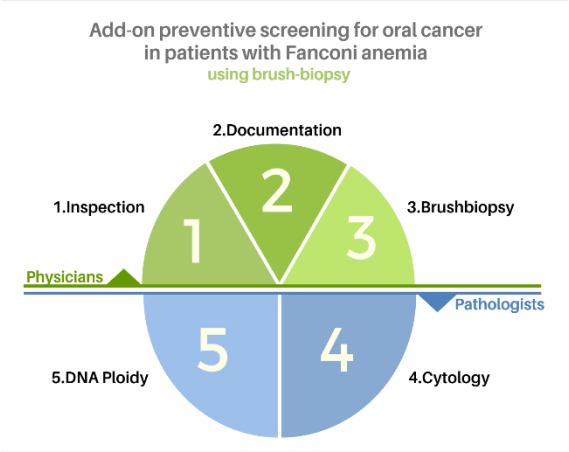
Standard operating procedures (SOP) for

“Add-on preventive screening for oral cancer in patients with Fanconi anemia using brush-biopsy”



- **SOP “Inspection” (Step 1-3):**
Oral inspection, documentation, taking brush biopsy (incl. mouth map)

- **SOP “Cytology” (Step 4):**
Brush biopsy-based cytology
(processing & cytological interpretation of samples)

- **SOP “Ploidy” (Step 5):**
DNA ploidy analysis
(staining, measurements & interpretation of results)

Standard operating procedure (SOP) "Inspection"
Oral inspection, documentation, taking samples, mouth map
for *„Add-on preventive screening for oral cancer in patients with Fanconi anemia using brush-biopsy"*:

Associated SOPs for „Add-on preventive screening for oral cancer in FA":

- **SOP "Inspection": oral inspection, documentation, taking samples, mouth map**
- SOP "Cytology": Brush biopsy-based cytology (processing & cytological interpretation of samples)
- SOP "ploidy": DNA ploidy analysis (staining, measurements & interpretation of results)

Preamble:

Patients diagnosed with Fanconi anemia (FA) present with a highly elevated risk for squamous cell carcinoma. These aggressive cancers arise **at much earlier age** in FA compared to the general population even if the FA patients do not expose themselves to the known carcinogens like alcohol and tabaco. Due to the underlying DNA repair defect FA patients are **hypersensitive to most of the classical standard chemotherapeutics.** Therefore, standard treatment protocols for the treatment of advanced oral cancers **cannot** be applied to FA patients.

Consequently, early detection of oral cancers and their precursors are of huge impact for these patients. Frequently, FA patients present with **multiple visible lesions** in the oral cavity which cannot all be biopsied. In spite of the elevated cancer risk, only a minority of visible oral lesions are premalignant. Recently, a prospective study confirmed the diagnostic accuracy of oral brush biopsy-based cytology for the early detection of oral cancer and precursors in FA. Accordingly, the **oral cancer screening** is based on three pillars, described in this SOP:
Inspection, documentation and oral brush biopsy.

Material needed:

- Cell collectors (= *brushes*)
  - CerviBrushes (CellPath Ltd, Newton, United Kingdom) or
  - Orcellex brushes (Rovers Medical Devices, Oss, The Netherlands)
- Collection tube with fixation medium (= *tubes*):
  - BD SurePath (BD Biosciences, Heidelberg, Germany)
- Digital documentation system
- Oral cavity map (= *mouth map*)

1. Inspection of the oral cavity:

Frequently, oral potentially malignant lesions in patients with Fanconi anemia are small and therefore easily to miss. Therefore, the inspection of the oral cavity should be performed **systematically**. Here is an example:

- Inspection of the outer gingiva starting with the upper right quadrant followed by the remaining in a clockwise manner. Include the retromolare trigone as well.
- Inspection of the inner part of the gingiva following the same procedure
- Inspection of the palate, the palatal arches and the tonsils.
- Inspection of the tongue: dorsum of the tongue, including base and tip of the tongue, lateral borders of the tongue, ventral surface of the tongue
- Inspection of the floor of the mouth

- Inspection of the right and left cheek
- Inspection of the lips

Squamous cell carcinoma in patients with Fanconi anemia often arise in the visible areas of the oral cavity. The **tongue** and the **gingiva of the molars** are frequent locations. Visible oral lesions **are frequent** in patients with Fanconi anemia.

