F*ex*ISH® Practical Procedure – Technical Tips and Tricks:

Technical tips & tricks which should be considered before performing the F*ex*ISH® FISH method:

Preparatory Steps:

- Please check first all temperatures of the used technical devices with a thermometer; as we know these can widely differ from the temperatures which are seen on the display of the respective devices.

- Please, set in advance the following conditions: In order to standardize, it is recommended to always use 8 slides during the heat pretreatment and stringency washing (per 70 ml staining jar). If processing less slides, place empty slides in the jar as placeholders.

- Regularly change the ethanol and xylene solutions. We recommend changing these solutions every 150 to 200 slides. Otherwise, the deparaffinization might be insufficient interfering with the FISH results.

- Do not use a permanent marker to mark the tissue section on the glass slide. The marking color may dissolve during the dewaxing step in xylene and the dehydration step in ethanol and thus it might come in contact with the tissue sections which could result in an orange haze when evaluating the slides using a fluorescence microscope. Instead, use a diamond grinding pen to encircle the tissue section.

Preparation of Specimens:

- The specimen size should not exceed 0.5 cm^3. Fixation should be carried out in 10% neutrally buffered formalin (37% buffered formaldehyde solution is called formalin 100%; 10% formalin solution corresponds to 4% formaldehyde solution) for 24h-48h at room temperature (18-25°C) using premium quality paraffin. Infiltration and embedding should be carried out at temperatures below 65°C.
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- The sections should have of a thickness of 2-4 μm. Sections thicker than 4 μm may lead to difficult evaluation due to overlapping nuclei. Fixation of sections should be carried out for 2-16h at 50-60°C.

- The specimens should be drawn up onto positively charged microscope slides which are compatible with FISH (e.g. Histobond). Using slides that are not positively charged leads to very weak connection between tissue section and slide so that the tissue may float off during pretreatment. As an alternative, glass slides can be APES-treated (silanized) or coated by poly-L-lysine. Please note that unsuitable slides may also cause autofluorescence or a haze.

- Please make sure to avoid the usage of the following interfering fixatives for fixation as they are incompatible with FISH: Bouin's fixative, B5 fixative, acidic fixative (e.g., picric acid), Zenker's fixative, alcohols (when used alone), mercuric chloride, formaldehyde/zinc fixative, Hollande's fixative, and non-buffered formalin.

Proteolysis:

- The pepsin digestion is a crucial step and the digestion time needs to be adapted. For each tissue type, the optimal pepsin digestion time can differ due to specimen variability (tissue/cell type, nature and duration of fixation, thickness of section) and needs to be determined first. This can be done by performing a pepsin digestion time series: Try, e.g., 3 different pepsin digestion times (e.g., 10, 15, and 20 minutes) in order to find the most appropriate one.

- Incubate at 37°C, not at room temperature (the optimum temperature of pepsin is 37°C), using the pepsin directly out of the fridge.

- Please note that the incubation time and temperature of heat pretreatment, the incubation time for pepsin digestion and denaturation are settings that depend on each other. Altering one of these three steps might require adaptation of the other two steps as well.

Hybridization:

- Make sure that the specimens are completely dry before applying the probe. Otherwise, this can affect the tissue morphology and result in weak signals.

- Protect the probe from light during application and in all following steps. Incubations should now be carried out in the dark.

- Drying of the tissue/cell samples during hybridization and consequently a decreased signal intensity must be prevented. It is necessary to cover the specimens with a coverslip after applying the FISH probe and seal it completely with hot glue or rubber cement (e.g., Fixogum). Use humid conditions for hybridization. If using a hybridizer, wet the strips of the hybridizer or fill the respective water tanks.

- After hybridization it is necessary to remove the coverslip before performing the stringency washing step!

Stringency Washing:

- The exact stringency washing temperatures and times are mandatory for optimal FISH results:

Implementation Kit	Buffer	Temperature	Time
F*lex*ISH-Tissue Implementation Kit	1x F*lex*ISH Wash Buffer	72°C	10 min
Zyto*Light* FISH-Tissue Implementation Kit	1x Wash Buffer A	55°C	2x 5 min
Zyto*Light* FISH-Cytology Implementation Kit	Cytology Stringency Wash Buffer SSC	70°C	2 min

Evaluation:

- Avoid mixing of DAPI/DuraTect™ Solution with immersion oil as this might cause turbid overlay. Thus, it is recommendable to use large coverslips (24mm x 60mm). Make sure to use immersion oil approved for fluorescence microscopy. Expired immersion oil might become opaque.

- The appropriate filters specific for the fluorochrome excitation and emission wavelengths should be used. If you need help to choose the correct filters, please tell us the specifications of your filters (excitation and emission wavelengths).

- Beside filter sets, the excitation source used has an impact on the signal quality. We recommend a 120 watt mercury lamp which yields expanded light intensity near the ultraviolet spectrum and has a long lifetime.

- For further information refer to „Microscopy and Filters – Technical Tips and Tricks“