Ex vivo fluorescence confocal microscopy: prostatic and periprostatic tissues atlas and evaluation of the learning curve

Laura Bertoni¹, Stefano Puliatti²,³, Luca Reggiani Bonetti⁴, Antonino Maiorana⁴, Ahmed Eissa²,⁵, Paola Azzoni¹, Luigi Bevilacqua², Valentina Spandri², Shaniko Kaleçi¹, Ahmed Zoeir²,⁵, Maria Chiara Sighinolfi², Salvatore Micali², Giampaolo Bianchi², Giovanni Pellacani⁶, Bernardo Rocco², Rodolfo Montironi⁷

Received: 6 September 2019 / Revised: 17 December 2019 / Accepted: 22 December 2019 / Published online: 6 January 2020
© Springer-Verlag GmbH Germany, part of Springer Nature 2020

Abstract
Ex vivo fluorescence confocal microscopy (FCM) is an optical technology that provides fast H&E-like images of freshly excised tissues, and it has been mainly used for “real-time” pathological examination of dermatological malignancies. It has also shown to be a promising tool for fast pathological examination of prostatic tissues. We aim to create an atlas for FCM images of prostatic and periprostatic tissues to facilitate the interpretation of these images. Furthermore, we aimed to evaluate the learning curve of images interpretation of this new technology. Eighty fresh and unprepared biopsies obtained from radical prostatectomy specimens were evaluated using the FCM VivaScope® 2500 M-G4 (Mavig GmbH, Munich, Germany; Caliber I.D.; Rochester NY, USA) by two pathologists. Images of FCM with the corresponding H&E are illustrated to create the atlas. Furthermore, the two pathologists were asked to re-evaluate the 80 specimens after 90 days interval in order to assess the learning curve of images’ interpretation of FCM. FCM was able to differentiate between different types of prostatic and periprostatic tissues including benign prostatic glands, benign prostatic hyperplasia, high-grade intraepithelial neoplasm, and prostatic adenocarcinoma. As regards the learning curve, FCM demonstrated a short learning curve. We created an atlas that can serve as the base for urologists and pathologists for learning and interpreting FCM images of prostatic and periprostatic tissues. Furthermore, FCM images is easily interpretable; however, further studies are required to explore the potential applications of this new technology in prostate cancer diagnosis and management.

Keywords Fluorescence confocal microscope · Prostate cancer · Atlas · Learning curve
Ex vivo fluorescence confocal microscopy (FCM) is a novel optical technology that allows the examination of freshly excised tissue to provide a quasi-histological view of the tissues in few minutes. FCM is based on the combination of two types of lasers to allow the examination of tissue specimens using the reflectance mode (785 nm) that depends on the difference of the refractive indices of various cellular structure, and the fluorescence mode (488 nm) that utilizes a contrast agent to allow the visualization of microstructures [20].

To date, FCM has been used in healthy skin [12] as well as in cutaneous lesions such as melanoma [10, 11], basal cell carcinoma, and squamous cell carcinoma [9, 17]. Furthermore, it was used for diagnosis of cutaneous inflammatory skin lesions [3]. Moreover, Ragazzi M et al. [26] examined the use of FCM on different surgical specimens such as breast, lymph node, thyroid, and colon, demonstrating that neoplastic tissues were easily distinguishable from normal structures.

The main advantage of the FCM is its ability to provide quasi-histological images that are similar to hematoxylin and eosin (H&E) staining, which render the interpretation of images easier for pathologist novice to this technology. Prostate cancer (PCa) is predicted to be the most commonly diagnosed male cancer in the USA in 2019 [29]. The gold standard diagnosis of PCa is based on the histopathological examination of prostatic tissues. Infiltrative growth pattern, absence of basal cell layer, and nuclear atypia (nuclear enlargement and prominent nuclei) represent the main pathological criteria for PCa diagnosis [19]. However, histopathological examination of tissues is a time-consuming procedure that may require up to 2 days in the highest quality pathological laboratories [1]. Currently, there is an increased interest in “real-time” pathological examination of prostatic tissues either during prostate biopsies or during radical prostatectomy [28, 31]. In this setting, several options were introduced for “real time” pathological examination of prostatic and periprostatic tissues including frozen section [28], light reflectance spectroscopy [21], multiphoton microscopy [30], confocal laser endomicroscopy [18], video-rate fluorescence-structured illumination microscopy [32], and FCM [24].