2. Documentation of a visible lesions in the oral cavity

The **photo**-documentation of a visible oral lesion is indispensable to evaluate **changes** (*e.g.* size, color, composition and texture) of the frequently persisting lesions over time. Moreover, an exact **anatomical** documentation is required. The use of an **oral cavity map** (= *mouth map*) in addition to the description of the anatomical location proved to be very reliable. The exact anatomical documentation enables:

- Performing an invasive biopsy at the exact same location in case of a suspicious cytological result
- Better assignment of pictures and brush-biopsies in case of multiple visible oral lesions.

The oral cavity map should stay with the phyisican optaining the brush-biopsy and the inspection. Both documentation systems enable higher visibility and comparability in the exchange with colleagues and the patient.

An **oral cavity map** is attached to this SOP.

3. Oral brush biopsy of a visible lesion in the oral cavity

- Turn brushes at least 15 times either clockwise or anti-clockwise on the visible oral lesion.
- Transfer brush into the collection tube.
- If needed (*e.g.* if taking a sample at a difficult anatomical site) brush the same lesion using a second brush and transfer it in the same collection tube as brush number 1.
- Turn the two brushes inside of the collection tube against each other.
- Detach the brushes from their holders and close the collection tube.
- Gently shake the collection tube to allow cells resolving from the brushes.

Add-on preventive screening for oral cancer in patients with Fanconi anemia

Mouthmap for documentation of visible lesions in the oral mucosa

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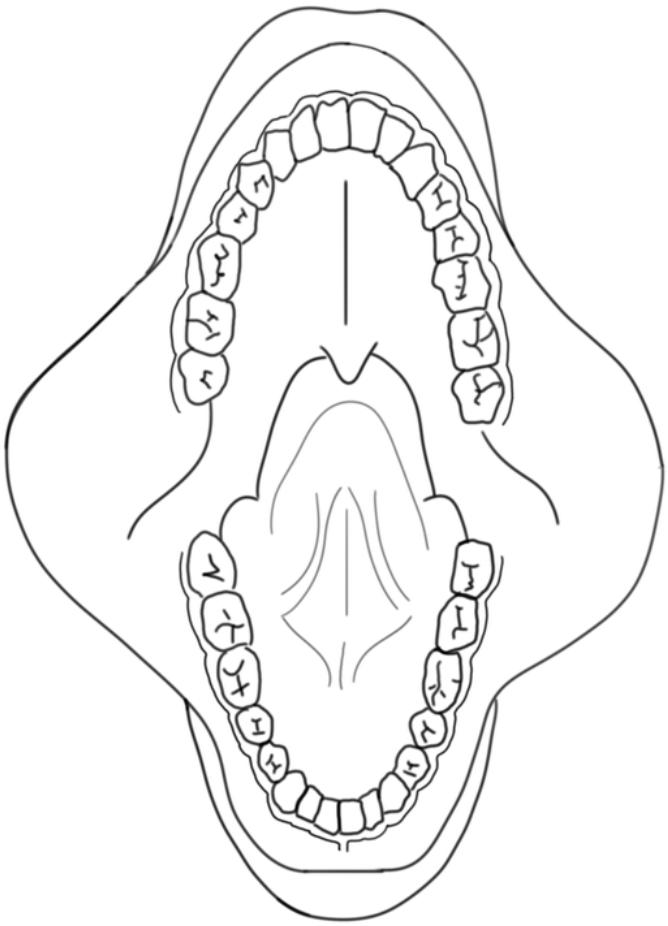
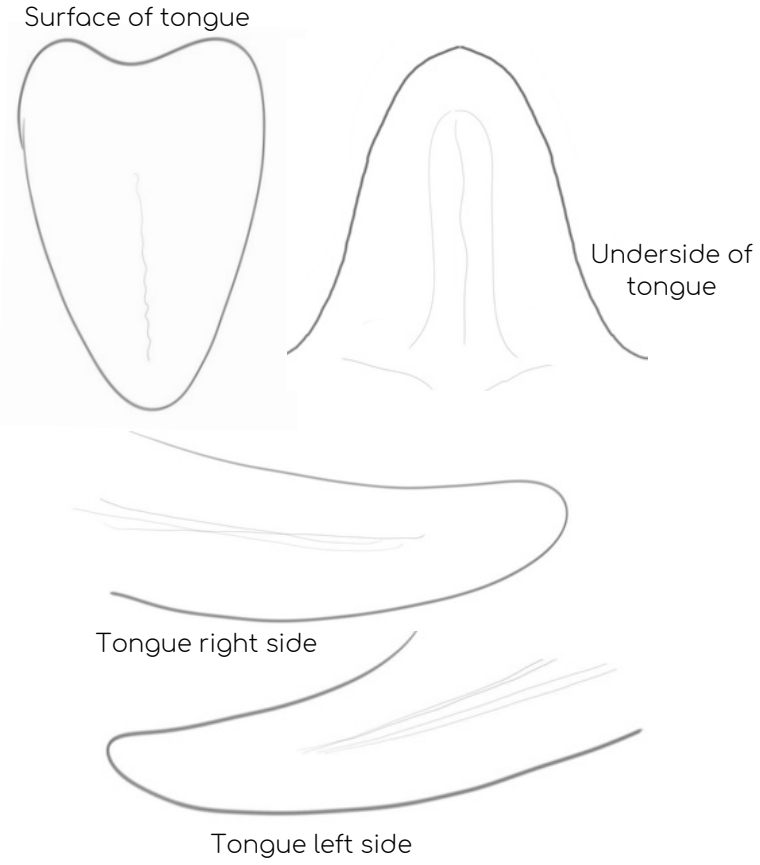
Please mark the location of visible lesions/brush biopsies on the map. Label them additionally (e.g. gingiva buccal regio 34). Please don´t forget to also make a photo-documenation.

