FISH SCORING IN ONCOLOGY

Guidelines produced from a Best Practice Workshop held in London, October 2003

Workshop Organiser: Dr Fiona Ross, Wessex Regional Genetics Laboratory

Secretary: Dr John Swansbury, Royal Marsden Hospital

Guidelines ratified by ACC Council on 2nd December 2003

CONTENTS

1 Quality and Verification of Probes 3
2 Material to Test 4
3 Quality of FISH 5
4 Analysis 6
5 Checking 7
6 Policy for Undertaking FISH Tests 7
7 Reporting 8
8 Training 9
Appendix 1 Participants 10
Appendix 2 Examples 11
Appendix 3 Abbreviations Used 13
RECOMMENDATIONS FOR FISH SCORING IN ONCOLOGY

FISH is a rapidly evolving technology so that service requirements change year by year. The recommendations given here are from a consensus of practices that have been developed in UK laboratories and are intended to give help in approaching a difficult problem. They are essentially concerned with factors affecting interphase FISH scoring, although Section 6 also considers situations where it may be appropriate to carry out metaphase FISH studies. Interphase FISH studies are often carried out because of the absence of sufficient metaphases. However, if metaphases are present, they can add significant information to the analysis (see Section 4.3). Section 6 repeats that these guidelines are intended for those who use FISH rather than molecular analysis. In many instances RT-PCR could be used in place of FISH; this document does not attempt to lay down any guidelines for whether to use FISH or molecular.

1 Quality and Verification of Probes

(This applies to both commercial and “in-house” probes.)

1.1: Several instances of incorrect probe packaging are known, therefore it is recommended that each batch is tested on metaphases to verify chromosomal location.

Explanatory note: If metaphases are present in the first sample to be tested, this provides an adequate control, but otherwise a small quantity of a control suspension with a sufficient mitotic index should be hybridised simultaneously with the first sample.

1.2 Controls

1.2.1: It is not necessary to test dual fusion probes against multiple negative controls due to the workload implications and essentially zero false positive rates expected. NB if these dual fusion probes are used in diagnostic as well as monitoring situations results from negative patients can act as negative controls to provide reassurance that the laboratory scoring policy does result in zero false positives.

1.2.2: Single copy or single fusion probes will have very variable false positive rates. It is recommended that situations where reporting of low numbers of positive cells for these probes is likely should be avoided unless specific projects are being undertaken where a negative control series must be run. Low level positive results always require careful assessment of relevant factors before reporting. This must include some idea of the likely false positive level for the type of probe used and the uncertainties inherent in setting any cut-off level.
1.3: It is expected that any reports based on in-house probes will be issued with caution until extensive experience has been gained of results they produce in a wide variety of cases.

1.4 Expiry dates on commercial probes usually have a wide safety margin. If high hybridisation efficiencies and strong signals are sustained, then probes may be used beyond the expiry date.

2 Material to Test

2.1 Diagnostic cases

2.1.1: Bone marrow is acceptable in those diseases where high level involvement is expected, e.g. AML, ALL, CGL, MDS and MPD.

2.1.2: Blood is an acceptable alternative tissue for diagnostic FISH in CGL, in CLL, and in high count acute leukaemias or leukaemias with blasts in the circulation. It is acceptable for some MPDs, for example when there is a requirement to exclude BCR/ABL positivity.

2.1.3: Bone marrow and blood are generally not suitable for FISH studies of solid tumours, lymphomas or MM, unless there is extensive infiltration (at least 20%) identified by morphology. Laboratories would therefore be justified in declining to test such samples, since unnecessary testing is not good practice. An alternative sample should be considered, such as a bone marrow smear, tumour touch prep, tumour aspirate, separated cells, or, if the laboratory already has sufficient experience, a paraffin embedded tissue section or cells released from such a section.

Explanatory note: Haemodilution of the BM sample sent for cytogenetics results in a substantially lower proportion of neoplastic cells present compared to the slide assessed for morphology. Thus, all negative reports in these circumstances should include mention of the possibility that there were too few relevant cells present to obtain a result.

2.1.3.2: For the disorders described in 2.1.3, it would be acceptable for a laboratory to process a BM sample but to wait until it had been notified that the tissue was adequately infiltrated before doing FISH.

