

# Towards a single cell cancer diagnosis. Multimodal and Monocellular Measurements of Markers and Morphology (5M)

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Dear Professor Reith,

We would like to draw the attention of the readers towards recent developments in diagnostic cytopathology, which deserve interest by pathologists, cytologists, biologists, clinicians, engineers and public health economists: The increasing possibilities to diagnose different malignant tumors in early stages painlessly and without bloody biopsies or surgery on a comparably low number of cells.

## 1. Aims of diagnostic cytopathology

The aims of diagnostic cytopathology mainly are:

- to early identify either so far unknown (pre-)malignant lesions via screening (e.g., for early cancers of the lungs, of the urinary bladder, or of the uterine cervix) or
- to decide on the nature of clinically already identified suspicious lesions of various organs (e.g., in the thyroid, salivary glands, lungs, liver, pancreas, prostate or in the conjunctiva, oral or oesophageal mucosa);

- to decide on the effect of an applied therapy (therapy-monitoring).

Cytopathological diagnoses mostly do not substitute histopathological investigations, as tumor cell-positive lesions are mostly operated upon. They rather help to decide on the following diagnostic steps and thus help to reduce the number of unnecessary bioptic or surgical procedures.

## 2. How much tissue to remove?

It is an ethic goal not to remove more cells or tissues from a patient than is necessary to establish a requested diagnosis as this may cause pain, complications and defects. As obtaining tissues via biopsies or operations may cause discomfort and complications [2,3,5] this should be avoided, whenever the diagnosis needed as a first step can also be obtained non-invasively on a few hundred or thousand cells. The still sometimes heard desire of pathologists to obtain as much tissue as possible to make a valid histological diagnosis, is therefore not in agreement with the demand to hurt the patient as little as possible. If a cancer diagnosis can be made on a few cells, why should we ask the clinician to remove more?

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### 3. Specificity of bioptic procedures

It has to be realized that the majority of bioptically or surgically removed tissues do neither contain cancer nor precancer. As 96% of resected thyroid nodules [5] do not contain cancer, 53% of resected breast lumps [9], and 83% of resected oral lesions [11] a corresponding percentage of operations could be avoided if the nonexistence of cancer or precancer could have been established with noninvasive methods. It is on the other hand evident, that for exclusion of neoplastic cells, a representative sample has to be investigated.

### 4. Sample representativity

The question of sample representativity affects diagnosis, typing, and grading malignant tumors. For these purposes a representative sampling must be performed before relying on a given tumor type or grade. The procedure of needling during aspiration biopsy mostly yields more representative material than a single punch biopsy (e.g., in the prostate, liver or salivary glands). If the diagnosis of a malignant tumor can be achieved on the basis of some hundred or thousand representative neoplastic cells, this will be sufficient if an operation with subsequent histological typing will follow. To establish the diagnosis of malignancy on the basis of a few cells only does not mean that only a few have to be sampled. But if a few cells are sufficient for an unequivocal cancer diagnosis the sample as a whole can comprise fewer cells as before when pathologists asked for as much tissue for their diagnosis as possible. If one or a few cells are principally sufficient for a diagnosis of cancer, the probability to identify tumor cells in a given sample is higher. If 1000 millilitres of ascites contain 250 tumor cells, there is still a good chance to identify one malignant cell in 5 millilitres if one is prepared to do so and to rely an unequivocal diagnosis thereon.

### 5. Multistep cancer diagnosis

The process of cancer diagnosis is a multistep procedure. The clinician as a first step in the diagnostic workup of a patient with suspicion of a malignant tumor does not need an exact, definite histogenetic typing. The first step rather is to identify a lesion as (pre)-malignant or not. Depending on the suspected type of the tumor the following step is either surgery, which is

followed by an exact histologic tumor typing or radiation/cytostatic therapy. In the latter cases only an exact preoperative typing and grading of the cells or tissues from a given tumor is requested. In those cases in which cytological typing is not possible or doubtful (as, e.g., often in malignant lymphomas) a biopsy may be necessary. Following this diagnostic approach many of unnecessary operations could be avoided as many lesions can be identified as non neoplastic without bloody biopsies or operation. This is especially true for cancers of the cornea/conjunctiva, thyroid, salivary glands, oral mucosa, uterine cervix and prostate.