Date
Name





Standard operating procedure (SOP) "Cytology"
Taking samples, processing & cytological interpretation of samples
for *„Add-on preventive screening for oral cancer in patients with Fanconi anemia using brush-biopsy":*

Associated SOPs for „Add-on preventive screening for oral cancer in FA":

- SOP "Inspection": oral inspection, documentation, taking samples, mouth map
- ➢ **SOP "Cytology": Brush biopsy-based cytology (processing & cytological interpretation of samples)**
- SOP "ploidy": DNA ploidy analysis (staining, measurements & interpretation of results)

Brush biopsy-based cytology

a. Material needed:

- Cell collectors (= *brushes*)
  - CerviBrushes (CellPath Ltd, Newton, United Kingdom) or
  - Orcellex brushes (Rovers Medical Devices, Oss, The Netherlands)
- Collection tube with fixation medium (= *tubes*):
  - BD SurePath (BD Biosciences, Heidelberg, Germany)
- Cytospins: Cellspin I (Tharmac, Wiesbaden, Germany)

b. Processing the liquid-based oral brushings:

- Shortly vortex the collection tube
- To determine the amount of the cells obtained with the brush, transfer the content to a 15mL Falcon tube
- Centrifuge for 5min at 500g at room temperature
- Remove carefully the supernatant using a pipette
- Resuspend 5 µL of the cell pellet in a small amount of the supernanant to prepare a cytospin with approx. 3 cm^2
- For example, use Cellsin I (Tharmac, Wiesbaden, Germany) centrifuge and coated slides with rectangular funnels to prepare rectangular spots of 2.2 x 1.4 cm.
- Centrifuge 10 min at 1800 rpm at room temperature (Cellsin I, Tharmac, Wiesbaden, Germany)
- Let the slides air dry
- Stain the slides according to Papanicolaou

c. Cytological interpretation of the samples:

- negative: indicate normal squamous cells and reactive or inflammatory changes. Normal cytological results include hyperkeratotic changes, ulcers and/or erosions with or without regeneration, different degrees of mucosal inflammation, and candidiasis.

- atypical: indicate atypical cells present. Atypical cytological results include abnormal regenerating squamous epithelial cells that may demonstrate

prominent nucleoli, hyperchromasia, coarse chromatin, and irregular nuclear contours with an inflammatory background and fibrin deposits, or superficial to intermediate cell dyskaryosis with a slight to moderately altered nuclear-to-cytoplasmic ratio and enlarged hyperchromatic nuclei.

