The EGFR FISH Assay in Metastatic Colorectal Carcinoma: A Practical Guide for Analysis and Interpretation

Goal:
Standardization and Reproducibility of EGFR FISH assay among laboratories

Aspects to be Addressed

I. Specimen Preparation

II. Assay conditions and Instruments

III. Criteria for Microscope Analysis

- Quality Assessment
- Selection of Tumor Foci
- Selection of Nuclei to Score
- Imaging for Permanent Record

IV. Signal Enumeration and Recording

- Counting Signals
- Defining Gene Amplification

V. Definition of FISH pattern and Reporting

I. Specimen Preparation

Formalin-fixed, paraffin-embedded tissue according to guidelines proposed for HER2 FISH in breast carcinomas

1. **Time from tissue acquisition to fixation**: as short as possible
2. **Tissue fixation**: 6-48 hours in 10% neutral buffered formalin
3. **Storage of fixed tissue**: Protected from light at room temperature
4. **Thickness of sections**: 4μm +/- 1μm
5. **Mounting**: on charged (coated) glass slides, sample attached ~ 1/3 distal to the frosted edge, all slides from each specimen with similar orientation
6. **Section age:** Preferentially freshly cut (<3 months), maintained protected from light at room temperature
7. **Material requested by the FISH laboratory**
    a. One H-E stained and 2 unstained sections,
    b. Preferentially from the diagnostic specimen.
    c. Tumor content confirmed before transfer to FISH laboratory

II. Assay Conditions and Instruments

1. Probe: LSI EGFR SpectrumOrange/CEP7 SpectrumGreen (Abbott Molecular)

2. Protocol: Flexible as soon as quality of the results is excellent

3. Microscope features:

a. Epifluorescence
b. Equipped with single band pass filters (TR, FITC, DAPI). Double and triple band pass filters highly desirable
c. Objectives with high NA

4. Imaging features: CCD camera and Z-stacking capability

III. Criteria for Microscope Analyses

A. Quality Assessment of the FISH Specimen

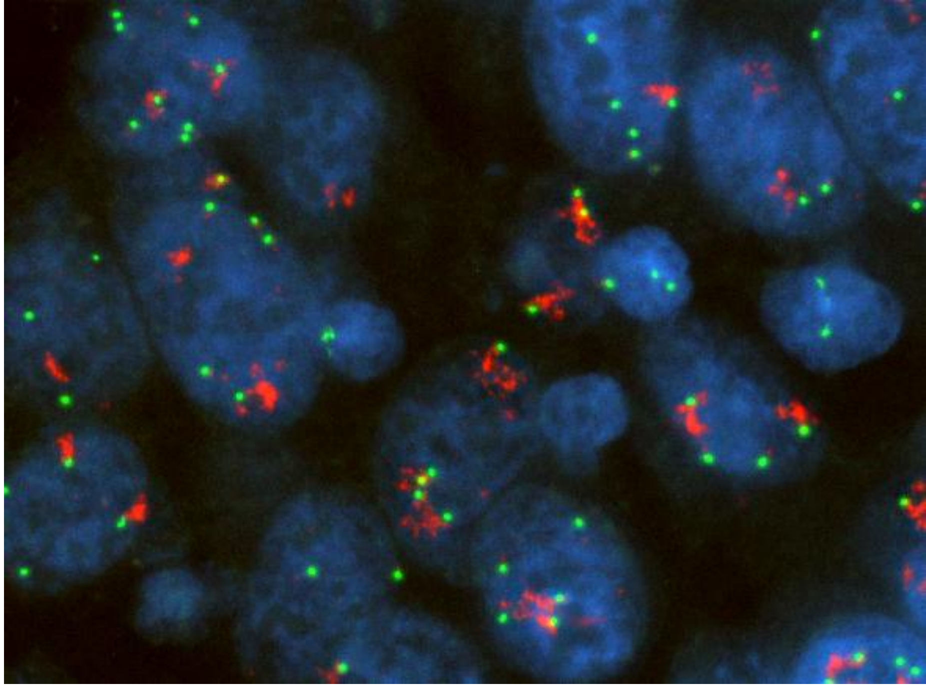
1. Assess the **adequacy of the specimen for analysis** according to the requisites listed below, using high power objectives (40x, 63x or 100x). All requisites must be reached in **at least 70% of tumor cells**, otherwise specimen is classified as unsatisfactory and troubleshooting must be performed.