Explanatory note: If there are difficulties in obtaining this extra information, it is acceptable to inform the clinician that the sample will be stored until a specific test is requested.

2.1.4: Histological sections can be used for FISH studies but these have both technical and interpretation problems. This type of service should not be offered by the laboratory until it has sufficient experience.

Guidance notes: FISH of sections requires that the tissue was properly and promptly fixed to reduce DNA degradation, that the section is thin enough to
minimise cell crowding but thick enough to minimise signal loss due to the cells being incomplete and that the section be pretreated with a proteinase. 4 micron sections are recommended, but laboratories should evaluate whether under their local conditions thinner sections give clearer results. Where appropriate, confirmation of the presence of the relevant cell type for analysis should be sought.

2.2 Follow-up studies

2.2.1: In haematological disorders, follow-up studies are best carried out on the same tissue that was used at diagnosis. Follow-up samples on a tissue different from that previously tested cannot be reported without either obtaining a baseline sample for comparison, or issuing a rider explaining the difficulties of interpretation of results under these circumstances. (NB occasionally blood rather than bone marrow will be used for diagnosis of some leukaemias. It is acceptable to use BM for follow-up in these cases).

2.2.2: In some situations it is appropriate to score only selected cells. Where this is done it must be made clear on the report.

Explanatory note: When scoring in lymphoid malignancies it is accepted that neutrophils can be excluded. More recently it has been suggested that it may be appropriate to score only neutrophils if performing follow-up analyses of Glivec-treated CGL patients on blood (Rheinhold et al. Leukemia (2003) 17, 1925-1929, and results from the Birmingham lab).

2.2.3: When using FISH for follow-up studies, it is important that that the signal pattern of the diagnostic sample is known. This can be established from stored diagnostic material at the time of follow-up. If no diagnostic sample is available and anything other than a standard abnormal result is obtained, then the report must explain the difficulties of interpretation.

2.2.4: FISH studies are suitable for assessing initial response to treatment, but are not sensitive enough for detecting minimal residual disease at a clinically significant level.

3 Quality of FISH

3.1: It can be difficult to provide a reliable result from a case that has a low hybridisation level, due to a high proportion of dead cells, low probe hybridisation efficiency, etc. If relevant, the hybridisation should be repeated to try to improve the result. If a more reliable result cannot be obtained the report must be qualified to explain the possibility that the appropriate cells were not assessed.

3. 2: It can be difficult or impossible to score cells with highly-fragmented signals. These seem to occur more often in positive cases than negative, so the possibility of bias by exclusion must be taken into account.
3.3: Culture time can have a significant effect on the proportion of malignant cells present, especially in lymphoid disorders, so FISH should be done on cells cultured for the shortest time. It may be appropriate to consider setting up a direct culture or making smears specifically for FISH.

Explanatory note: the most severe recorded example of this is in Ph+ve ALL; several instances of childhood ALL are known where overnight culture reduced the visible Ph+ve level by FISH to around 5%. Good documentation of the problems with other types of cells is not available but it has to be accepted that even short culture periods can have a drastic effect on any cell type with poor viability in vitro. This is much less likely to be a significant problem in diseases where standard cytogenetic cultures usually show good quality abnormal metaphases.

3.4: Samples where the signals are not visible to the naked eye should not usually be reported when the result is negative. It is acceptable to report unequivocally abnormal results obtained by image enhancement. If image enhancement can capture several focal planes then unequivocally normal results with at least 100 cells scored can also be reported.

4 Analysis

4.1.1: Analysis must always be done with the full knowledge of the probe(s) used and of the potential signal patterns, both normal and abnormal, and any variants.

4.1.2: It is preferable that analysis is done with full knowledge of the material being tested; for example, tumour or lymphoma samples may consist mainly of a mixture of normal and reactive cells.

Explanatory comment: A sample may have a small total population of abnormal cells but these may have a characteristic morphology. Thus 4 cells out of 100 with apparent positive signals but which are much larger than (or a different shape from) the rest of the population should instigate a search for more cells of that size or shape before completing the analysis. Even a single abnormal cell with an unusual morphology and characteristic signal pattern should be considered highly suspicious in any tissue where the relevant cells might be in a low proportion. Minimum recommended cell numbers may not be adequate under such circumstances.

