

ORIGINAL ARTICLE

Complementary value of DNA flow cytometry and image morphometry in detection of malignant cells in effusion fluids

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Abstract

Background: In cytologic evaluation of body cavity effusions, the morphologic changes exhibited by reactive mesothelial cells often confound the diagnosis. This study investigates the role of DNA flow cytometry (DNA FCM) and image morphometry (IM) in improving diagnostic accuracy. **Methods:** 53 pleural and 47 ascitic fluid samples were evaluated cytologically. All were also subjected to flow cytometry to assess DNA ploidy. Image morphometry was used to measure nuclear diameter, nuclear perimeter and nuclear area. **Results:** On cytomorphology 79% cases were diagnosed as benign, 19% as malignant and 2% as suggestive of malignancy. DNA FCM showed aneuploidy in 13 of 19 malignant cases and diploidy in 6 cases. The mean nuclear area of the benign group was $60.14 \pm 39.91 \mu\text{m}^2$ and that of malignant cases was $190.54 \pm 56.06 \mu\text{m}^2$. Using DNA FCM and IM, one of the two cases “suggestive of malignancy” was placed in the benign group and the other in the malignant group. Also, these modalities were able to pick up one case of malignancy that was diagnosed as benign on cytology. **Conclusions:** Cytomorphology remains the foremost diagnostic modality in detecting malignant cells in effusions. DNA flow cytometry and image morphometry hold a valuable complementary value.

Keywords: effusion, cytomorphology, DNA ploidy, image morphometry

INTRODUCTION

Cytologic evaluation of body cavity fluids is routinely performed to detect malignancy. However, a definitive diagnosis cannot always be made on cytologic evaluation alone. The differentiation between hyperplastic mesothelial cells, neoplastic mesothelial cells and metastatic adenocarcinoma is often impossible due to their cytologic similarities.¹ Effusion can be the first indication of a malignancy. In treated cancer patients or in patients undergoing treatment, effusion often represents the first manifestation of recurrent disease. Identification of malignant cells in effusion samples, therefore, becomes significant from the therapeutic as well as prognostic points of view. Apart from the misinterpretation of mesothelial cells as malignant cells, substantial false-negative rates exist for adenocarcinoma cells in effusion samples. Thus, there is a need for complementary methods to identify tumour cells within body cavity fluids.² This study is an attempt to determine the value of two such

techniques namely DNA flow cytometry (DNA FCM) and image morphometry (IM) for the diagnosis of malignant cells in fluids.

MATERIALS AND METHODS

The effusions of 100 patients, irrespective of their age, sex, site of disease and treatment modality were included in the study. Pyogenic effusions, fluids from cystic lesions and specimens containing blood clots were excluded from the study. The samples which were slightly haemorrhagic were processed as usual for cytology, IM and DNA FCM. All the specimens were received fresh, without anticoagulant or fixative, in the Cytopathology laboratory. The fluid samples were divided into two parts; one part was processed for making slides for cytology and IM, and the other part for DNA FCM.

Cytologic examination

Air dried smears were stained with May Grünwald Giemsa (MGG) and alcohol fixed

smears with hematoxylin and eosin (H&E) or Papanicolaou (Pap). Special cytochemical stains like mucicarmine were performed wherever required. The cytological examination was performed for identification and, wherever possible, subclassification of tumour.

Flow cytometric analysis (FCM)

The specimen was run on multiparameter four colour flow cytometer (FACSCalibur: Becton, Dickinson & Co.; San Jose, CA). CycleTEST™ PLUS DNA reagent kit was used to treat the cell pellet. The analysis was performed using CellQuest Pro software based on forward scatter and side scatter. To analyse the list mode data and calculate the DNA index, software program (ModFit LT Verity Software House, Inc.; Topsham, ME), was used. The ploidy peaks, fraction of cells in respective phases of the cell cycle and the coefficient of variation for aneuploid cases were noted.

Image morphometry (IM)

Image-Pro® Plus version 6.1 designed to run under the Microsoft® Windows 2000 or XP Professional operating system was used. For each case, MGG stained smears that contained well dispersed cell population were selected. Photomicrographs of the areas of interest were taken using DP20-DRV Version, Olympus Corporation at 40X magnification. These areas included fields with good cellularity, showing well dispersed cells or cell clusters with discernible cell morphology. Nuclear diameter, nuclear perimeter, and nuclear area of 100 cells in each case were measured. The data thus collected was transferred to Microsoft excel sheet.