The aim of the current study is to create an atlas for the FCM images demonstrating its ability to identify different architectural structures in prostatic specimens (such as muscle, nerves, vessels, and adipose tissue) with differentiation of inflammatory changes from malignant transformation. Furthermore, among the important factors, determining the feasibility and superiority of any new imaging technology is the accurate and easy interpretation of its images. This can be demonstrated by assessing the learning curve of this new technology [15]. Thus, the learning curve for FCM images interpretation was evaluated as a secondary aim.
used to improve association with cellular nuclei and non-
cellular structures, respectively [8]. In this study, a self-
written ImageJ software (https://imagej.nih.gov/ij/) was used
to the standard digital image staining approach. Zoom
capabilities enable an enhanced visualization of cell
morphology details. The staining and imaging processes of
each specimen were completed within 5 min.

**FCM images’ evaluation**

All FCM images were stored in a dedicated database. Two
pathologists (general pathologist and an expert genitour-
pathologist) from two different centers (University of Modena
and Reggio Emilia and University of Ancona) were integrated
in the assessment of the FCM learning curve. The images were
randomly displayed to each of the two pathologists while being
blinded to the histopathologic diagnoses. After more than
90 days, they were asked to re-evaluate the specimens. All
examinations were performed independently and included the
evaluation of the tumor and non-neoplastic tissue comprised in
the samples with particular regard to normal prostatic glands
and soft tissue components (muscle bundles, nerve, adipocytes,
vessels) as well as other non-neoplastic conditions such as in-
flammation and benign glandular hyperplasia.

**Histopathological examination**

From the biopsies included in paraffin and processed accord-
ing to the standard protocol [27], we performed 3 consecutive
sections of 3 μm thickness, starting from the most superficial
portion without discarding material. The sections were H&E
stained.

Diagnostic criteria for carcinoma included atypical acini
arranged in one or more patterns of growth, combined with
atypical cytologic findings including prominent nucleolus or
pale enlarged and irregular nuclei, were useful criteria for
cancer, particularly when small, irregular, abortive acini with
primitive lumens and absence of basal layer were visible. The
tumor grade groups were assessed according to International
Society of Urological Pathology (ISUP) grading [6].

Immunohistochemical analysis included racemases/34betaE12
cytkeratin cocktail stain and were used for 8 spec-
imens with suspicious small foci of cancer detected on H&E-
stained slides.

H&E-stained slides were compared with the images obtain-
ed by FCM techniques in order to describe overlaps or discor-
dant features.

**Statistical analysis**

The Cohen’s kappa (κ) statistic was used to measure the
agreement between histopathologic diagnoses and the FCM
diagnosis for each pathologist in each reading. We selected κ
statistic as the measure of agreement as our variable of interest
is binary. Kappa is a measure of this difference, standardized
to lie on a −1 to 1 scale, where 1 is perfect agreement, 0 is
exactly what would be expected by chance, and negative
values indicate agreement less than chance, i.e, potential sys-
tematic disagreement between the observers [5].

The Cohen’s kappa can be interpreted as following: (κ < 0)
is less than chance agreement, (κ = 0.01 to 0.20) represents
slight agreement, (κ = 0.21 to 0.40) is fair agreement, (κ =
0.41 to 0.60) is moderate agreement, (κ = 0.61 to 0.80) is
substantial agreement, while, (κ = 0.81 to 0.99) is considered
almost perfect agreement. The interpretation of reproducibility
is marginal (κ = 0.00 to 0.40), good (κ = 0.40 to 0.75), and
excellent (κ > 0.75) [7].