4.2: The standard number of cells recommended for a FISH study is 100. However, it is recognised that an adequate positive result can often be obtained with fewer (particularly when expecting an all-or-nothing result, e.g. in CGL), and a suspicious finding may need more (see 4.1.2).

4.3: In all diagnostic FISH studies, a positive effort should be made to examine a few metaphase cells, if present, and not depend entirely on interphase nuclei. In normal metaphases this confirms that the correct probes were used and abnormal metaphases can be invaluable in interpreting unusual signal patterns.
4.4: A diagnostic laboratory has limited resources to pursue inconclusive results. If a single repeat experiment or an additional experiment with a readily available probe could shed significant light on the interpretation, this should be pursued. Otherwise it is adequate to issue a qualified result explaining the difficulties of interpretation.

5 Checking

5.1: At least two people should score a substantial number of cells in each study. A minimum of 50 each is recommended. These people should work “blind”, i.e. being unaware of each other’s scores. Where very low numbers of abnormal cells are found references must be noted so that the same cells can be agreed by both analysts.

5.2: At least one of the scorers must be a state registered Clinical Cytogeneticist.

5.3: Where hybridisation is carried out on large numbers of cells it is acceptable to report a combined score whether or not a positive effort was made to screen different parts of the slide, as the chance of both scorers assessing the same cells is small.

5.4: If the two primary scores differ significantly then a third person (if necessary from another laboratory) should be called in to provide a resolution. This person should normally be informed of the previous scores.

6 Policy for Undertaking FISH Tests

The range of abnormalities being tested, and the variety of probes available change frequently. However, the following very general best practice recommendations should be valid for a significant time. (See Appendix 2 for a list of examples valid in October 2003). There are many instances of FISH testing for which molecular analysis could be substituted. The choice of FISH or molecular tests depends on the laboratories and their users. The following apply if FISH is chosen.

6.1: Provided that suitable commercial probes are available, FISH should be performed in all situations where:

a) the relevant cells may not yield metaphases or cryptic abnormalities are known to occur, AND
b) the presence or absence of the abnormality has an unequivocal effect on treatment.

Point b) refers to either establishing the correct diagnosis or placing the patient in the correct trial stratification group. Where particular abnormalities are associated with a specific morphology, it is not essential to screen all patients with normal or failed karyotypes, only those with a reasonable index of suspicion. This information should be supplied by the referring clinician. Where there is no associated morphology then all patients with failed karyotypes should be screened, but screening is only
necessary in those with normal karyotypes if there is a strong likelihood of the relevant cells failing to divide

6.2: In most haematological disorders, it is preferable to use FISH at diagnosis as an adjunct to, and not in place of, a conventional cytogenetic study. The exceptions include those diseases where it is difficult to obtain relevant mitoses, e.g. CLL and myeloma, although full cytogenetics is always likely to give more information than FISH alone. Where no fresh material is available, FISH may be the only possibility. This is particularly true for lymphomas and solid tumours.

6.3: It is not recommended that FISH be used routinely to confirm cytogenetically visible abnormalities although it should be used to check uncertain variants of diagnostic or prognostic significance. It may also be appropriate to check apparently classical abnormalities in the context of an atypical presentation.

6.4: Further FISH techniques such as painting or M-FISH are rarely necessary unless it is suspected that a complex abnormality is masking something of clinical significance that cannot be detected by locus-specific FISH.

6.5: FISH can be much simpler and more accurate for follow-up than conventional cytogenetics but there is no situation in which it is mandatory, although it should be offered if conventional cytogenetics fails. Use of FISH for monitoring should be determined by individual laboratories on the basis of convenience.

6.6: A combination of both FISH and cytogenetics should not be routinely used in follow-up studies. The cost of two tests cannot be passed on to the referring centre without agreement that both tests are specifically required.

7 Reporting

7.1: It is not essential to use current FISH ISCN in interphase analysis reports. If not using ISCN the results should be described as simply as possible. Terms such as ‘nuc ish’ should be avoided as they could give the impression that an approved nomenclature was being used.