RESULTS

The age of patients ranged from 14 to 89 years, with mean age of 48.76 ± 17.43 years. There were a total of 49 males and 51 females. The male to female ratio was 1:1.08. The 100 samples examined included 53 pleural fluids and 47 ascitic fluids.

Cytologic examination

On cytologic examination, the cases were categorised as benign, malignant and suggestive of malignancy. Seventy-nine cases (45 pleural and 34 ascitic) were diagnosed as benign, 19 cases (8 pleural and 11 ascitic) as malignant and 2 cases (ascitic) were suggestive of malignancy. The sensitivity and specificity of cytomorphology were 95.04% and 98.75%, respectively.

In the present study, the amount of effusion fluid or the amount of tumour cells in the effusions did not make any difference to the tumour cell positivity and the results. However, the exact quantification of the tumour cell burden was not done.

Flow cytometric analysis (FCM)

All 100 effusion samples were run on flow cytometer to assess DNA ploidy. Eighty-six cases showed diploid DNA histograms and 14 cases showed aneuploid DNA histograms.

Out of 79 benign cases on cytological examination, 78 (98.75%) were diploid and 1 (1.25%) was aneuploid. The one aneuploid case in the benign group was a 35-year-old male who was suspected to have hepatocellular carcinoma. The abdominal ultrasound of this patient showed large echogenic masses in both lobes of the liver and the serum alpha fetoprotein was elevated (558 ng/ml). The morphometric values of this case were in the malignant range. Thus, both DNA FCM and IM strongly supported the clinical data suggesting malignancy. The patient succumbed to his illness before confirmatory investigations could be performed.

Amongst the 19 malignant cases diagnosed on cytology, 13 (65.0%) were aneuploid and 6 (35.0%) were diploid. DNA indices of the benign cases had a mean of 1.01 ± 0.1 whereas that of malignant cases was 1.52 ± 0.6 . The co-efficient of variation of all the DNA peaks in this study was less than 5%. The sensitivity and specificity of DNA FCM were derived as 68.0% and 99.0%, respectively.

There were two cases diagnosed as suggestive of malignancy on cytology. One case showed aneuploidy on FCM and morphometric values in the malignant range. This 60-year-old female patient was diagnosed with gallbladder adenocarcinoma on histopathological examination. The second case showed exuberant reactive mesothelial cell proliferation, leading to the misdiagnosis (Fig.1a). DNA FCM showed diploid peak and the morphometric values were in the benign range (Figs. 1b, 1c). Repeat sampling of ascitic fluid was benign on cytology, the same being supported by DNA FCM and IM. The final diagnosis in this case was rendered as benign and the patient is healthy till date.

Image morphometry (IM)

Morphometry was performed on 100 cell nuclei in each of the 100 cases. The mean nuclear area, mean nuclear perimeter and mean nuclear

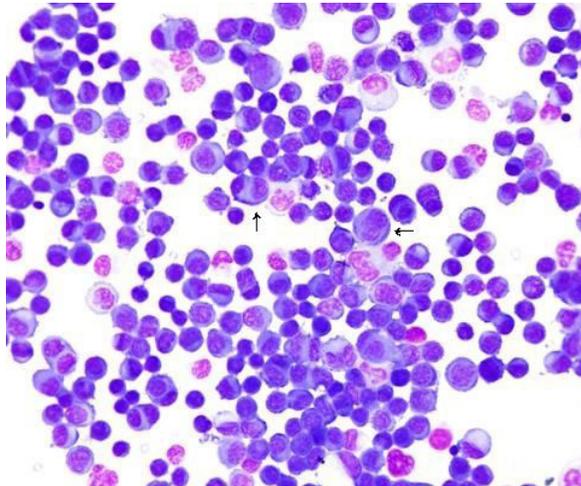


FIG.1a: Smear from ascitic fluid shows marked proliferation of reactive mesothelial cells. Few cells show bi- and multi-nucleation (arrows) (MGG, x200).