The process of deciding whether the numerical results
quantifying hypothesized relationships between variables,
are acceptable as a description of the data, is known as vali-
dation. We select k-Fold Cross-Validation, as it reduces the
risk of errors induced by bias by utilizing the full-data set for
training and for validation. In K Fold cross validation, the data
is divided into k subsets. Now the holdout method is repeated
k times, such that each time, one of the k subsets is used as the
test set/validation set and the other k-1 subsets are put together
to form a training set. The error estimation is averaged over all
k trials to get total effectiveness of our model [13].

**Results**

**FCM atlas**

Samples from the biopsies were classified as non-neoplastic
tissue (NNT), high-grade prostatic intraepithelial neoplasia
(HG-PIN), and prostatic acinar adenocarcinoma (PAA).

**Non-neoplastic prostatic and extraprostatic tissues**

In some samples, probably taken from the periphery of the
prostatic gland, a mix of connective and soft adipose tissue,
crossed by thin muscle bundles, were detected as well as bun-
dles of nerves (Fig. 1a–c). Chronic prostatitis typically shows
an aggregation of lymphocytes and plasma cells within the
stroma (Fig. 1d). The different components of prostatic and
periprostatic soft tissue were easily recognized at the corre-
sponding FCM images (Fig. 1e–h). NNT included prostatic
glands lined by typical two cell layers represented by an outer
layer of low cuboidal cells and an inner layer of tall columnar
mucin-secreting epithelium, organized into non-infiltrating
pattern of growth. The glands are surrounded by a thin con-
nective tissue and, sometimes, contained corpora amylacea
(Fig. 2a, b). As shown in Fig. 2d, e, in FCM mosaics, different
cell layers of the prostatic glands as well as corpora amylacea
were appreciable. Hyperplastic prostatic glands with taller and
enlarged columnar secretory cells, with pale to clear cytoplasm and enlarged nuclei without atypia, are visible in the samples. The glands are evidently surrounded by basal cells (Fig. 2c, f).
High-grade prostatic intraepithelial neoplasia and prostatic acinar adenocarcinoma

HG-PIN included glands composed of crowded and irregularly spaced multi-layered epithelial cells, with enlarged, hyperchromatic and pleomorphic nuclei, with prominent nucleoli. Basal cells surrounded the glands (Fig. 3a). At FCM, we observed the prominent nucleolus and the stratified epithelium surrounded by evident basal cells (Fig. 3e).

PAA was composed of closely packed irregular glands variably in size and shape and with back-to-back distribution. Few or no interposing stroma was intermingled through the atypical glands. Enlarged round, hyperchromatic nuclei and prominent nucleolus are visible. Basal cells were absent. These tumors were graduated as grade group 1 [GG1] (Fig. 3b). Tumor composed by predominantly well-formed glands and exhibiting a lesser component of cribriform glands were classified as grade group 2 [GG2] (Fig. 3c); whereas the cribriform glands were predominant; the tumors were classified as grade group 3 [GG3]. When sheet of undifferentiated cell and poorly formed/fused glands were detected, we classified the tumors as grade group 4 [GG4] (Fig. 3d). The corresponding FCM images demonstrated the atypia of the nuclei, the irregular morphology of the glands, as well as the typical pattern of growth of the glands (Fig. 3f-h). Figure 4a and b emphasize the infiltrating growth of PAA within a nerve and the atypia of the glands with particular regard among the prominent nucleolus and the absence of the basal cell layer. These features
Fig. 3 Histological images and the corresponding FCM images of high-grade prostatic intraepithelial neoplasia (a–e) and prostatic acinar adenocarcinoma grade group 1 (b–f), grade group 2 (c–g), and grade group 3 (d–h). (Scale bar, 100 µm)
were well represented by the corresponding FCM images (Fig. 4c, d).