7.2: Since FISH testing is now widely used in UK laboratories and in accordance with professional custom, it is no longer necessary that FISH reports carry a rider stating that the commercial probes have not been licensed for diagnostic use.

7.3: Routine disclaimers about the limitations of FISH analysis are not necessary. See sections 1.2, 1.4, 2.1.3, 2.2.1, 2.2.3 and 3.1 for the occasions where specific problems do warrant further explanation.

7.4: The exact probe set used and number of cells studied should be included in the report.
8 Training

Many laboratories have reported that even quite experienced cytogeneticists had significant problems when first analysing interphase FISH oncology preparations. It is clear, therefore, that a proper training programme is necessary for all new FISH scorers. This should include the following:

8.1: The trainee must be made aware of basic quality issues such as that scoring should only be carried out on areas where the cells are adequately separated (for accurate signal determination) and where there is minimum background noise.

8.3: The trainee must be aware that cells are rarely two dimensional and that changing focus is usually necessary to see all relevant signals. This means that it may be impossible to capture accurate signal patterns by image enhancement unless the equipment can capture more than one focal plane.

8.4: The trainee must understand the expected probe signal patterns, including common variations. (S)he must also understand which region(s) of the chromosomes hybridise to the probe so that completely unexpected signal patterns can be interpreted correctly. (S)he must be aware of the potential for atypical fusion signals, particularly those where one component is weak and may be masked by the other colour.

8.5: The trainee must be aware that dual and triple filters reduce the intensity of the signals. Any resulting uncertainties of interpretation can often be resolved by assessing each signal with the appropriate single filter.

8.6 The trainee must note when a high proportion of cells are not scoreable, and assess whether this is due to general or specific problems which could affect the validity of the result.

8.7: The trainee must have a basic understanding of the diseases that they are examining, particularly their implications for the relevance of the material being tested.

8.8: The trainee must be able to distinguish between lobulated single nuclei and aggregations of mononuclear cells.

8.9: There should be a significant period of monitoring of new scorers until it is established that their results for all types of probes used by the laboratory are sufficiently similar to those found by more experienced scorers.
Appendix 1
Participants at the Meeting on 8th October 2003
to Determine these Recommendations

Chair
Fiona Ross, Wessex Regional Genetics Laboratory

Secretary
John Swansbury, Royal Marsden Hospital

Speakers
Denise Sheer, CRUK London Research Institute,
Christine Harrison, LRF UKCCG ALL Database
Nick Bown, Northern Genetics Service
Susan Rose, West Midlands Regional Genetics Laboratory
Roland Ventura, LRF Immunohaematology Group, Oxford
Lyndal Kearney, LRF Molecular Cytogenetics Group, ICR, London

Participants
Miriam O’Connor, Human Genetics Unit, Dundee
Norma Morrison, West of Scotland Regional Genetics Service
Eddie Maher, South East of Scotland Genetics Service
Mervyn Humphreys, Northern Ireland Regional Genetics Service
Sharon Keogh, Cytogenetics Department, National University of Ireland, Galway
Alison Dunn, Yorkshire Regional Genetics Service
Una Maye, Merseyside & Cheshire Genetics Service
Nick Telford, Oncology Cytogenetics Service, Manchester
Ann Watmore, North Trent Genetics Service
Adele Calvert, Nottingham Centre for Medical Genetics
Karen Marshall, Leicestershire Genetics Centre
Mark Crocker, Oxford Regional Genetics Service
Mark McKinley, Haematology Cytogenetics Laboratory Cardiff
Peter Thompson, Institute of Medical Genetics, Cardiff
Clare Kitchen, Regional Cytogenetics Centre, Bristol
John Pearson, Norwich Cytogenetics Service
Sarah Ryley, The Kennedy Galton Centre
Debra Lillington, Cytogenetics Laboratory, Barts
Karen Saunders, Cytogenetics, King’s College Hospital
Lucy Hill, South West Thames Regional Genetics Centre
Steve Chatters, Cytogenetics, University College Hospital
Julie Howard, Cytogenetics Section and MRD Unit, Hammersmith Hospital
Helena Kempski, Institute of Child Health
Sarah Mould, Cytogenetics, Royal Bournemouth Hospital

