A novel method for isolation of tumor and healthy cells from FFPE carcinoma samples improves genetic analysis by next-generation sequencing

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Introduction

Formalin-fixed paraffin-embedded (FFPE) tissue samples represent an invaluable and easily accessible resource of biological specimens for retrospective large-cohort studies on the identification of neoantigens or biomarkers by next-generation sequencing (NGS).

Although handling and storage of FFPE samples is convenient, there are also drawbacks when analyzing these samples. For most FFPE samples there is no healthy cell reference available to distinguish between tumor and non-tumor cell–specific characteristics, e.g., in ploidy or mutational analysis. Moreover, besides cancer cells, samples of solid tumors contain various amounts and proportions of non-cancer cells, depending on the tumor entity, disease stage, and prior tumor treatment. Non-cancer cells can influence molecular analysis of the cancer cells considerably by diluting the ratio between non-neoplastic and neoplastic cells in the sample. In tumors with a dense fibrotic stroma, such as pancreatic tumors, the percentage of tumor cells is low. In such tumors, enrichment of tumor cells prior to their analysis is of critical importance.

We developed a complete workflow for the efficient dissociation of FFPE samples, which preserves epitopes of cytokeratin and vimentin and therefore allows for the discrimination of tumor cells and non-tumor cells in flow cytometry analysis and the isolation of tumor cells by flow sorting. In this workflow, vimentin$^+$ non-tumor cells serve as an intrinsic control to reveal cancer cell–specific abnormalities. Moreover, the workflow increases the accuracy of detecting cancer-specific genomic mutations by NGS significantly.

Results

1 Automated FFPE tissue dissociation does not compromise DNA stability

We developed a workflow that allows for the efficient dissociation of FFPE carcinoma samples for subsequent isolation of tumor cells and non-tumor cells by flow cytometry–based cell sorting and MACS® Antibodies (fig. 1). FFPE carcinoma tissue sections (50 µm) are dissociated into single-cell suspensions using the FFPE Tissue Dissociation Kit and the gentleMACS™ Octo Dissociator with Heaters. The protocol includes deparaffinization and rehydration of FFPE samples, heat-induced antigen retrieval to reverse formalin-induced modifications, and the enzymatic and mechanical dissociation process. Heat-induced antigen retrieval has been described to enhance immunohistochemical staining of FFPE samples[1], and mild enzymatic treatment has been previously used in the context of FFPE tissue dissociation for flow cytometry[2].

To test whether the tissue dissociation procedure has an effect on DNA quality, we compared the fragmentation patterns of DNA that was prepared directly from FFPE pancreas carcinoma tissue using the QIAamp® DNA FFPE Tissue Kit (Qiagen) and DNA prepared from dissociated FFPE pancreas carcinoma tissue based on our new workflow. DNA from the dissociated tissue was also prepared using the same kit, omitting the deparaffinization step. The fragmentation patterns were similar between the two DNA samples (fig. 2).