**Learning curve assessment**

Both pathologists achieved very encouraging levels of agreement between FCM evaluation and the gold standard histopathological diagnosis of prostatic biopsies (Table 1). The percentages of agreement become higher from the first evaluation 86% and 92% ($\kappa = 0.68$ and $\kappa = 0.79$ substantial agreement) to the second one, reaching 95% ($\kappa = 0.87$ almost perfect agreement) for both the pathologists. Furthermore, the reproducibility was excellent ($\kappa > 0.75$) for both raters in the second evaluation. Besides high accuracy, Table 1 also indicates a promising result in terms of specificity and sensitivity. Out of 59 negative biopsies, 50 and 57 have been correctly identified as negative by the first pathologist in the first (specificity 84.7%) and second evaluations (specificity 96.6%), respectively. For the second pathologist, 58 negative biopsies have been correctly identified both in the first and the second evaluations which correspond to 98.3% specificity. On the other hand, 19/21 positive biopsies have been correctly

**Table 1** First and second FCM evaluation of prostatic biopsies compared to histopathological diagnoses, the percentage of correct diagnoses, $\kappa$ value, the level of agreement, the sensitivity, the specificity, and ROC area for both raters

| Histopathological diagnoses | % of correct diagnosis | $\kappa$ value | Level of agreement | Sensitivity | Specificity | ROC area |
|-----------------------------|------------------------|---------------|-------------------|------------|------------|----------|
| Negative                    | 50                     | 2             | 86%               | 0.68       | Substantial| 90%      | 85%      | 0.87     |
| Positive                    | 9                      | 19            |                   |            |            |          |          |          |
| First evaluation Rater 1    | Negative               | 57            | 2                 | 95%        | 0.87       | Almost perfect| 90%      | 97%      | 0.93     |
| Positive                    | 2                      | 19            |                   |            |            |          |          |          |
| Second evaluation Rater 1   | Negative               | 58            | 5                 | 92%        | 0.79       | Substantial| 76%      | 98%      | 0.87     |
| Positive                    | 1                      | 16            |                   |            |            |          |          |          |
| First evaluation Rater 2    | Negative               | 58            | 3                 | 95%        | 0.87       | Almost perfect| 86%      | 98%      | 0.92     |
| Positive                    | 1                      | 18            |                   |            |            |          |          |          |
| Second evaluation Rater 2   |                        |               |                   |            |            |          |          |          |

Virchows Arch (2020) 476:511–520 517
classified by the first pathologist in both evaluations which leads to a sensitivity value of 90.5%. The second pathologist correctly identified 16/21 positive samples during the first evaluation and 18/21 in the second evaluation (sensitivity 76.2% and 85.7% respectively). Moreover, the area under the receiver operating characteristic (ROC) curve, that describes the relationship between the sensitivity and specificity, becomes higher from the first to the second evaluation (from 0.87 to 0.92–0.93, respectively) for both raters.

Discussion

Surgeons show growing interest in the real-time pathological examination as it may potentially allow a more precise and patient-tailored surgery [25]. In PCa, real-time pathological examination plays an important role as it may be used for assessing the surgical margins (apical, bladder neck, and neurovascular bundle) during radical prostatectomy thus allowing complete resection of the tumor without compromising the functional outcomes [18, 22, 30]. It can be also used during prostate biopsy to decrease the need for additional biopsies [31]. Furthermore, it has been used in the histopathological screening for prostate cancer in organ donors, especially with the increasing age of organ donors [2]. The gold standard technique for real-time pathological examination is the frozen section, which, is debatable due to its associated drawbacks including the technical difficulty, the complexity of the procedure that requires multiple dedicated personnel, and being resource and time-consuming technique [22]. Other options include light reflectance spectroscopy [21], multiphoton microscopy [30], confocal laser endomicroscopy [18], and video-rate fluorescence structured illumination microscopy [32]. Furthermore, we have previously proved that FCM has a diagnostic accuracy of 91% compared to the standard histopathological examination with AUC, sensitivity, and specificity of 0.884, 83.33%, and 93.53% for the pathological examination of prostatic tissues [24], which render FCM as a promising option for “real-time” pathological examination of prostatic tissue.