### 6. New techniques for representative cell sampling

The development of sophisticated clinical procedures to obtain sufficient cellular material from suspicious, often hidden mucosal and parenchymal lesions encourages cytopathologists to further develop methods for the achievement of accurate diagnoses on cells. Tiny brushes are available to obtain a few thousand cells from mucosal surfaces, like the cornea, conjunctiva or oral mucosa [6,16]. Via CT lesions nearly everywhere in the body can be reached with thin needles with an accuracy of  $\pm 3$  mm (e.g., in the lungs or the liver [1,3]). Via sonographic control subcutaneous tumors (e.g., in the thyroid, salivary glands or breast) can be punctured with an accuracy of  $\pm 5$  mm. Using endosonographic fine needle aspiration biopsies small tumors within the mediastinum, liver or pancreas can be punctured, thus saving operations or dangerous cutting needle biopsies [21].

The reduction of the number of cells required for an unequivocal cancer diagnosis achieved by the combined application of adjuvant diagnostic methods on the other hand may encourage clinicians to further promote procedures for sampling cells instead of tissues.

### 7. Economic aspects

From the economic point of view cytopathological diagnoses help to save money as they often avoid costs for biopsies, endoscopies, X-rays or operations and for days of hospitalisation during which patients and clinicians wait for histological diagnoses. In most cancers therapy of early stages is much cheaper than of late stages.

## 8. Adjuvant methods increase diagnostic accuracy

Diagnostic cytopathology is able to compete with histopathology in some fields as its diagnostic accuracy has dramatically increased during the last ten years through the introduction of adjuvant, mostly quantitative methods. These are: DNA-image-cytometry (DNA-ICM), AgNOR-analysis, immunocytochemistry, chromatin-pattern analysis, chromosomal FISH and Polymerase Chain Reaction (PCR). For these methods alone high diagnostic accuracies have been reported:

1. Nadjari et al. [13] diagnosed malignant melanomas and squamous cell carcinomas of the cornea and conjunctiva with a sensitivity and specificity of 100% each on brush-biopsies applying DNA-ICM.
2. Sudbø et al. [18] could predict the development of invasive oral cancers out of dysplasias with a positive predictive value of 90% within five years by applying DNA-ICM.
3. Remmerbach et al. [16] achieved a sensitivity of 98.2% and specificity of 100% for the non-invasive cytological early diagnosis of oral cancer using brush-biopsies and DNA-ICM. Applying AgNOR-analysis they reached a sensitivity of 100% [17].
4. Nadjari et al. [13] identified thyroid neoplasias in FNAB's with a positive predictive value of 100% applying DNA-ICM.
5. Grote et al. [7] could predict histologically proven malignancy within the next three months with a positive predictive value of 43% using DNA-ICM in ASCUS and L-SIL.
6. Tribukait [19] applying DNA-flow cytometry on FNAB's of the prostate could identify cancer patients with peridiploid DNA contents who did not die earlier than healthy men of the same age, even if untreated.
7. Motherby et al. [10], achieved a 95.4% sensitivity and 100% specificity for the identification of cancer cells in effusions using BerEP4 immunocytochemistry.
8. Pomjanski et al. [15] reported a sensitivity of 95% for mesothelioma cell identification in effusions applying AgNOR-analysis.
9. Bubendorf et al. [4] detected invasive bladder cancers with 100% sensitivity and 97% specificity applying chromosomal FISH on voided urines.

## 9. Markers referred to single cells

Cytopathology has another advantage over histopathology: all applied biological markers (as DNA-content, AgNOR-counts or chromosomal-FISH) can be related directly to individual, whole cells or nuclei. In histopathology the counted nuclear structures or measured markers have to be related to sections of unknown thickness. The so called "tomato salad phenomenon" prevents to refer the results to individual, whole cells or nuclei. Thus, a large number of sectioned nuclei has to be measured and statistical procedures have to be applied to obtain diagnostically valid results. This is not necessary if counted structures can be related directly to individual whole nuclei as they occur in cytological specimens.

## 10. Combination of adjuvant methods improves diagnostic accuracy

It could be repeatedly demonstrated that by combining two or more of these adjuvant methods on the same material, diagnostic accuracies could be increased.