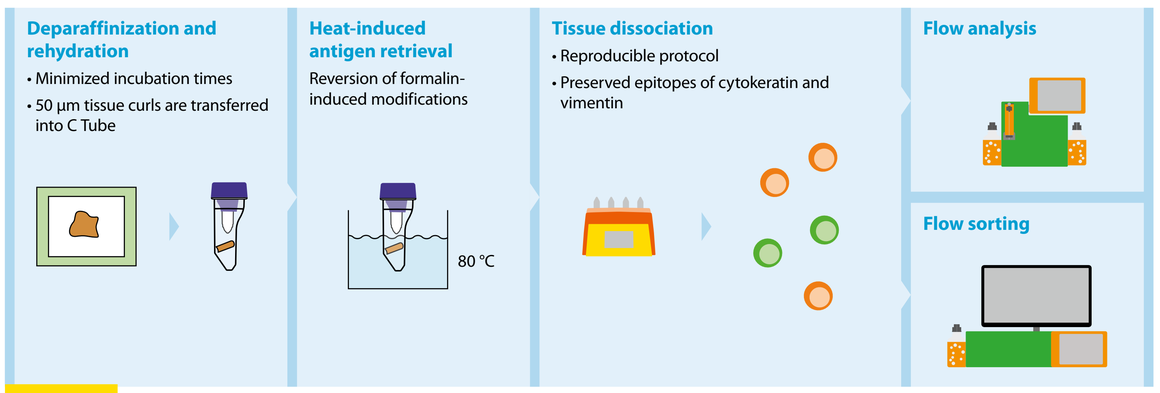

Figure 1

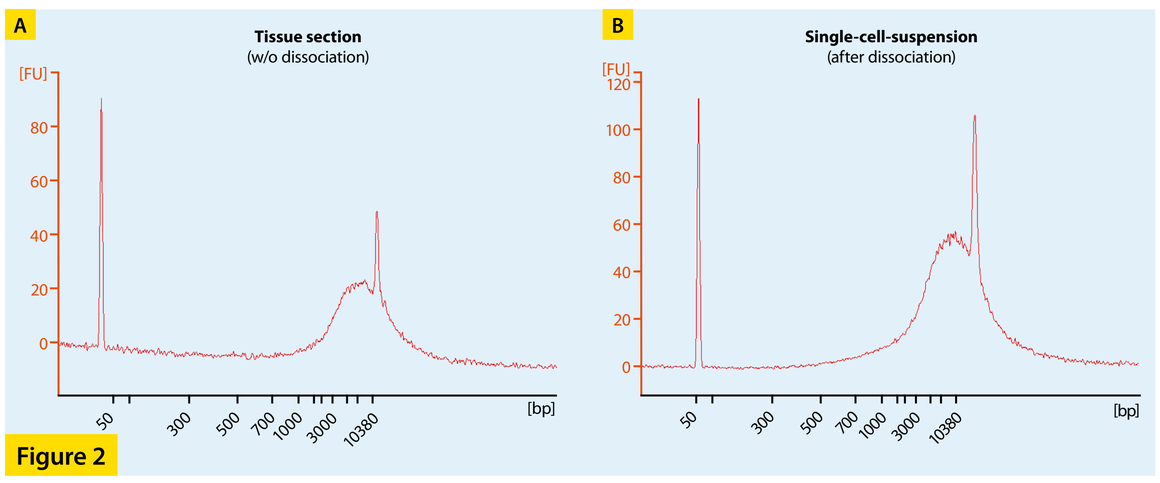

Figure 2

2 Ploidy analysis of carcinoma cells using vimentin$^+$ cells as internal control

DNA ploidy can serve as a biomarker for different tumor entities. The extent of chromosome loss or gain (aneuploidy) depends on the tumor entity and the malignant potential. Tissue dissociation by the gentleMACS Octo Dissociator with Heaters and the FFPE Dissociation Kit allowed for the discrimination of cytokeratin$^+$ carcinoma cells and vimentin$^+$ non-carcinoma cells by flow cytometry. Concurrent DAPI staining and measurement of the mean fluorescence intensity enabled the assessment of the DNA content for each cell type. Vimentin$^+$ non-carcinoma cells served as an internal control defining the normal DNA content of a cell. The DNA index (DI), i.e., the ratio of DNA content in carcinoma (yellow histogram) vs. non-carcinoma cells (green histogram), specifies the aneuploidy status of the carcinoma cells. A ratio of <1 indicates loss of chromosomes, whereas a ratio of >1 means a gain of chromosomes. The DI for a large population of colon carcinoma cells was >1, whereas the DI of prostate tumor cells was close to 1 (fig. 3, table 1). This indicates that the malignant potential of the analyzed colon carcinoma cells was higher compared to the pancreatic tumor cells.

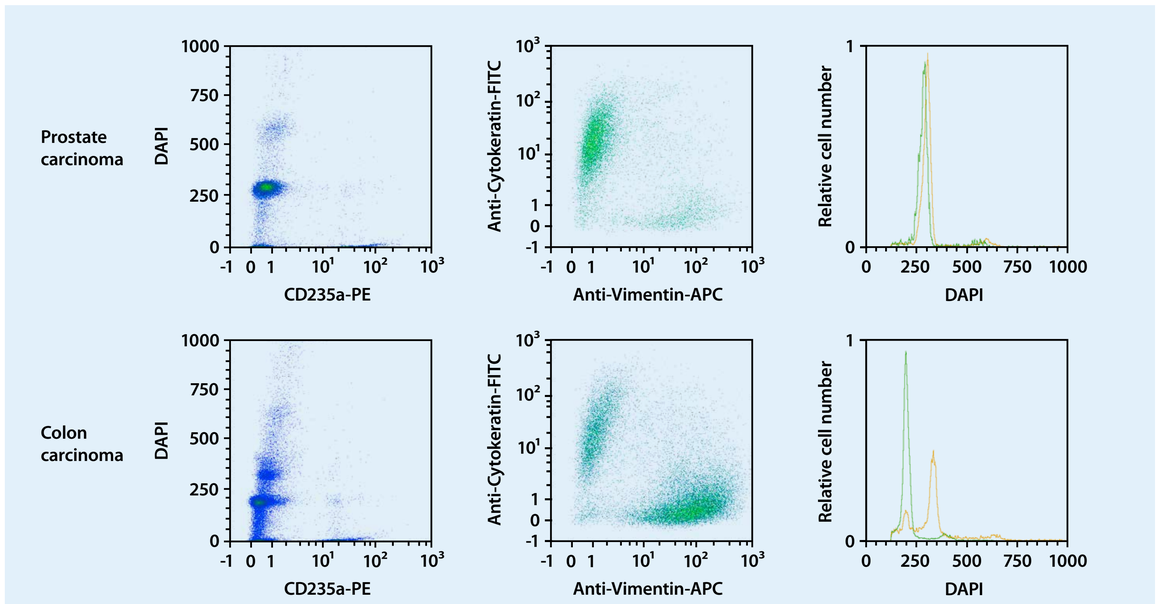

Figure 3

Sample	Cytokeratin$^+$ population	
	DNA index peak 1	DNA index peak 2
Prostate carcinoma	1.08	–
Colon carcinoma	1.03	1.74