In the current study, FCM was able to provide a rapid, and high-resolution quasi histological images of the freshly excised, non-formalin fixed prostatic and periprostatic specimens. The primary aim of our study was to create an atlas for the FCM images for prostatic and periprostatic tissue examination; however, throughout the process of collecting representative images for the atlas, FCM demonstrated a promising results in identifying cellular and nuclear characters of prostatic and periprostatic tissues with almost perfect agreement ($\kappa = 0.87$) compared to the gold standard histopathological examination after a short learning curve. Furthermore, it was able to accurately differentiate benign tissues and inflammatory changes from malignant transformation in the prostatic specimens.

However, we did not assess the potentiality of FCM to assess the specimens grade group; FCM was able to identify the tumor growth pattern of specimens used for creating this atlas. Currently, we have an ongoing study that will assess the ability of FCM to identify the group grade.

The learning curve is among the most important limitations of any new technology. It is important to examine the learning curve of image interpretation before any new cellular imaging technology is introduced in the clinical practice [14, 16]. In these setting, we evaluated the learning curve of FCM images. We intentionally integrated a general pathologist and an expert genitourinary pathologist in the evaluation of the learning curve to provide a more realistic assessment. We hypothesized that the great similarity between the FCM images and the gold standard H&E histopathological examination will render them easily interpretable by pathologists novice to FCM. Our results supported this hypothesis, since the agreement between the FCM reading and the histopathological diagnosis increased from 86 to 92% in the first reading for the first and the second pathologists, respectively, to reach 95% for both pathologists in the second reading. Noteworthy, 8 samples (10%) required immunohistochemistry for diagnosis, which, may be responsible for the persistent 5% disagreement obtained by both pathologists on the second reading. Interestingly, Bertoni et al. [4] demonstrated that FCM is capable of providing immunofluorescent images of special markers using fluorochrome-conjugated antibodies, which may enhance the diagnostic performance of FCM of doubtful cases; however, in their study, they used paraffin-fixed sections and not freshly excised specimens. Panarello D et al. [23] reported that the learning curve for the confocal laser endomicroscopy images interpretation seems to be short; however, this was not assessed in their study.

FCM provides several advantages including the H&E-like images which facilitate the interpretation of images, the fast preparation of specimens and rapid image acquisition (less than 5 min per specimen in our experience), and the preservation of the specimens’ integrity not only for subsequent histopathological examination but also for ancillary studies like immunohistochemistry [25]. Furthermore, FCM represents a step forward towards digitalized pathology, as the specimen preparation is simple and can be performed by the treating surgeon, and it provides a digital image that can be sent online to a remote pathologist for interpretation. Despite the promising level of agreement between FCM evaluation and the histopathological examination of prostatic and periprostatic tissues, there were some pitfalls (Supplementary Fig. 1); one of the specimens with prostatic acinar adenocarcinoma on the H&E was misdiagnosed as HG-PIN due to the presence of mild nuclear atypia associated with crowded nuclei in the FCM images. Moreover, adenocarcinoma cells were
misdiagnosed as a result of the presence of technical artefact that masqueraded mild atypia in another specimen. Finally, in one specimen, a focus of atypical grade group 4 single cells were misdiagnosed as inflammatory cells.

The main limitation of the current study is the small sample size. On the other hand, our study has many strengths as it is the first study (to our knowledge) to provide a comprehensive description of the FCM images of the prostatic and periprostatic tissue and to assess the learning curve of this technology in the field of PCa.

Conclusion

FCM is an optical technology that can be used for “real-time” pathological examination of prostatic tissue. This atlas can serve as the base for urologists and pathologists for learning and interpreting FCM images of prostatic and periprostatic tissues. Further studies are required to define the potential applications of FCM in PCa.