1. Planz et al. [14] achieved a 94% sensitivity and 100% specificity for the cytological identification of urinary bladder cancer in urine applying the combination of immunocytology and DNA-ICM.
2. Motherby et al. [10] achieved a 88.9% sensitivity and 95% specificity to detect malignant cells in cytologically doubtful serous effusions combining DNA-image cytometry and immunocytochemistry.

## 11. Diagnosis, grading and typing of malignancy with adjuvant methods

To establish the diagnosis and perform a grading of tumor malignancy DNA-ICM, AgNOR and chromosomal analysis can be used. Immunocytochemical markers on the other hand contribute to tumor typing.

Applying these methods to identical cells offers the possibility, not only to rely the diagnosis of malignancy on more than one marker, but also to type and grade these tumor cells additionally. Thus complex and complementary information obtainable on individual cells allows to more accurately diagnose, grade and type them and thus to reduce the number of required cells.

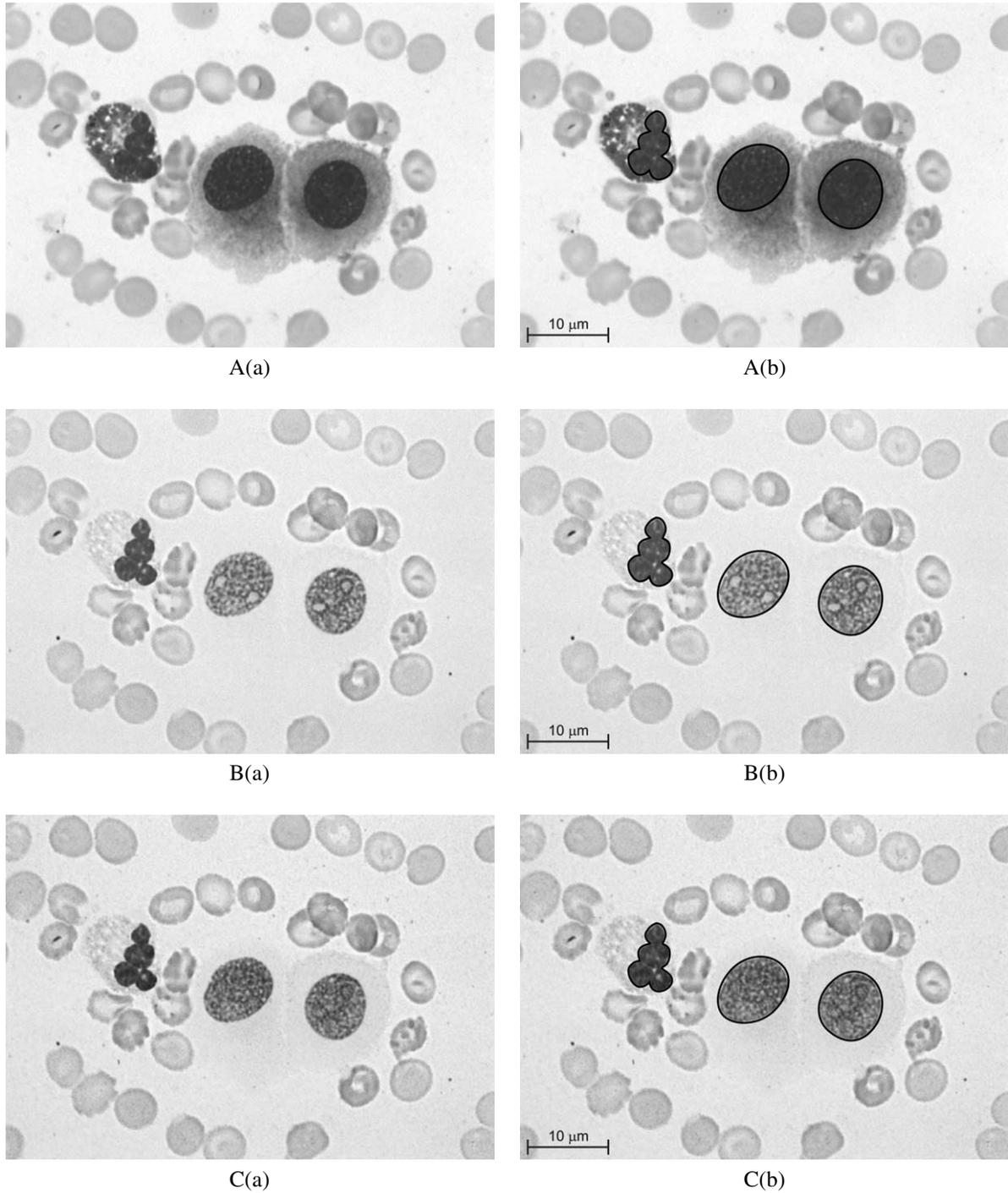


Fig. 1. Multimodal cell analysis on two identical malignant epithelial mesothelioma cells and one neutrophilic granulocyte sequentially stained first according to May–Grünwald–Giemsa (MGG) for subjective evaluation (figure series A), secondly according to Feulgen for DNA-image cytometry (figure series B) and thirdly with silver nitrate for AgNOR-analysis (figure series C). The cells were repeatedly automatically relocated using a Leica DMLA microscope and a  $63\times$  objective, n.a. 1.25, controlled by a self-developed software for precise cellular relocation (a) after conventional staining only, (b) after nuclear segmentation, (c) after nuclear segmentation and AgNOR-detection: overlay of AgNOR-masks on MGG- and Feulgen-images, (d) after IOD-measurement and AgNOR counting with DNA content in c-units and number of single AgNOR's and -clusters. An animated sequence of these images can be seen at our website: [Sanfte-Krebsdiagnostik.de](http://Sanfte-Krebsdiagnostik.de).

