity purified F(ab')2 fragment antibody (1:200, # 711-605-152, Jackson ImmunoResearch Laboratories), respectively. For tissue clearing, immunolabeled samples were incubated in methanol, dichloromethane, and finally dibenzyl ether.\textsuperscript{6} Cleared tumours were imaged using a custom-built light-sheet microscope.\textsuperscript{13}

Amira (FEI) software was used for 3D volume rendering, vessel segmentation, and quantification.\textsuperscript{14} Images were processed and normalised using Amira and ImageJ (National Institutes of Health, Washington, DC) software. Lymphatics were segmented according to the LYVE-1 immunosignal level\textsuperscript{15} using an intensity-based threshold and spatial graph view algorithms of the Amira suite, which also calculated the vessel length and radius. Every vessel was automatically separated to the next branch as one segment and used for the analyses. The spatial heterogeneity feature of the LYVE-1 expression was examined by calculating the kurtosis, skewness, and variance of the LYVE-1 expression density for each 5-μm Z-section.\textsuperscript{5}

Statistics
The values are given as the mean ± SE, median and interquartile range (IQR) for continuous variables, and frequency with percentage for categorical variables. Variables between groups were compared using the Mann-Whitney U-test. To assess the ability of the DIPCO pipeline, we carried out a receiver operating characteristic (ROC) curve analysis to distinguish cancers with advanced stages and vascular or lymphatic system invasion, i.e., lymphovascular invasion plus positive lymph node involvement. Finally, an area under the curve (AUC) value with a 95% confidential interval (CI) was determined for discrimination. Statistical significance was accepted for P values < 0.05. All analyses were performed using the SPSS version 22.0 statistical software package.

RESULTS AND DISCUSSION
Clinical FFPE samples from a cohort of 30 patients with bladder cancer, of which 20% had low-grade tumours and 80% had high-grade tumours, were assessed. The pathological T stage for Ta-1,
| Parameter                      | Sample storage time   | P value                  |
|-------------------------------|-----------------------|--------------------------|
|                               | All patient (n = 30)  | ≤12 mo vs >12 <60 mo (n = 14) | >60 mo (n = 10) | ≤12 mo vs >60 mo | >12 & ≤60 mo vs >60 mo |
| 2D imaging                    |                       |                          |                |                  |                        |
| 2D LYVE-1 density, mean (%)   | 164 ± 1.1             | 15.7 ± 2.6               | 15.2 ± 1.1     | 18.6 ± 2.4       | 0.621                  |
|                               |                       | 0.588                    | 0.349          |                  |                        |
| 3D imaging                    |                       |                          |                |                  |                        |
| 3D LYVE-1 density, mean (%)   | 144 ± 2.7             | 11.1 ± 2.2               | 12.1 ± 1.1     | 19.9 ± 7.8       | 0.602                  |
|                               |                       |                          |                |                  | 0.263                  |
| Lymphatic vessel length, mean (μm) | 60.4 ± 0.8          | 60.2 ± 2.1               | 60.2 ± 0.9     | 60.9 ± 1.6       | 0.621                  |
|                               |                       |                          |                |                  | 0.562                  |
| Lymphatic vessel radius, mean (μm) | 3.8 ± 0.1           | 3.8 ± 0.2                | 3.8 ± 0.1      | 3.9 ± 0.2        | 0.934                  |
|                               |                       |                          |                |                  | 0.958                  |
| LYVE-1 density kurtosis, mean | 3.7 ± 0.9             | 3.5 ± 1.5                | 5.1 ± 1.5      | 1.9 ± 1.2        | 0.621                  |
|                               |                       |                          |                |                  | 0.313                  |
| LYVE-1 density skewness, mean | 0.8 ± 0.2             | 0.3 ± 0.4                | 1.1 ± 0.4      | 0.6 ± 0.3        | 0.138                  |
|                               |                       |                          |                |                  | 0.368                  |
| LYVE-1 density variance a10, mean | 5.0 ± 0.9            | 3.7 ± 0.9                | 6.9 ± 1.8      | 3.0 ± 0.9        | 0.458                  |
|                               |                       |                          |                |                  | 0.428                  |
|                               |                       |                          |                |                  | 0.079                  |

The values are given as mean ± SE. aThe Mann-Whitney U-test.

Table 1. Sample storage time and parameters tested in the study

T2 and T3 were in 53% and 27%, respectively. The median storage time was observed in five patients (17%). The median storage time was observed in five patients (17%).
Fig. 2 Predictive abilities and comparisons between 2D and 3D microscopy methods. ROC curve analysis of 30 FFPE bladder tumours for detecting pT3 tumours or greater (a) and positive vascular or lymphatic system invasion (b). c Table of AUC (95% confidence interval (CI)) values summarising the ROC-AUC analysis in (a) and (b). LVI lymphovascular invasion, LNI lymph node involvement

plans for cancer patients. When treating patients with bladder cancer, accurate cancer staging prior to cystectomy is important to select the correct candidates for neoadjuvant chemotherapy before surgeries. We believe that applying the DIPCO pipeline could enable pathologists to characterise typical cancer hallmarks and perform multi-region 3D analyses of tumours, resulting in more exact cancer diagnoses.

In summary, these results show the capacity of light-sheet microscopy to phenotypically characterise intact bladder tumours and to improve accuracy of cancer staging. The limitations of this study are its retrospective nature as well as the small cohort and the lack of survival data with treatment annotations, with the exception of three patients who were deceased.

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AUTHOR CONTRIBUTIONS
N.T. and P.U. designed the research, analysed the data, and wrote the manuscript. N.T., D.K., and S.K. performed the research. C.S., P.M., A.S., A.M., and P.W. provided conceptual advice.

ADDITIONAL INFORMATION
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