Table 1

3 Molecular analysis of carcinoma versus non-tumor cells

To test whether the tissue dissociation procedure had an effect on DNA mutation frequencies, we compared DNA that was prepared directly from FFPE samples (table 3, blue) and DNA prepared from the dissociated FFPE tissue before cell sorting (table 3, green). The mutation frequencies were similar between the two DNA samples.

Flow cytometry–based cell sorting yielded a cytokeratin$^+$ carcinoma cell population and a vimentin$^+$ non-tumor cell population (table 2). High purities of the isolated populations (fig. 4) enabled sensitive analysis of cancer-associated genetic alterations. Several mutations were significantly enriched in isolated cytokeratin$^+$ cells, e.g., CYP2B6 (chr19:41512841; table 3, red). Moreover, as DNA from carcinoma cells was analyzed without interference by DNA from non-tumor cells, certain alterations, e.g., KRAS (chr12:25398284) and TP53 (chr17:7577120), could be specifically attributed to the tumor cells, which indicates the presence of somatic mutations. Alterations that were also detectable in DNA from non-tumor cells (table 3, yellow) indicated germline mutations.

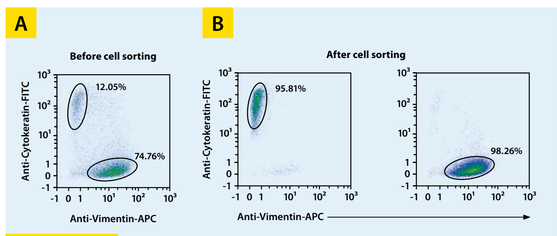

Figure 4

	DAPI$^+$cytokeratin$^+$ cells	DAPI$^+$vimentin$^+$ cells
Cell number	2.1×10^5	2.5×10^5
Extracted DNA (µg)	1.7	2.7

Table 2

Gene	Location	AA change	Codon change	Mutation frequency				Mutation type
				Non-dissociated	Unsorted	Vimentin$^+$	Cytokeratin$^+$	
TP53	chr17:7579472	p.P72R	c.215C>G	100.0%	99.5%	99.6%	99.4%	germline
ABCB1	chr7:87160618	p.S893A	c.2677T>G	99.8%	100.0%	99.8%	99.5%	germline
TAS2R38	chr7:141673345	p.A49P	c.145G>C	99.8%	99.8%	100.0%	100.0%	germline
TAS2R38	chr7:141672604	p.I296V	c.886A>G	99.6%	99.7%	99.7%	99.5%	germline
CYP2B6	chr19:41512841	p.Q172H	c.516G>T	53.6%	57.2%	48.5%	87.0%	germline
FGFR4	chr5:176520243	p.G388R	c.1162G>A	43.8%	45.0%	46.8%	55.8%	germline
CYP2B6	chr19:41515263	p.K262R	c.785A>G	39.3%	36.0%	29.2%	81.8%	germline
KRAS	chr12:25398284	p.G12V	c.35G>T	13.9%	14.7%	<1.0%	52.3%	somatic
TP53	chr17:7577120	p.R273H	c.818G>A	11.9%	13.8%	<1.0%	80.5%	somatic
BAX	chr19:49458970	p.E41fs	c.121insG	1.3%	<1.0%	<1.0%	1.5%	noise

Table 3

Conclusion

- FFPE Tissue Dissociation Kit and gentleMACS Octo Dissociator with Heaters enable fully automated FFPE tissue dissociation of multiple samples at the same time. The dissociation process results in single-cell suspensions with preserved markers for flow cytometry of FFPE carcinoma samples.
- The isolation of tumor cells from bulk tumor enhances the sensitivity of genetic analysis. Tumor-specific signals are enriched and can be easily analyzed and distinguished from background signals, e.g., by NGS.
- No additional healthy tissue or blood sample is required as a control because vimentin$^+$cytokeratin$^-$ cells provide an ideal reference to distinguish between DNA signals from tumor and non-tumor cells in ploidy analysis and somatic from germline mutations in NGS.
- Preservation of cytokeratin and vimentin may allow for the separation of tumor cells of different epithelial-mesenchymal transition statuses to obtain information on tumor heterogeneity.

References
1. Shi, S.R. *et al.* (1991) J. Histochem. Cytochem. 39: 741–748.
2. Corver, W.E. and ter Haar, N.T. (2011) Curr. Protoc. Cytom. Chapter 7: Unit 7.37.