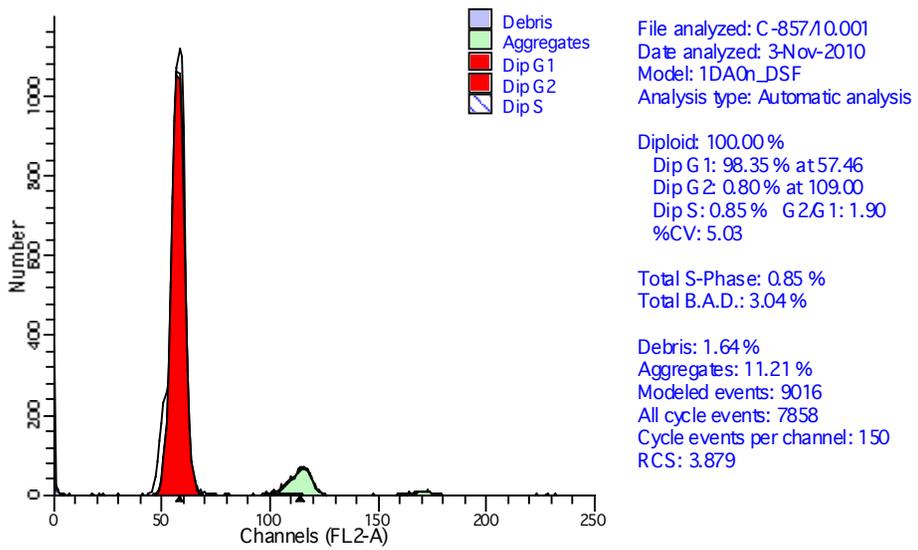


FIG.1b: DNA FCM histogram shows diploid peak.

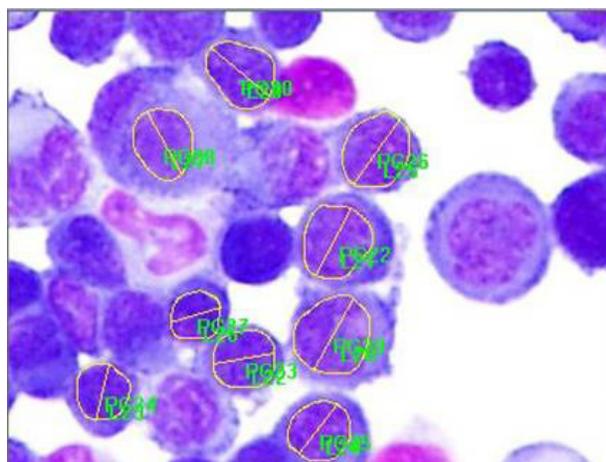


FIG.1c: Zoomed-in image of selected mesothelial cell nuclei for morphometric analysis (MGG).

TABLE 1: Morphometry of cytologically benign versus malignant effusions

Feature	Benign (n=80)	Malignant (n=20)	Significance
Mean nuclear area (μm^2)	60.14±39.91	190.54±56.06	p = <0.001
Mean nuclear perimeter (μm)	31.46 ±14.48	57.72±7.16	p = <0.05
Mean nuclear diameter (μm)	9.51±3.94	18.67±2.84	p = <0.05

diameter were evaluated (Table 1). Using regression analysis, out of the three variables, mean nuclear area (p = <0.001) was found to be the most accurate measure to contrast benign versus malignant cells.

In the cytologically benign and diploid group comprising 78 cases, there were 14 cases with morphometric values out of the morphometric benign range, two of them actually falling in the morphometric malignant range. On reviewing, the two cases with malignant range on morphometry comprised reactive mesothelial

cells with marked mesothelial cell proliferation on cytological examination. These two cases did not show any evidence of malignancy on follow-up.

There were 6 cases in the cytologically malignant but FCM diploid group. The morphometric values of all these cases were in the malignant range. These included 4 cases of metastatic adenocarcinoma, one case of mixed germ cell tumour of the ovary and one case of anaplastic carcinoma (Figs. 2a, 2b). Three of the metastatic adenocarcinoma cases, i.e., 2 cases of

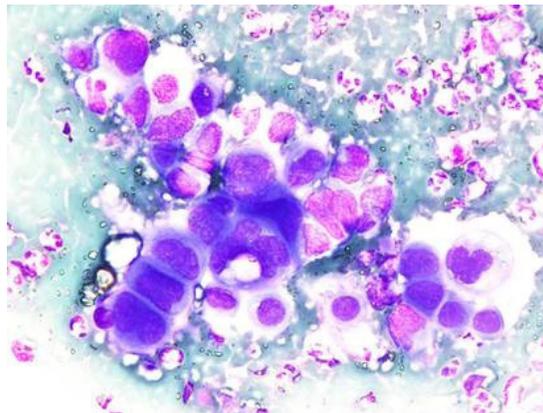


FIG.2a: Smear from pleural fluid in a case of anaplastic carcinoma showing pleomorphic tumour cells in a hemorrhagic background (MGG x400).