- suspicious: indicate dyskaryotic cells of the parabasal cell type or only a few malignant cells present. Suspicious cytology includes dyskaryosis of the parabasal cell type with a high nuclear-to-cytoplasmic ratio, irregular nuclear contours, and hyperchromatic nuclei or sometimes only very few malignant squamous cells in a specimen that are insufficient for a positive diagnosis.

- positive: indicate malignant cells present. Specimens in the positive category demonstrate cytomorphology that is typical of an SCC with necrotic debris; scattered or small irregular groups of frankly malignant cells with cyanophilic cytoplasm; a high nuclear-to-cytoplasmic ratio; enlarged, hyperchromatic nuclei with nucleoli; and coarse chromatin. Keratinizing SCC may demonstrate bizarre configurated eosinophilic cytoplasm.

- not sufficient: indicate few or poorly preserved squamous cells present or obscuring bacterial colonization

Standard operating procedure (SOP) "Ploidy"
Staining, measurements and interpretation of results for DNA ploidy analysis on liquid-based cytology for samples
for *„Add-on preventive screening for oral cancer in patients with Fanconi anemia using brush-biopsy"*:

Associated SOPs for „Add-on preventive screening for oral cancer in FA":

- SOP "Inspection": oral inspection, documentation, taking samples, mouth map
- SOP "Cytology": Brush biopsy-based cytology (processing & cytological interpretation of samples)
- ➢ **SOP "ploidy": DNA ploidy analysis (staining, measurements & interpretation of results)**

DNA ploidy analysis

Specimens with atypical or suspicious cytology and specimens with negative cytology but with a discrepant clinical impression should be analyzed for DNA ploidy using preferably manual image cytometry. Areas containing suspicious cells should be marked on the coverslip of the specimen and photocopied to restore the labeling after uncovering and re-staining according to Feulgen. Alternatively, the whole cytospin may be analyzed (for example, cytospins with a rectangular plane of 1.4 x 2.2 cm (approx. 3 cm^2)).

a. Feulgen Staining

Uncover Papanicolaou-prestained slides in xylene and subsequently destain and restain in a temperature-controlled staining machine with Schiff reagent. After rehydration in decreasing ethanol concentrations and refixation in buffered 10% formalin, apply 5 N HCl for acid hydrolysis at 27°C for 1 hour, followed by staining in Schiff reagent for another hour, followed by rinsing in SO_2-water and dehydration at increasing ethanol concentrations.

Complete procedure:

1	Xylene	15 min
2	99,5 % ethanol	5 min
3	96% ethanol	5 min
4	10% buffered formalin	50 min
5	Distilled water	10 min
6	Distilled water	10 min
7	5 N HCl (~ 27°C, HCL-resistant cuvette)	60 min
8	Distilled water	2 min
9	Distilled water	2 min
10	Distilled water	2 min
11	Schiff's reagent	60 min
12	SO_2-water	5 min
13	SO_2-water	5 min
14	SO_2-water	5 min
15	Distilled water	1 min
16	Distilled water	1 min
17	70% ethanol	10 min
18	96% ethanol	10 min
19	99,5% ethanol	10 min
20	Xylene	15 min

Cover the slides with Entellan (Merck) and store in the dark.

Of note: SO_2-water must be freshly prepared. Use Schiff's reagent only once. Stain maximum 20 slides simultaneously. Control the temperature carefully during hydrolysis with 5N HCL. Do not forget the slides in Xylene after the staining procedure, as this will change the color of the Feulgen stain.

Staining roboter	**Order number**	**manufacturer**
Cell Stain,		Tharmac, Wiesbaden, Germany
Reagent		
Ethanol 70%; 96%; 99,5%		
10% buffered formalin	HT501128	Sigma Aldrich
5 N HCl	1.09911.0001	Merck
Schiff's reagent	1.09033.0500	Merck
1 N HCl	1.09970.0001	Merck
Potassium disulfite	1.05057.1000	Merck
Xylol	1.08685.2500	Merck
Distilled water		

SO_2 stock solution (1l):
- Resolve 100 g potassium disulfite (K2S2O5) in 1L distilled water
- Use a brown or dark bottle
- Label with the date of production
- best for 6 months

SO_2-water (2l) (must be freshly prepared for Feulgen staining)
- 1800 ml distilled water
- 100 ml 1 N HCl
- 100 ml SO_2 stock solution
- Mix vigorously on a magnetic mixer and fill up the cuvettes for staining

b. Measurements of nuclear DNA content (= c).

