## Case Report

# Fluorescence *In Situ* Hybridization Method in Isolated Single Nuclei extracted from Paraffin-Embedded Hepatocellular Carcinoma Tissues

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***Background:*** *Genetic analysis using the fluorescence in situ hybridization (FISH) method applied to intact tissue sections of formalin-fixed paraffin embedded (FFPE) tissue is well known to be relatively difficult. The frequent technical problems include unsuccessful hybridization as a result of poor probe penetration, excessive probe requirement, excessive background, auto-fluorescence, and overlapping or incomplete nuclei. These problems lead to absence or insufficiency of fluorescent signals, resulting in an inaccurate analysis. Formalin-fixed paraffin embedded tissue can be analyzed either as intact tissue sections or as a suspension of disaggregated, but intact, nuclei. Intact tissue sections have the advantage of preserved tissue architecture and morphology but have the intrinsic disadvantage of poor probe penetration, overlapping or incomplete nuclei and auto-fluorescence, accordingly reducing the accuracy of fluorescent signals evaluation.*

***Objective:*** *To present the effective FISH method applied to isolated of single nuclei and the procedures for isolation of a single nuclei from formalin-fixed paraffin embedded tissues of hepatocellular carcinoma.*

***Material and Method:*** *Ten paraffin-embedded blocks of hepatocellular carcinoma tissues from the department of pathology, Ramathibodi hospital, Thailand were studied. Isolated single nuclei were extracted from 10- m sections of paraffin-embedded blocks of hepatocellular carcinoma tissue and hybridized with alpha-satellite centromeric DNA enumeration probes for chromosomes X (CEP X, spectrum green) and satellite III for chromosomes Y (CEP Y, spectrum orange). The signal of, at least, 200 interphase nuclei were counted from each specimen.*

***Results:*** *The efficacy of this method has been evaluated in 10 formalin-fixed paraffin embedded tissue of hepatocellular carcinoma. The results showed bright, planar and an easy to score signal.*

***Conclusion:*** *FISH procedure described here is particularly suitable for retrospective studies of genetic aberration applied to formalin-fixed paraffin embedded tissues.*

***Keywords:*** *Fluorescence in situ hybridization (FISH), Isolated single nuclei, Formalin-fixed paraffin embedded tissues, Hepatocellular carcinoma*

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Conventional cytogenetics is not always useful for analysis of cytogenetic aberrations, owing to difficulties in obtaining metaphases necessary for karyotyping[1-4]. Fluorescence *in situ* hybridization (FISH) is a valuable technique for detection of genetic aberration such as amplifications, deletions, or translocations in interphase or metaphase nuclei[5]. The technique is based on the hybridization of labeled probes to complementary sequences in the DNA or RNA of the cells. Interphase FISH is most often applied on cytological material but the method is also used to study genetic changes in formalin-fixed paraffin-embedded tissue. Paraffin-embedded tissue can either be analyzed as an intact section of tissue or as a suspension of disaggregated, but intact, nuclei. FISH analysis in intact section of paraffin-embedded tissue preserves tissue architecture and morphology, but interferes with the scoring of individual nuclei[6]. FISH analysis of paraffin embedded sections has been proved to be more difficult than the analysis of conventional cytogenetic preparations. Problems have included unsuccessful hybridization as a result of poor probe penetration, excessive probe requirement, excessive background, auto fluorescence, and sectioned or incomplete nuclei[7,8]. Therefore, the authors presented the effective method to isolate single nuclei from paraffin-embedded tissue of hepatocellular carcinoma which have been mapped with H&E and the FISH procedure applied to the isolated single nuclei which produced bright, planar and an easy to score signal.

## Material and Method

### *Samples*

Ten paraffin-embedded blocks of hepatocellular carcinoma tissue from the pathology department of Ramathibodi Hospital, Thailand.