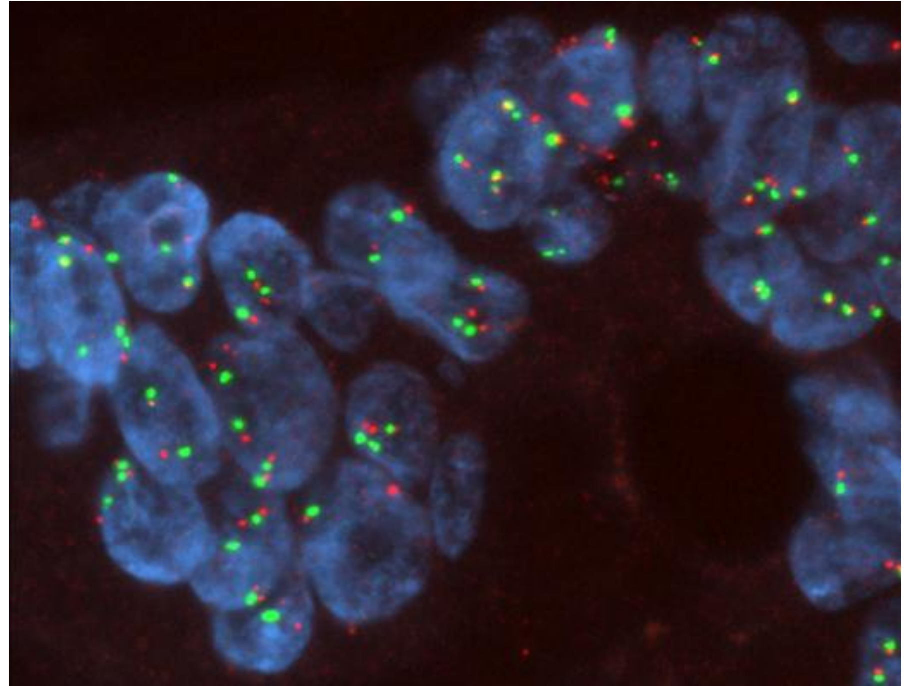
a. **Use the DAPI filter to verify integrity of the tumor nuclear morphology**. In adequate specimens, chromatin in the tumor cells is not over-treated to the point of preventing clear identification of nuclear border and is not missing from nuclear areas, and tumor nuclei are not covered by a cloudy yellowish layer or obscured by autofluorescent structures.

b. **Use single and double pass interference filters to inspect quality of the hybridization** in tumor and non-tumor cells (stroma reactive cells, etc). In adequate specimens, green signals (CEP 7) are bright, compact (occasionally slightly stringy or diffuse), oval shapes and red (EGFR) signals are bright, small round shapes, commonly adjacent to CEP 7 signals. The CEP 7 signal is larger and brighter than the EGFR red signal. Signals may not be fuzzy or very patchy.

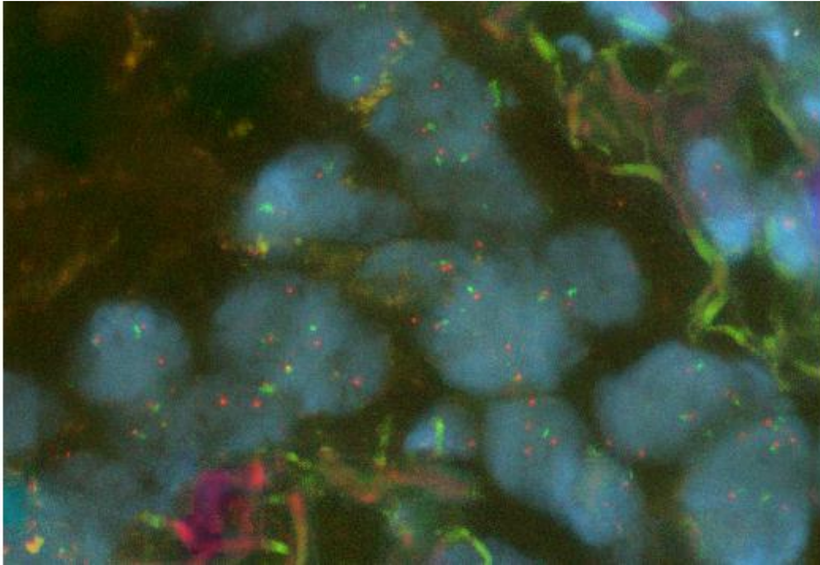
c. **Use single and double pass interference filters to inspect the background**. It should appear dark and free of fluorescence particles or haziness.