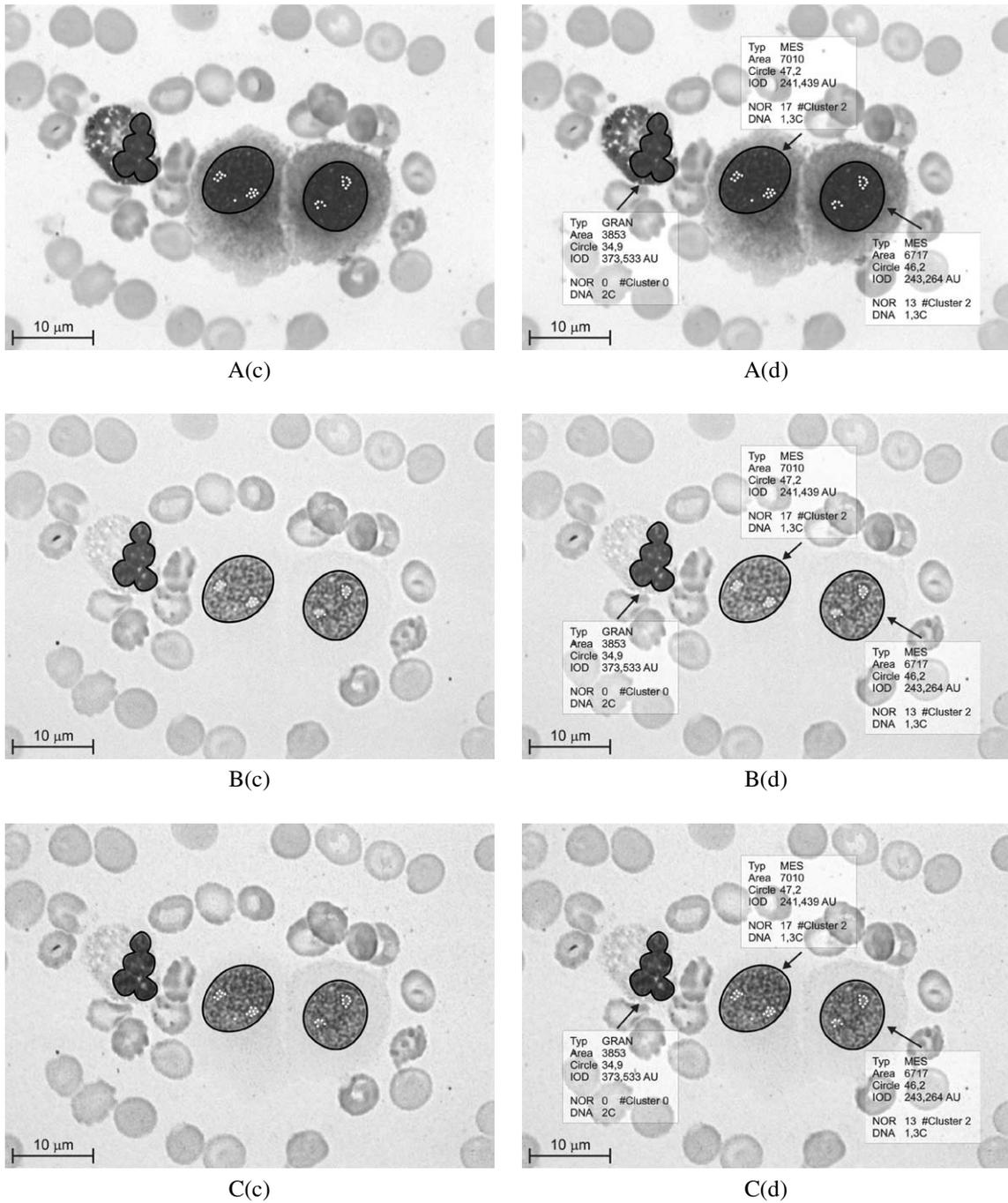


Fig. 1. (Continued.)

1. Supposed that immunocytochemical markers identify 300 morphologically suspicious cells in serous effusion as mesothelial in origin (e.g., by Calretinin-positivity and BerEP4-negativity) image-cytometry identifies abnormal DNA-contents > 9c and a peridiploid stemline, then

these cells most likely derive from a malignant mesothelioma [10].

2. Supposed that 30 abnormal cells in a peritoneal effusion are BerEP4 positive and one reveals a DNA-content > 9c, then the unequivocal diagnosis or a peritoneal carcinosis can be made [10].

3. Supposed that 20 morphologically suspicious urothelial cells in urine reveal an abnormal number of chromosomes 3 and 17 [4] then these most likely represent urothelial carcinoma cells.
4. Supposed that 300 squamous cells of the oral mucosa reveal an aneuploid DNA-stemline and more than five AgNOR's per nucleus, this is enough information to unequivocally identify them as squamous carcinoma cells [17].
5. Supposed that 300 abnormal cells from a conjunctival lesion are HMB 45- and S-100-positive and reveal a DNA-stemline at 3c, then the diagnosis of a malignant melanoma can be made [13].
6. Supposed that 300 abnormal cells from a FNAB of the pancreas are chromogranin- and synaptophysin-positive and reveal multiple DNA-stemlines, then the diagnosis of a neuroendocrine carcinoma can be made [20].

## 12. Multimodal cell analysis reduces number of required cells

A new type of multimodal cytodiagnostics approach is possible if identical cells fixed on glass slides are repeatedly relocated and remeasured after different stainings and marker demonstrations have been applied (Fig. 1). As these cells remain their positions, up to four different stainings and analytical procedures can be performed sequentially. The results can be attributed to the same cell via TV image analysis. Thus, the cytopathologist obtains much more morphological, biological and functional information on one cell than previously. Yet, subjective morphology is still the leading method for interpretation and the pathologist remains master of the process of this multimodal cell analysis (Multimodal and Monocellular Measurements of Markers and Morphology, 5 M).

Thus, in the future sophisticated cytopathological cancer diagnoses, including typing and grading of malignancy, may be possible on a few cells only. New markers as p16 [8] may be integrated as soon as they come up and are diagnostically validated. Thus, many cancer diagnoses can be established or excluded without hospitalisation and without hurting the patient. As the compliance of patients will be higher for such non-invasive diagnostic methods as compared to bloody biopsy procedures, most likely more of them will seek for doctors help in earlier stages of their tumor diseases. Mortality from different cancers may therefore

fall, because more tumors will be detected in earlier stages.

We therefore would like to encourage the above mentioned groups to continue their development and validation of the respective analytical methods for diagnostic application to a few cells only.

Parts of this concept were presented during the "Ploem-Lecture": "Towards a single cell cancer diagnosis" held on the 7th ESACP congress in Caen, France, April, 2001, by A. Böcking.

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