### *Nuclei extraction from paraffin-embedded tissue*

Three sequential 10- m sections were cut from a paraffin-embedded block, which had been previously selected by a pathologist. The sections were mounted onto slides. One section was stained with H&E by using standard techniques. The pathologist used H&E slide for mapping another slides. The desired areas after mapping were scraped and put in 1.5 ml microcentrifuge. The paraffin was dissolved at room temperature with two changes of xylene: 1.0 ml xylene for 30 minutes and 500 l xylene for 10 minutes. The tissue was then rehydrated with 500 l of 95%, 75%, 50% ethanol and sterile water for 2 minutes each. The enzymatic digestion was then performed by adding 200 l of proteinase K solution (0.005% proteinase K, 30 U/mg protein, in 0.05 mol/L Tris hydroxymethyl aminomethane hydrochloride (pH 7.0), 0.01 mol/L ethylenediaminetetraacetic disodium salt, and 0.01 mol/L sodium chloride) to the microcentrifuge tube. The specimen was incubated at 37 C for 2 hours to aid with enzymatic digestion, the sample was vortexed frequently during this incubation period. Nuclei were pelleted by using centrifugation at 6000 rpm for 10 minutes. Proteinase K was carefully removed with a micropipetter and the nuclei washed by resuspension with vortexing in 100 l of phosphate-buffered saline (PBS). The PBS solution was removed and the nuclei fixed by resuspension with vortexing in two changes of freshly prepared fixative (three parts methanol and one part glacial acetic acid). The nuclei were resuspended in 30 l of fixative. Slides were made by dropping 8 l of nuclei suspension. Those slides were then dried in a 65 C oven for 15 minutes and kept in a slide box at -70 C until it was convenient to perform FISH studies.

### *Pretreatment and FISH*

Slides were incubated in 2x standard saline citrate (SSC) at 75 C for 20 minutes. Slides were then transferred to a Coplin jar containing 0.4% pepsin solution (0.16 g pepsin in 40 ml 0.9% pH 1.5 sodium chloride) and incubated at 37 C for 8 minutes. The slides were then dipped in water, rinsed in 2xSSC for 5 minutes and air-dried.

### *FISH and Posthybridization Wash*

The alpha-satellite centromeric DNA enumeration probes for chromosomes X (CEP X, spectrum green) and satellite III for chromosomes Y (CEP Y, spectrum orange) from Vysis, Inc., Downers Grove, IL, USA were prepared as follows: 3 l of probe solution prepared by manufacturer's recommendation was applied to the pretreated slide, covered with cover slip and sealed with rubber cement. Probe and target were co-denatured using a hot plate at 80 C for 5 minutes, followed by an overnight hybridization at 37 C in a humidified chamber. Posthybridization washing was carried out at 73 $\pm$ 1 C in 0.4XSSC/0.3%NP-40 for 2 minutes and in 2XSSC/0.1%NP-40 at room temperature for 1 minute. The slides were air-dried then counterstained with DAPI II (Vysis) and cover-slipped.

### *Microscopy*

Analysis was done using a fluorescence microscope (Nikon Labophot-2, Kawasaki, Japan) equipped with a 100-W mercury lamp. To view signals

a single pass spectrum green, single pass spectrum Orange was used. At least a total of 200 interphase nuclei were scored from each specimen.

***Statistical analysis***

To determine the cut-off levels for the detection of numerical chromosomal aberrations by using alpha-satellite centromeric DNA enumeration probes for chromosomes X (CEP X) and satellite III for chromosomes Y (CEP Y), 1,600 normal, male hepatocytes and 2,698 normal, female hepatocytes from autopsy were analyzed. According to Ward et al[9], the thresholds for gains and losses were calculated as the mean + 3 SD.

**Result**

***Determination of cut-off levels for male.***

Analysis of 1,600 normal male hepatocytes with the probe specific for X (CEP X) and Y chromosome (CEP Y) showed zero signal at 0.06%, 0.70% of the cells (SD 0.13,0.58%) and two or more signals at 2.90%, 2.44% of the cell (SD 1.20%, 0.95%). The cut-off levels (mean + 3 SD) were determined as 0.46%, 2.40% for losses and 6.50%, 5.31% for gains of chromosome X and Y respectively. The details are shown in Table 1 and Fig. 1.