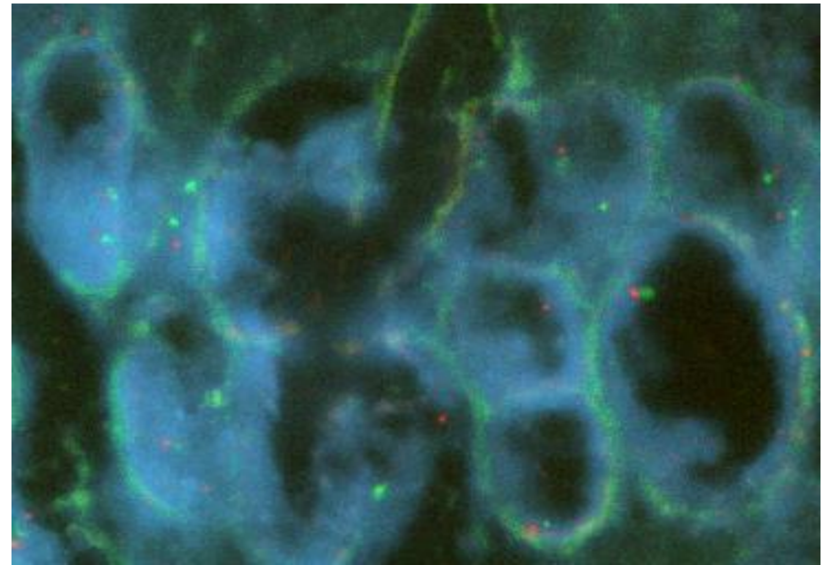
Adequate Specimen - Passed Quality Assessment