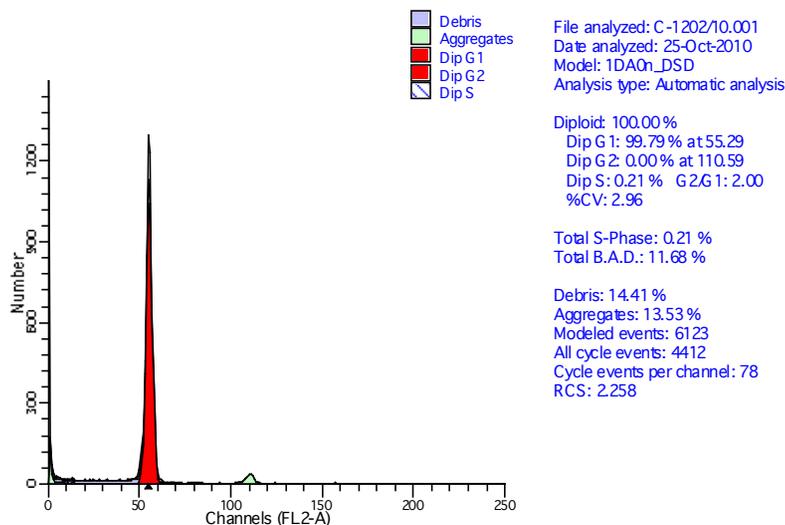


FIG.2b: DNA FCM histogram shows a diploid peak

ovarian carcinoma and one case of gallbladder carcinoma, were on chemotherapy.

The cytologically malignant and FCM aneuploid group had 13 cases. Morphometric values were in malignant range in 12 cases. One case had morphometric values in the benign range. On reviewing the cytology slides of this case, the predominant malignant cell population comprised small-sized tumour cells with coarse chromatin and small 2-3 nucleoli in some (Fig.3a). DNA FCM showed 3 aneuploid peaks (Fig.3b). The small size of tumour cells could have resulted in a lower mean value on morphometry.

Follow-up of all the 19 patients diagnosed

as malignant on cytomorphology was available. Histopathology was available in 11 cases and is summarised as follows: 2 cases of papillary adenocarcinoma ovary, 2 cases of infiltrating duct carcinoma breast, one case each of mixed germ cell tumour of the ovary, metastatic adenocarcinoma to bilateral ovaries, uterine adenocarcinoma, adenocarcinoma gallbladder, signet cell adenocarcinoma of the rectum, adenocarcinoma lung and adenocarcinoma colon. One case showed adnexal mass on ultrasonography and had increased CA 125 levels (1428 µg/ml). One case was diagnosed as adenocarcinoma lung on trans-bronchial needle aspirate. One case showed squamous cell

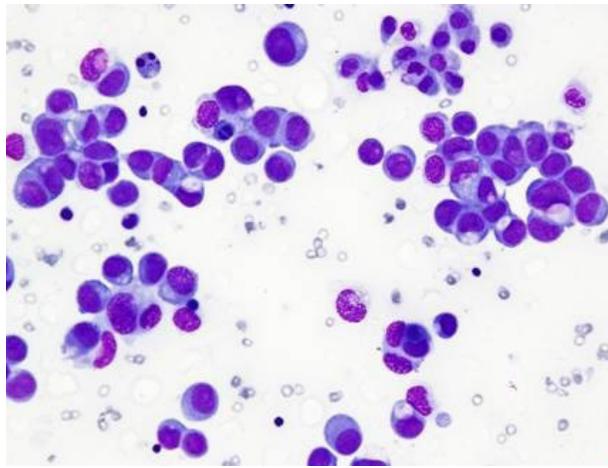


FIG.3a: Smear from metastatic adenocarcinoma in ascitic fluid shows small clusters and singly scattered tumour cells with large, vesicular nuclei and vacuolated cytoplasm (MGG, x400).