Authors Contributions

Bertoni L, Pulitelli S, Eissa A, Sighinolfi MC, Bianchi G, Pellacani G, Rocco B, Montironi R: Conception and Design. Bertoni L, Pulitelli S, Reggiani Bonetti L, Maiorana A, Azzoni P, Bevilacqua L, Spandri V, Montironi R: Acquisition of Data. Kaleci S: Data Analysis. Bertoni L, Pulitelli S, Reggiani Bonetti L, Maiorana A, Eissa A, Azzoni P, Sighinolfi MC, Micali S, Rocco B, Montironi R: Interpretation of Data. Bertoni L, Pulitelli S, Reggiani Bonetti L, Eissa A, Zoëir A, Sighinolfi MC: Drafting and writing. Micali S, Bianchi G, Pellacani G, Rocco B, Montironi R: Revision

Compliance with ethical standards

This study was approved by the Ethical Committee in the university of Modena & Reggio Emilia (protocol number 0018091/18) and written informed consent was obtained from all patients.

Conflict of interest

Eissa A has a temporary contract of consultation with MAVIG GmbH.

References

1. Alshieban S, Al-Surimi K (2015) Reducing turnaround time of surgical pathology reports in pathology and laboratory medicine departments. BMJ Qual Imp Rep 4:u209223–w203773. https://doi.org/10.1136/bmjquality.u209223-w203773
2. Avellini C, Baccarani U, Orsaria M, Adani GL, Bresadola V, Lorenzin D, Bresadola F, Beltrami CA (2009) Evaluation of prostate cancer staging in organ donors: intraoperative histology on periglandular soft tissues—a proposal. Transplant Proc 41:1099–1103. https://doi.org/10.1016/j.transproceed.2009.03.089
3. Bertoni L, Azzoni P, Reggiani C, Pisciotta A, Carnevale G, Chester J, Kaleci S, Reggiani Bonetti L, Cesinaro AM, Longo C, Pellacani G (2018) Ex vivo fluorescence confocal microscopy for intraoperative, real-time diagnosis of cutaneous inflammatory diseases: a preliminary study. Exp Dermatol 27:1152–1159. https://doi.org/10.1111/exd.13754
4. Bertoni L, Pisciotta A, Azzoni P, Bertani G, Reggiani Bonetti L, Pulitelli S, Farnetani F, Carnevale G, Pellacani G (2018) Use of ex vivo fluorescence confocal microscopy for detection of tissue specific markers. Biomed J Sci & Tech Res 10. https://doi.org/10.26717/BJSTR.2018.10.002003
5. Edgar B (2004) Applied nonparametric statistical methods. P. Sprent and N. C. Smeeton, Chapman & Hall/CRC, London, England, 2001. No. of pages: ix+461. Price:£29.99. ISBN: 1-58488-8145-3 Statistics in medicine 23:1988-1989. https://doi.org/10.1002/sim.1755
6. Epstein JI, Egevad L, Amin MB, Delahunt B, Srigley JR, Humphrey PA (2016) The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma: definition of grading patterns and proposal for a new grading system. Am J Surg Pathol 40: 244–252. https://doi.org/10.1097/PAS.0000000000000530
7. Fleiss JL (1981) The measurement of interrater agreement. Statistical Methods for Rates and Proportions, 2nd Edition.Edn. Wiley, New York. pp 212–236