***Determination of cut-off levels for female***.

Analysis of 2,698 normal female hepatocytes with the probe specific for X (CEP X) chromosome showed one signal at 11.58%(SD 5.59%) and three or more signals at 1.95% (SD 1.51%). The cut-off levels (mean + 3 SD) were determined as 28.35% for losses and 6.49 for gains. The details are shown in Table 2 and Fig. 1.

In the present study ten formalin-fixed, paraffin-embedded hepatocellular carcinoma tissue

**Table 1.** Control studies to determine the cut-off level for gains and losses of chromosome X, Y in males (n = 1600)

| | Signal/nucleus, % | | | |
|---|---|---|---|---|
| | Zero signal | | Two or more signals | |
| Probe | CEPX | CEPY | CEPX | CEPY |
| Male hepatocyte | | | | |
| Mean | 0.06 | 0.70 | 2.90 | 2.44 |
| SD | 0.13 | 0.58 | 1.20 | 0.96 |
| Cut-off level* | 0.46 | 2.46 | 6.50 | 5.31 |

* Mean + 3 SD

**Table 2.** Control studies to determine the cut-off level for gains and losses of chromosome X in females (n = 2698)

| | Signal/nucleus, % | |
|---|---|---|
| | One signal | Three or more signals |
| Probe | CEPX | CEPX |
| Female hepatocyte | | |
| Mean | 11.58 | 1.95 |
| SD | 5.59 | 1.51 |
| Cut-off level* | 28.35 | 6.49 |

* Mean + 3 SD

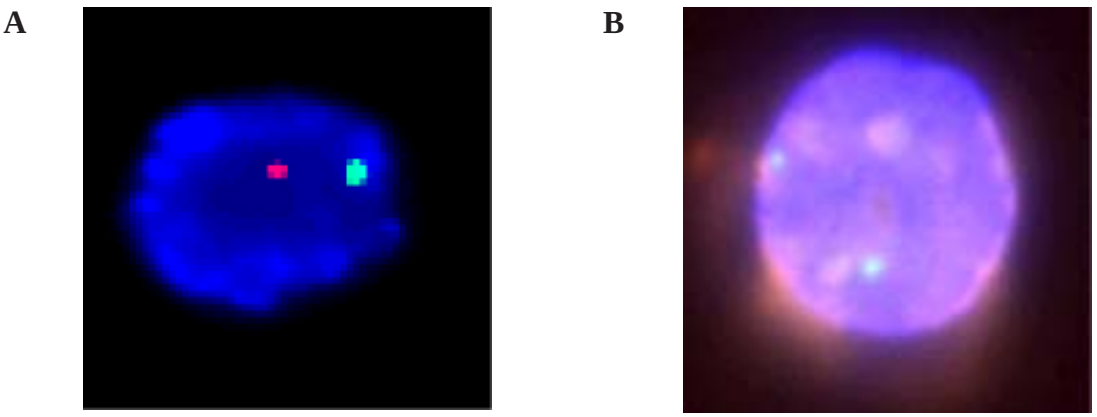


**Fig. 1** FISH analysis of single nuclei isolated from formalin fixed, paraffin embedded tissue using CEP X (green), CEP Y (orange) probe. (A) normal male hepatocyte from autopsy. (B) normal female hepatocyte from autopsy

**Table 3.** Percentage of hybridization signals of each isolated nuclei from paraffin-embedded HCC tissue with CEPX and CEPY probe