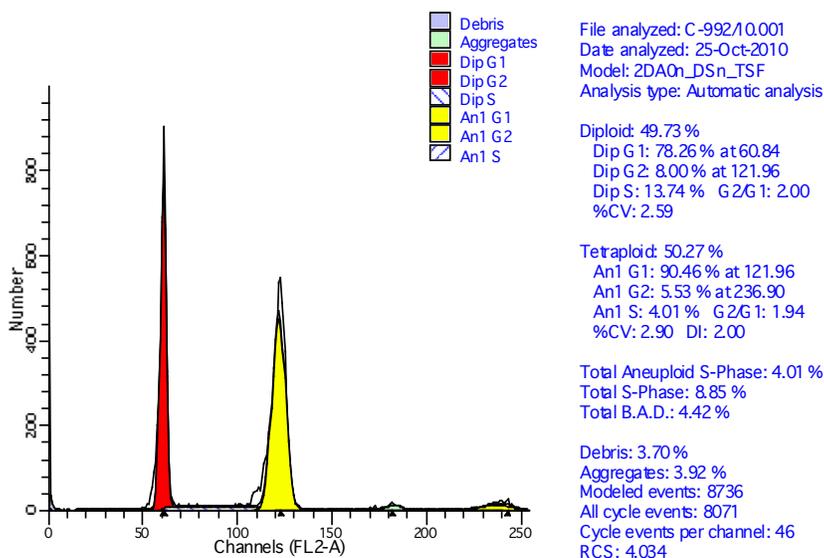


FIG.3b: DNA FCM histogram showing tetraploid peak.

carcinoma on bronchial brushings. Four patients with metastatic adenocarcinoma and one patient with anaplastic carcinoma, in whom primary sites of malignancy could not be determined, expired during hospital stay.

The sensitivity, specificity, diagnostic accuracy and positive and negative predictive values for the three diagnostic modalities are shown in Table 2. All these parameters had higher value for cytology when compared with DNA FCM or morphometry. In comparison to DNA FCM, the morphometric mean nuclear variables had higher sensitivity (97.72%, 94.25% and 97.67% for mean nuclear area, perimeter and diameter, respectively) than DNA FCM (sensitivity of 68.0%). In contrast, the specificity of the same morphometric variables was considerably lower (8.33%, 7.69% and 7.14%, respectively) compared to DNA FCM (99.0%). Scatter plots of dispersion of nuclear areas around the mean value for benign and malignant cases, respectively, are shown. This indicates that though morphometry is superior to DNA FCM in identifying true positive cases, it lags behind in detecting true negative cases.

DISCUSSION

In the current study, cytomorphology of effusions gave the first indication of malignancy in 12 out of 19 malignant cases. The sensitivity and specificity of cytomorphology alone was 95.04% and 98.75% respectively. The sensitivity of cytomorphology in other studies has been reported to be in the range of 43%-81%.³⁻⁹

DNA flow cytometry showed aneuploidy in 14% cases. DNA histogram was interpreted as aneuploid if there was a distinct DNA aneuploid peak, i.e., any peak other than the G_1 ($2n$) and G_2M ($4n$) phase peaks, DNA index (DI) >1.0 and coefficient of variation of the peak(s) $<5\%$.

There was one cytologically benign case with aneuploid DNA peak. It was false negative for

malignancy on cytology and was later confirmed to be malignant. The malignant tumours with diploid DNA histograms in this study were three cases of ovarian carcinoma, one case each of gallbladder carcinoma, bilateral ovarian metastasis and anaplastic carcinoma. Two cases of ovarian carcinoma and one case of gallbladder carcinoma were receiving chemotherapy at the time of ascitic fluid evaluation. The effect of chemotherapy on DNA ploidy of tumours has been investigated in some solid tumours like gastric and colorectal carcinomas.¹⁰ DNA ploidy pattern in gallbladder carcinoma examined by flow cytometry in a study, showed DNA aneuploidy in 46.3% cases and diploid pattern in 53.7% cases.¹¹ The gallbladder carcinoma in this study also showed diploid pattern.