8. Gareau DS, Li Y, Huang B, Eastman Z, Nehal KS, Rajadhyaksha M (2008) Confocal mosaicing microscopy in Mohs skin excisions: feasibility of rapid surgical pathology. J Biomed Opt 13:054001. https://doi.org/10.1117/1.2981828
9. Hartmann D, Krammer S, Bachmann MR, Mathemeier L, Ruzicka T, Bagci IS, von Braunmühl T (2018) Ex vivo confocal microscopy features of cutaneous squamous cell carcinoma. J Biophotonics 11: e201700318. https://doi.org/10.1002/jbio.201700318
10. Hartmann D, Krammer S, Ruini C, Ruzicka T, von Braunmühl T (2016) Correlation of histological and ex vivo confocal tumor thickness in malignant melanoma. Lasers Med Sci 31:921–927. https://doi.org/10.1007/s11013-016-1936-5
11. Hartmann D, Krammer S, Vural S, Bachmann MR, Ruini C, Sardy M, Ruzicka T, Berking C, von Braunmühl T (2018) Immunofluorescence and confocal microscopy for ex-vivo diagnostic of melanocytic and non-melanocytic skin tumors: a pilot study. J Biophotonics:11. https://doi.org/10.1002/jbio.201700211
12. Hartmann D, Ruini C, Mathemeier L, Dietrich A, Ruzicka T, von Braunmühl T (2016) Identification of ex-vivo confocal scanning microscopic features and their histological correlates in human skin. J Biophotonics 9:376–387. https://doi.org/10.1002/jbio.201500124
13. Hastie T, Tibshirani R, Friedman J (2009) The elements of statistical learning data mining, inference, and prediction. Springer Verlag, Secaucus
14. Jain M, Pulijal SV, Rajadhyaksha M, Halpern AC, Gonzalez S (2018) Evaluation of bedside diagnostic accuracy, learning curve, and challenges for a novice reflectance confocal microscopy reader for skin cancer detection in vivo. JAMA Derm 154:962–965. https://doi.org/10.1001/jamadermatol.2018.1668
15. Kuiper T, Kiesslich R, Ponsioen C, Fockens P, Delker E (2012) The learning curve, accuracy, and interobserver agreement of endoscope-based confocal laser endomicroscopy for the differentiation of colorectal lesions. Gastrointest Endosc 75:1211–1217. https://doi.org/10.1016/j.gie.2012.01.040
16. Liu J, Li M, Li Z, Zuo XL, Li CQ, Dong YY, Zhou CJ, Li YQ (2014) Learning curve and interobserver agreement of confocal laser endomicroscopy for detecting precancerous or early-stage esophageal squamous cancer. PLoS One 9:e99089. https://doi.org/10.1371/journal.pone.0099089
17. Longo C, Borsari S, Pampena R, Benatti E, Bombonato C, Raucci M, Mirra M, Di Stefani A, Peris K, Pellacani G (2018) Basal cell carcinoma: the utility of in vivo and ex vivo confocal microscopy. J Eur Acad Dermatol Venereol 32:2090–2096. https://doi.org/10.1111/jdv.14984
18. Lopez A, Zlatev DV, Mach KE, Bui D, Liu JJ, Rouse RV, Harris T, Leppert JT, Liau JC (2016) Intraoperative optical biopsy during robotic assisted radical prostatectomy using confocal...
endomicroscopy. J Urol 195:1110–1117. https://doi.org/10.1016/j.juro.2015.10.182