DNA cytometer:
Recommendation: Manual MotiCyte DNA workstation (Motic, Xiamen, China) with Motic BA400 microscope, 40x objective, 12-bit colour CCD camera with a resolution of 1360x1024 pixels (MoticamPro 285A) and the MotiCyte-DNA image cytometry software which provides shading- and glare correction.

Systems that provide shading and glare correction are highly recommended. The guidelines for diagnostic interpretation and quality assurance of the consensus reports of the European Society for Analytical Cellular Pathology should be considered.

Internal calibration/reference:
For every measurement, at least 30 normal squamous epithelial cells (preferred), lymphocytes, or granulocytes should be used for internal calibration (2c reference value).

Cells to be analyzed:
Select approximately 300 (at least 100) nuclei of interest in the previously labeled areas and measure the integrated optical density in c units (with c indicating nuclear DNA content) and plot them in a DNA histogram.

c. Interpretation of results:

Definition of DNA stemline:
A population of cells with a similar DNA content accompanied by values approximately at its 2-fold DNA content.

Euploid or euploid polyploidy:
2n chromosomal sets (*e.g.*, diploidy, tetraploidy). DNA stemline(s) are within normal ranges (1.80c - 2.20c and 3.60c - 4.40c). Benign condition.

DNA aneuploidy:
- modal value of a DNA stemline in c units has a position outside normal peridiploid or peritetraploid ranges <1.80c or >2.20c and <3.60c or >4.40c (= stem line aneuploidy) or
- at least 1 cell with a DNA content of >9c (= single-cell aneuploidy)
  Note: The diagnosis “single-cell aneuploidy” should only be applied if accompanied by a population of cells with abnormal nuclear details such as enlargement, irregular shape, coarse chromatin structure, or hyperchromasia. This is necessary due to euploid polyploidization that occurs in many different organs that exhibit inflammation, regeneration, and reactive changes, such as lichen planus of the oral mucosa.

Suspected DNA aneuploidy:
If the interpretation of the measurement is inconclusive. This diagnostic criterion may distinguish oral SCCs that may have a near-tetraploid DNA content.
- an aneuploid DNA stemline consisted of only few cells (<60 cells) or
- single-cell aneuploidy is accompanied by bland cytomorphological features in the Feulgen stain or
- a stemline is very broad-based but with a modal value within the normal peritetraploid range. This criterion should only be applied if accompanied by the above mentioned abnormal nuclear features in the Feulgen stain.

Note: This diagnostic category is not covered by the ESACP consensus reports but represents diagnostic experience during the prospective cohort study on oral brush biopsy-based cytology in Fanconi anemia (Velleuer et al. 2020).

Literature:

Feulgen staining

- Feulgen R, Rossenbeck H. Mikroskopisch-chemischer Nachweis einer Nucleinsaure vom Typus der Thymonnucleinsaure und die darauf beruhende elektive Farbung von Zellkernen in mikroskopischen Praparaten. Hoppe Seylers Z Physiol Chem. 1924;135:203-248
- Chatelain R, Willms A, Biesterfeld S, Auffermann W, Bocking A. Automated Feulgen staining with a temperature-controlled staining machine. Anal Quant Cytol Histol. 1989;11:211-217

DNA ploidy analysis:

- Remmerbach TW, Meyer-Ebrecht D, Aach T, et al. Toward a multimodal cell analysis of brush biopsies for the early detection of oral cancer. Cancer. 2009;117:228-235
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- Velleuer E, Dietrich R, Pomjanski N, et al. Diagnostic accuracy of brush biopsy-based cytology for the early detection of oral cancer and precursors in Fanconi anemia. Cancer Cytopathol. 2020 Jun;128(6):403-413.

Consensus reports of the European Society for Analytical Cellular Pathology (ESACP) covering standard requirements of technical instruments, software, guidelines for diagnostic interpretation and quality assurance:

- Böcking A ,Giroud F, Reith A: Consensus report of the ESACP task force on standardization of diagnostic DNA image cytometry. European Society for Analytical Cellular Pathology. Anal Cell Pathol. 1995;8:67-74.
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