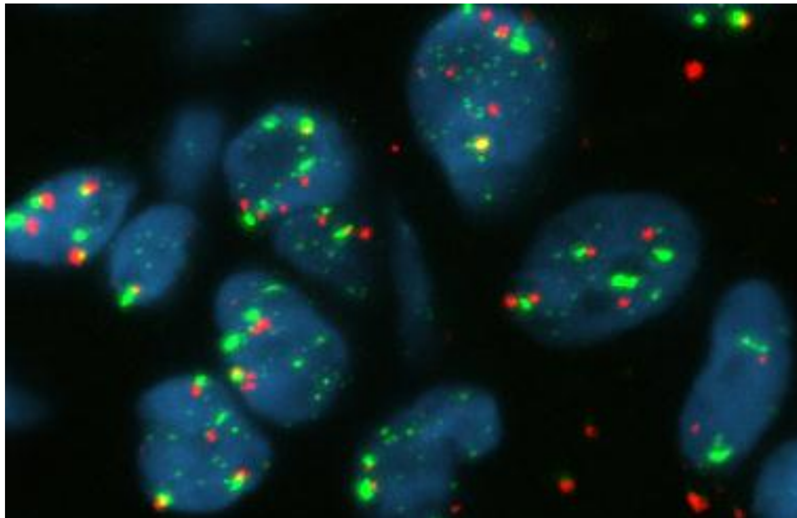


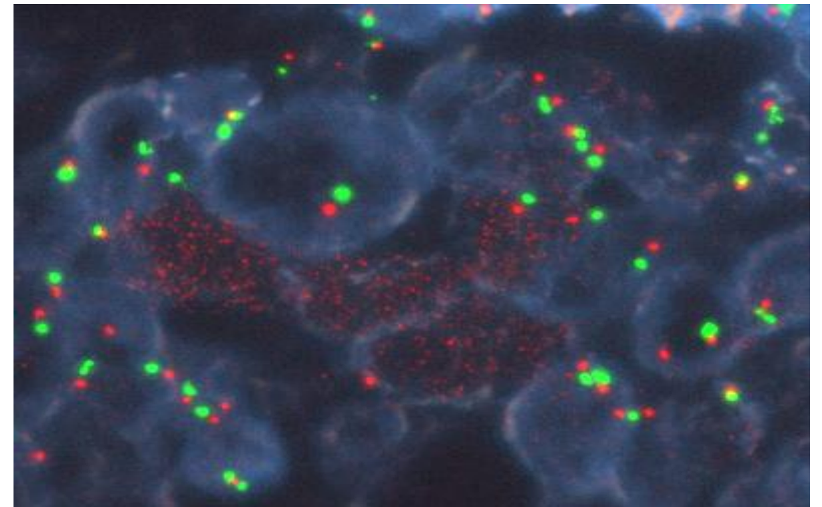


Inadequate Specimens: Quality Assessment Failure


Poor signal intensity; background haziness


Missing chromatin


Cross-hybridization of CEP 7 signals

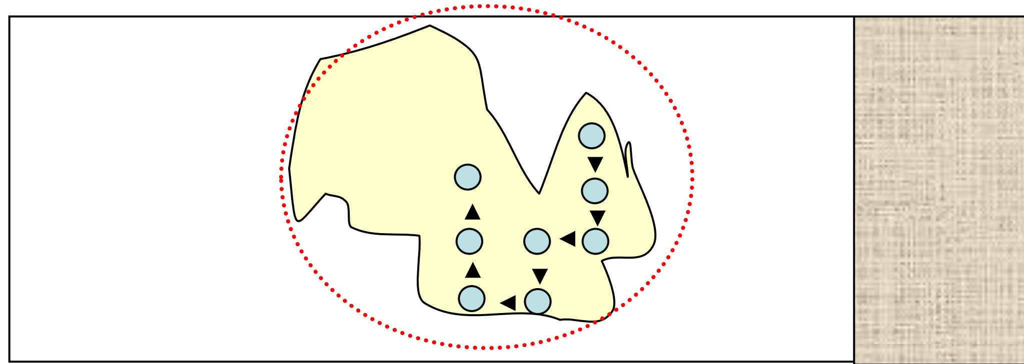

Patchy red background

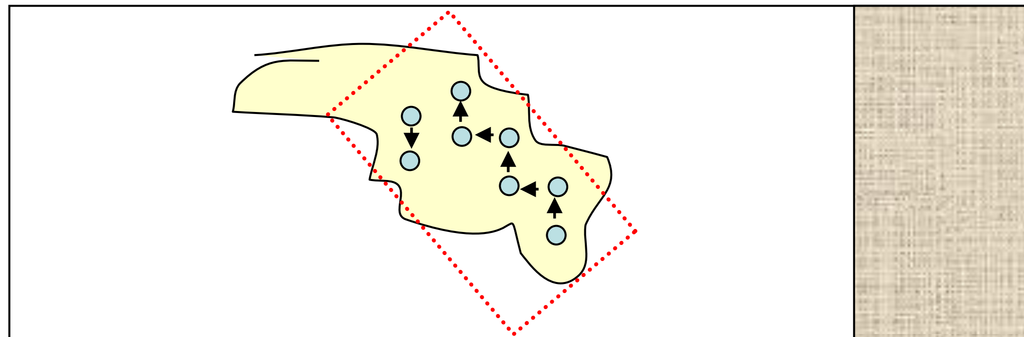
III. Criteria for Microscope Analyses

B. Selection of Tumor Foci: 3 mm strategy for Heterogeneous Tumors

1. Review grossly the H&E and FISH slides searching for areas with tumor material. Select a “starting corner” (for instance, NE), identify a distinct tumor area, switch to the 100x objective and analyze approximately 10 representative cells.
2. Insert back the 40x objective, move the stage 3mm along the Y (north or south) axis, and in this 2nd location select approximately 10 cells for analysis.
3. To reach the 3rd location, repeat the sequence of steps above. After the 3rd reading in the same axis.
4. For the next 3 locations (4th to 6th), move again the stage 3 mm along the Y axis (north or south) axis.
5. Choose the next 2 locations in different X axis locations, following same schema described, up to complete the 8 tumor areas and 80 tumor cells scored.
6. When the 3 mm strategy leads to a non-tumor area do the following:
   a. Scan the new field $\pm$ 1 mm (X or Y) to find a scorable area and resume the previous schema from that point on.
   b. If there is not tumor area close, return to the previous location and attempt moving 3mm in a different X or Y direction.
   c. If no sufficient tumor area is found within 3mm of the previously analyzed location, move to a completely different analyzable section of the specimen, score approximately 10 cells and then proceed with the 3mm strategy again.
7. In small biopsies or specimens with small tumor areas, the 3 mm strategy may have to be reduced to either a 1 mm or 2 mm strategy. If a different strategy has been used for analysis, it must be noted in the comments section of the analysis worksheet.

B. Selection of Tumor Foci: 3 mm Strategy for Heterogeneous Tumors





III. Criteria for Microscope Analyses

C. Selection of Nuclei to Score

1. **Select approximately 10 tumor nuclei for analysis in 2-3 microscope fields, in each of the 8 selected tumor areas**.
   a. **Select nuclei to be scored using the DAPI filter.** Choose only nuclei that shows:
      1. Median to large diameter in comparison with other tumor nuclei in the specimen (to reduce effect of the nuclear truncation).
      2. Unambiguous borders (no overlapings, disruptions, etc).
   b) **Confirm selection using the single or dual band pass filters**. Verify that nuclei shows:
      1. Objectively interpretable signals.
      2. At least one signal for each target.