Diploidy in malignant cases may be due to the primary tumour being diploid or minor chromosomal aberrations (1-3%) missed by DNA FCM.¹² Also, a small aneuploid peak formed by a small number of malignant cells may be overshadowed by a large number of diploid reactive mesothelial cells. Considerable admixtures of benign mesothelial cells with scanty malignant cells may create a problem in getting a prominent aneuploid peak.¹³

Benign cases with aneuploid DNA histograms and malignant cases with diploid DNA histograms have been reported by other studies as well.¹⁴⁻¹⁸

It is noteworthy that 25% of all tumours are diploid.¹⁴ The diploid tumours are distinguished with difficulty from the non-tumourous diploid cells in the G_0/G_1 phase. The distinction depends on the number of malignant cells and the coefficient of variation of the peak. Some examples of known diploid tumours are breast cancer, lymphomas, certain lung tumours, most small blue-cell tumours and malignant mesotheliomas.¹⁹⁻²¹

Conflicting reports exist in the literature about DNA FCM improving the sensitivity and

TABLE 2: Measures of test quality of the three diagnostic modalities

Diagnostic test		Sensitivity	Specificity	PPV	NPV	Diagnostic accuracy
Cytology		95.04%	98.75%	95.04%	98.75%	98%
DNA FCM		68%	99%	93.0%	93.0%	91%
Morphometry	MNA ^a	97.72%	8.33%	88.65%	33.34%	87%
	MNP ^b	94.25%	7.69%	87.23%	16.67%	83%
	MND ^c	97.67%	7.14%	84.65%	33.34%	85%

a-mean nuclear area (MNA); b- mean nuclear perimeter (MNP); c- mean nuclear diameter (MND)

specificity of conventional cytology. One study concluded that when DNA FCM is combined with cytology to detect malignant cells in effusions, the sensitivity improved to 100% from 83% but the specificity reduced to 94% from 100%.² Similarly, in another study, sensitivity increased from 57.1% to 71.4% but there was no improvement in specificity that remained at 93.5%.²²

The mean values of the nuclear variables in the present study were comparable with other studies.²³⁻²⁶ The p value in this study for the mean nuclear variables was <0.001 (highly significant) while it was <0.05 (significant) in a study by Scott *et al.*²⁶ The sensitivity and specificity of mean nuclear area, mean nuclear perimeter and mean nuclear diameter were 97.72% and 8.33%, 94.25% and 7.69% and 97.67% and 7.14%, respectively. In another study, the sensitivity and specificity of mean nuclear area were 16% and 100%, respectively, while the sensitivity of mean nuclear diameter was 14% and specificity was 100%.²⁶ This result is in sharp contrast to our study, where the sensitivity was higher and specificity significantly lower. The explanation may lie in the fact that greater number of benign cases showed mean nuclear variable values out of the benign range in the present study. This indicates that image morphometry can detect true positive cases but its efficiency in detecting true negative cases is low. Also, morphometric analysis is prone to systematic and statistical errors.²⁷

Some problems were faced while using semi-automated image morphometric analysis. There was nuclear overlapping in many malignant cell clusters making tracing difficult. Hypocellular smears required time-consuming scrutiny to locate cells of interest. In a few cases, debris and degenerated cells obscured the cells of interest. Though there are significant differences between morphometric variables in benign and malignant effusions, these are still insufficient to separate reliably benign and malignant cases in the overlap zones for each variable.

Several other ancillary techniques are employed to detect malignant cells in effusion fluids. These include immunocytochemistry, electron microscopy, polymerase chain reaction, Argyrophilic nucleolar organiser region (AgNOR), and fluorescent *in situ* hybridisation.^{13,28} Application of immune markers is aided by preparation of cell blocks. Cell blocks also provide higher cellular yield and better architectural patterns.^{29,30}

Conclusion

The diagnostic accuracy of cytomorphology was highest (98%) followed by DNA FCM (91%), mean nuclear area (87%), mean nuclear diameter (85%) and mean nuclear perimeter (83%). DNA FCM and IM proved to be helpful in cases that were missed or had suspicious diagnosis on cytomorphology. However, it is ascertained that cytomorphology remains the foremost diagnostic modality, while DNA FCM and IM hold a complementary value only.

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