Additional contributions from those unable to attend
Janet Shipley, Institute of Cancer Research, Sutton
David Stevenson, North of Scotland Regional Genetics Service
Appendix 2
Examples of Specific Situations Relevant in October 2003

Example 1: Testing for AML1/ETO in FAB type M2, PML/RARα in M3, and CBFβ/MYH11 in M4Eo, should be performed in all AML cases with relevant morphology but with no cytogenetic evidence of the associated chromosome abnormality. It is considered to be the responsibility of the referring clinicians to notify the laboratory when the morphology suggests a cytogenetic aberration that was not found. If communication with clinicians is difficult, then a rider could be put on normal or failed reports indicating that further testing is available if appropriate, i.e. it is not considered essential to test all AML normal and failed cases for these abnormalities, although some labs may find it more efficient to do so.

Example 2: Identification of abnormalities of the MLL gene does not affect treatment stratification in AML at present and therefore a FISH test for MLL is not one that the laboratory should be required to perform routinely in patients with AML, although many laboratories do prefer to offer this in AML M4/M5 cases, particularly those that have failed. NB MLL is a required test for childhood and infant ALL, but this will be carried out by the LRF UKCCG ALL Database if not done by the original laboratory.

Example 3: 5q-/7q- abnormalities in AML/MDS are not likely to be cryptic so FISH tests to detect them are not necessary where an adequate cytogenetic result has been obtained. However, if a conventional cytogenetic analysis has failed then these abnormalities should always be looked for by FISH.

Example 4: Testing for BCR/ABL by FISH should be performed at diagnosis in all cases of ALL regardless of age, unless the results of the conventional cytogenetic study have proved positive. Current evidence suggests that the established rearrangements in ALL are mutually exclusive, therefore it may not be necessary to test for BCR/ABL in cases with e.g. a visible MLL rearrangement. However, this evidence is still preliminary and trial guidelines indicate that all cases should be tested for BCR/ABL.

Example 5: Testing for deletion of the der(9) at diagnosis of CGL is not required until its effect on prognosis is clarified. NB the changing understanding of this abnormality is a good illustration of the potential problems of reporting new abnormalities whose wider significance has not been clearly demonstrated.

Example 6: A FISH test for BCR/ABL rearrangement is required to distinguish between CGL and atypical CML if a cytogenetic study is normal.

Example 7: FISH is required to investigate MYCN involvement in neuroblastoma. However, if a secondary tissue such as bone marrow is supplied, FISH should not be undertaken without proof of involvement. Laboratories should also be aware that the current trial requires that MYCN results be demonstrated by two methods, both of which should be carried out in national referral centres. Unfortunately there is no current funding for a national referral centre for FISH in neuroblastoma.
Example 8: FISH studies of specific rearrangements in other paediatric tumours can provide a differential diagnosis. However, such studies should be regarded as research until more experience has been gained. Where they are attempted all normal results should be regarded with caution.

Example 9: While cytogenetic and FISH results can have an effect on choice of treatment in lymphoma, there is often difficulty in obtaining suitable material for testing. Routine requests for IgH/CCND1, IgH/BCL2 and MYC split apart on bone marrow or blood should not be processed without morphological evidence of significant involvement by malignant cells. Otherwise it cannot be determined whether a negative result is due to absence of the genetic abnormality or absence of malignant cells.

Example 10: The distinction between atypical CLL and mantle cell lymphoma by CCND1/IgH FISH is something that all labs should currently be offering as this differential diagnosis affects treatment. However, although FISH for specific CLL abnormalities can distinguish different prognostic groups, and is more effective than cytogenetics at doing this, there is not yet universal agreement about what to do with this information. Thus, this testing is not something that must be offered yet.
Appendix 3
Abbreviations Used

ALL:  Acute lymphoblastic leukaemia
AML:  Acute myeloid leukaemia
BM:   bone marrow (usually aspirate)
CGL:  Chronic granulocytic leukaemia
      (usually analogous with chronic myeloid leukaemia)
CLL:  Chronic lymphocytic leukaemia
FISH: Fluorescence *in situ* hybridisation
MDS:  Myelodysplastic syndromes
MM:   Malignant myeloma
MPD:  Myeloproliferative disorders