D. Imaging for Permanent Record

1. Document results capturing at least one image of each of the 8 areas using Z-stacking .
2. Annotate the location of the imaged fields using microscope coordinates or reference slide.
3. Image any and all atypical findings not addressed in these guidelines.

Selection of Nuclei to Score

* = selected nuclei

IV. Signal Enumeration and Recording

A. Counting Signals

1. Determine and record the number of EGFR (red) and CEP 7 (green) signals for each individual nucleus in the FISH analysis worksheet. Count signals according to the instructions below:

   a) Use **single red filter to enumerate red** signals and **single green filter to enumerate green** signals. Use dual red/green and triple red/green/blue filters to verify signal numbers.

   b) Scan the **focus through the entire depth** of the section to ensure that all signals are identified within each nucleus.

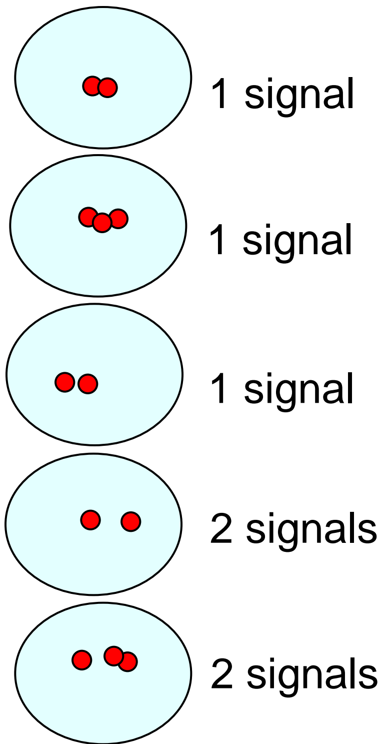
   c) Count as 1 signal any **doublets or triplets** that are physically connected (touching, linked by a tread) or adjacent (gap smaller than the diameter of the largest signal).

   d) Count as separate entities signals that are adjacent but separated by at least the diameter of the largest signal.

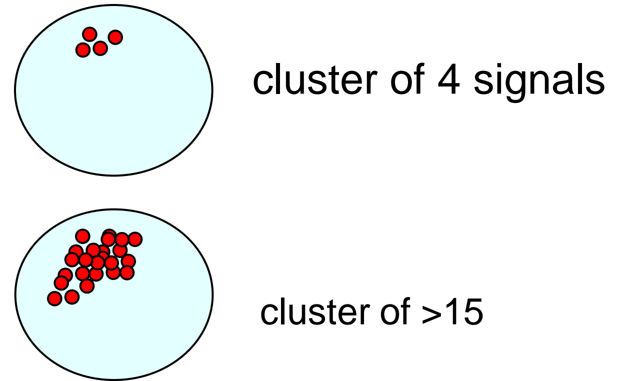
2. When nuclei do not exhibited clusters of signals, analyze at least **80 nuclei** per specimen. Report the assay as uninformative if the required number of nuclei is not available for analysis.

3. If clusters of the EGFR signal is present, proceed to (IV. B).

Counting Signals



Examples of clusters:



IV. Signal Enumeration and Recording

B. Defining Gene Amplification

1. Verify if consistently one or more copies of the **EGFR signal is atypically large** in tumor cells (brighter and larger than the CEP 7 signal) while showing the expected size in the non-tumor cells. Identify nuclei carrying this feature on the worksheet.
2. Verify presence of **clusters of signals for one or both probes**. If cells exhibit clusters of EGFR signals proceed as follows:
   a) if **clusters are small (4 -10 copies of signals)** or very tight and enumerable, follow instructions provided in IV.A.1 and IV.A.2.

   b) if **clusters are large (>10 copies),** count the signals in only 30 nuclei. Enumerate as many signals as possible. If more than 15 copies are present, annotate "16" in the worksheet.

   c) if clusters are present only in some cells or some tumor areas, identify nuclei carrying this feature on the records.

V. Definition of FISH Pattern and Reporting

1. Calculate for each specimen:
   a. % of cells showing ≤2, 3, ≥4 copies of the EGFR signal
   b. mean copy number per cell of EGFR signals and of CEP 7 signals
   c. ratio of the mean EGFR by mean CEP 7 signals
   d. % of cells with clusters of the EGFR gene with ≥4 signals or atypically large EGFR signals.