(%) of cells with the indicated number

| No. | Case No. | Surgical No. | Sex | Patho Dx. | Probe | No. of nuclei | Number of hybridization signals | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 0 | 1 | 2 | 3 | 4+ |
| 1. | 42 | S013436C2 | M | HCC | CEP X | 204 | | 94.1 | 5.4 | 0.5 | |
| | | | | | CEP Y | 204 | | 96.1 | 3.9 | | |
| 2. | 28 | S0113900D | F | HCC | CEP X | 201 | | 2.5 | 72.1 | 7.0 | 18.4 |
| | | | | | CEP Y | 201 | 100 | | | | |
| 3. | 38 | S0210547E2 | M | HCC | CEP X | 275 | | 48.0 | 50.2 | 0.7 | 1.1 |
| | | | | | CEP Y | 275 | | 97.8 | 2.2 | | |
| 4. | 40 | S0211064A2 | M | HCC | CEP X | 216 | | 72.7 | 26.9 | 0.5 | |
| | | | | | CEP Y | 216 | | 95.8 | 4.2 | | |
| 5. | 26 | S0316791A | M | HCC | CEP X | 276 | | 89.9 | 8.3 | 1.1 | 0.8 |
| | | | | | CEP Y | 276 | | 94.9 | 5.1 | | |
| 6. | 24 | S035737A | M | HCC | CEP X | 249 | | 77.9 | 18.9 | 2.0 | 1.2 |
| | | | | | CEP Y | 249 | 11.6 | 85.9 | 1.6 | 0.4 | 0.4 |
| 7. | 86 | S0412878E | M | HCC | CEP X | 206 | | 52.9 | 42.2 | 2.4 | 2.4 |
| | | | | | CEP Y | 206 | 58.3 | 41.3 | 0.5 | | |
| 8. | 90 | S046833C | M | HCC | CEP X | 204 | | 37.3 | 61.3 | 1.5 | |
| | | | | | CEP Y | 204 | | 35.3 | 63.7 | 1.0 | |
| 9. | 96 | S059926A | F | HCC | CEP X | 224 | | 12.1 | 83.9 | 2.7 | 1.3 |
| | | | | | CEP Y | 224 | 100 | | | | |
| 10. | 4 | S053410C | M | HCC | CEP X | 248 | | 98.4 | 1.6 | | |
| | | | | | CEP Y | 248 | 0.4 | 98.8 | 0.8 | | |

The counts found abnormal are printed in bold type

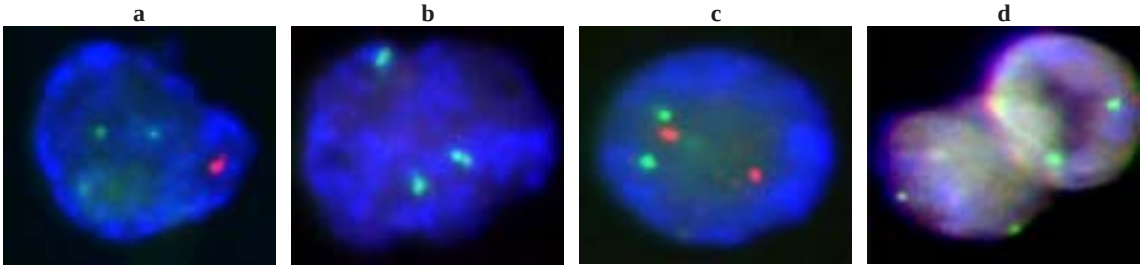


**Fig. 2** Representative nuclei extracted from paraffin-embedded HCC tissues
(a) Gain of chromosome X with normal chromosome Y on case No. 38
(b) Gain of chromosome X in female on case No. 28
(c) Gain of chromosome X and Y on case No. 90
(d) Gain of chromosome X but chromosome Y deletion on case No. 86

from 2001 to 2005 were selected. Single nuclei were successfully extracted from each with the present method. FISH method has been successfully evaluated in all specimens as shown in Table 3 and Fig. 2. In three cases (30%), the result showed normal chromosome X and Y. Gain of chromosome X was found in seven cases (70%): four cases with normal chromosome Y, two cases with loss of chromosome Y, and one case with gain of chromosome Y.

## Discussion

FISH uses fluorescently labeled probes for the visualization of DNA sequence on metaphase spreads or interphase nuclei. In the present study the authors demonstrated the effective method to prepare single nuclei from formalin fixed paraffin embedded

HCC tissues and the FISH procedure which produced a bright, planar and easy to score signal. The present method was evaluated in 10 formalin-fixed paraffin embedded HCC tissues. The authors found that isolated single nuclei can be obtained after two hours digestion. In the pre-treatments step, the digestion time can be varied with the type of tissue to be digested. The results of the present study suggested that the FISH procedure described here can detect chromosomal abnormality, particularly suitable for retrospective studies of archived tumor specimens.