19. Magi-Galluzzi C (2018) Prostate cancer: diagnostic criteria and role of immunohistochemistry. Modern pathology: an official journal of the United States and Canadian. Acad Pathol, Inc 31:112–121. https://doi.org/10.1038/modpathol.2017.139

20. MAVIG (2018) Datasheet VivaScope® 2500M-G4 https://www.vivascope.de/wp-content/uploads/2019/06/DS_VS-2500M-G4_287_0219-ohne-Mohs.pdf. Accessed 4 January 2020

21. Morgan MS, Lay AH, Wang X, Kapur P, Ozayar A, Sayah M, Zeng L, Liu H, Roehrborn CJ, Cadeddu JA (2016) Light reflectance spectroscopy to detect positive surgical margins on prostate cancer specimens. J Urol 195:479–483. https://doi.org/10.1016/j.juro.2015.05.115

22. Obek C, Saglican Y, Ince U, Argun OB, Tuna MB, Doganca T, Tufek I, Keskin S, Kural AR (2018) Intra-surgical total and reconstructible pathological prostate examination for safer margins and nerve preservation (Istanbul preserve). Ann Diagn Pathol 33:35–39. https://doi.org/10.1016/j.anndiagpath.2017.11.010

23. Panarello D, Comperat E, Seyde O, Colau A, Terrone C, Guillonneau B (2019) Atlas of ex vivo prostate tissue and cancer images using confocal laser endomicroscopy: a project for intraoperative positive surgical margin detection during radical prostatectomy. Eur Urol Focus. https://doi.org/10.1016/j.euf.2019.01.004

24. Puliatti S, Bertoni L, Piola GM, Azzoni P, Bevilacqua L, Elsherbiny A, Sighinolfi MC, Chester J, Rocco B, Micali S, Bagni I, Reggiani Bonetti L, Maiorana A, Malvehy J, Montironi R, Bianchi G, Pellacani G (2019) Ex-vivo fluorescence confocal microscopy: the first application for real-time pathologic examination of prostatic tissue. BJU Int. https://doi.org/10.1111/bju.14754

25. Ragazzi M, Longo C, Piana S (2016) Ex vivo (fluorescence) confocal microscopy in surgical pathology: state of the art. Adv Anat Pathol 23:159–169. https://doi.org/10.1097/pap.0000000000000114

26. Ragazzi M, Piana S, Longo C, Castagnetti F, Foroni M, Ferrari G, Gardini G, Pellacani G (2014) Fluorescence confocal microscopy for pathologists. Modern pathology: an official journal of the United States and Canadian. Acad Pathol, Inc 27:460–471. https://doi.org/10.1038/modpathol.2013.158

27. Samarutunga H, Montironi R, True L, Epstein JI, Griffiths DF, Humphrey PA, van der Kwast T, Wheeler TM, Srigley JR, Delahunt B, Egevad L (2011) International Society of Urological Pathology (ISUP) consensus conference on handling and staging of radical prostatectomy specimens. Working group 1: specimen handling. Modern pathology: an official journal of the United States and Canadian. Acad Pathol, Inc 24:6–15. https://doi.org/10.1038/modpathol.2010.178

28. Schlimm T, Tennstedt P, Huxhold C, Steuber T, Salomon G, Michl U, Heinzer H, Hansen J, Budala L, Steurer S, Wittmer C, Minner S, Haese A, Sauter G, Graefen M, Huland H (2012) Neurovascular structure-adjacent frozen-section examination (NeuroSAFE) increases nerve-sparing frequency and reduces positive surgical margins in open and robot-assisted laparoscopic radical prostatectomy: experience after 11,069 consecutive patients. Eur Urol 62:333–340. https://doi.org/10.1016/j.eururo.2012.04.057

29. Siegel RL, Miller KD, Jemal A (2019) Cancer statistics, 2019 CA Cancer J Clin 69:7–34. doi: https://doi.org/10.3322/caac.21551

30. Tewari AK, Shevchuk MM, Sterling J, Grover S, Herman M, Yadav R, Mudalair K, Srivastava A, Rubin MA, Zipfel WR, Maxfield FR, Xu C, Webb WW, Mukherjee S (2011) Multiphoton microscopy for structure identification in human prostate and periprostatic tissue: implications in prostate cancer surgery. BJU Int 108:1421–1429. https://doi.org/10.1111/j.1464-410X.2011.10169.x

31. Wang M, Kimbrell HZ, Sholl AB, Tulman DB, Elf er KN, Schlichenmeyer TC, Lee BR, Lacey M, Brown JQ (2015) High-resolution rapid diagnostic imaging of whole prostate biopsies using video-rate fluorescence structured illumination microscopy. Cancer Res 75:4032–4041. https://doi.org/10.1158/0008-5472.can-14-3806

32. Wang M, Tulman DB, Sholl AB, Kimbrell HZ, Mandava SH, Elf er KN, Luethy S, Maddox MM, Lai W, Lee BR, Brown JQ (2016) Gigapixel surface imaging of radical prostatectomy specimens for comprehensive detection of cancer-positive surgical margins using structured illumination microscopy. Sci Rep 6:27419. https://doi.org/10.1038/srep27419

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.