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## *เทคนิค ฟลูออเรสเซนซ์ อิน ซิตู ไฮบริไดเซชัน (Fluorescence in situ hybridization) ในเซลล์เดี่ยวที่สกัดจากเนื้อเยื่อมะเร็งตับซึ่งฝังอยู่ในพาราฟิน*

***อัญชลี ตันติเวทเรืองเดช, เรวัต พันธุ์วิเชียร, พัฒนา ศรมยุรา, โกเศศ ปิ่นประดับ, สุรศักด์ ลีลาอุดมลิปี***

***ภูมิหลัง:*** *เป็นที่ทราบกันดีว่า การวิเคราะห์โดยเทคนิค ฟลูออเรสเซนซ์ อิน ซิตู ไฮบริไดเซชัน ในเนื้อเยื่อที่ฝังอยู่ในพาราฟินนั้นค่อนข้างยาก ปัญหาทางเทคนิคอาจเนื่องมาจากตัวติดตาม ไม่สามารถเข้าไปในเซลล์ได้ หรือ ต้องใช้ตัวติดตามมากกว่าปกติ หรือ ขยะส่วนที่อยู่รอบ ๆ มากไป และในบางครั้งเนื้อเยื่ออาจจะเรืองแสงได้ด้วยตัวเอง และมีนิวเคลียสที่ไม่สมบูรณ์ จึงทำให้ไม่เห็นสัญญาณฟลูออเรสเซนซ์ หรือ ไม่สามารถนับสัญญาณฟลูออเรสเซนซ์ได้ถูกต้องแม่นยำ การวิเคราะห์โดยเทคนิค ฟลูออเรสเซนซ์ อิน ซิตู ไฮบริไดเซชัน ในเนื้อเยื่อที่ฝังอยู่ในพาราฟินนั้นสามารถวิเคราะห์ได้ทั้งในชิ้นเนื้อแผ่นบาง ๆ และในเซลล์เดี่ยว โดยที่การวิเคราะห์ในชิ้นเนื้อแผ่นบาง ๆ นั้นมีข้อดีที่ยังคงรักษาโครงสร้างและรูปร่างของเนื้อเยื่อไว้ แต่มักมีปัญหาในขั้นตอนการนับสัญญาณฟลูออเรสเซนซ์ เนื่องจากนิวเคลียสซ้อนกันและเนื้อเยื่ออาจจะเรืองแสงได้ด้วยตัวเอง ทำให้ความแม่นยำในการนับสัญญาณฟลูออเรสเซนซ์น้อยลง*

***วุตถุประสงค์:*** *เพื่อหลีกเลี่ยงปัญหาดังกล่าวในงานวิจัยครั้งนี้จึงได้เสนอวิธีการเตรียมเซลล์เดี่ยวจากเนื้อเยื่อมะเร็งตับที่ฝังอยู่ในพาราฟินและ ขั้นตอนในการวิเคราะห์ด้วยเทคนิค ฟลูออเรสเซนซ์ อิน ซิตู ไฮบริไดเซชัน ในเซลล์เดี่ยว*

***วัสดุและวิธีการ:*** *ผู้วิจัยใช้ขั้นตอนในการวิเคราะห์ดังที่เสนอไว้ในที่นี้ ในเนื้อเยื่อ 10 ราย*

***ผลการศึกษา:*** *พบสัญญาณฟลูออเรสเซนซ์ที่ชัดเจน ทำให้การนับสัญญาณฟลูออเรสเซนซ์ถูกต้องแม่นยำ นับว่ามีประโยชน์อย่างมากสำหรับการศึกษาวิจัยย้อนหลัง (retrospective studies)*

***สรุป:*** *เทคนิค ฟลูออเรสเซนซ์ อิน ซิตู ไฮบริไดเซชัน สามารถใช้ในการวิเคราะห์หาความผิดปกติทางด้านพันธุกรรมในเนื้อเยื่อที่ฝังอยู่ในพาราฟินได้*