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Early Lung Cancer Detection Project

Evaluation of 5,10,15,20 tetrakis (4-carboxyphenyl) porphine (H_2TCPP)
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DISCLAIMER

I. Abstract:

We evaluated a synthetic porphyrin, 5,10,15,20 tetrakis (4-carboxyphenyl) porphene (H_2TCPP) as a marker of carcinogenesis. H_2TCPP was compared with two other carcinogenesis markers evaluated in our laboratory for their ability to detect exfoliated sputum cells undergoing transformation to lung cancer.

Solid tumors arise through a process (carcinogenesis) which depends on a series of genetic changes, often point mutations, which activate protooncogenes and inactivate tumor suppressor genes resulting in the expression of peptide gene products. We have evaluated the detection of these gene products as pulmonary carcinogenesis markers from exfoliated airways epithelial cells. The critical step of marker validation is accomplished through linking of marker expression on preclinical sputum epithelial cells to those on subsequent lung tumor specimens. Specimen banks of prospectively collected exfoliated airways epithelial cells and subsequent tumor have been developed (or continue in progress) during our longitudinal studies. For each of these banks, an associated prospective database includes demographic information, potential carcinogen exposure and other risk factors. The ability provided by these specimen banks to directly link marker expression on premalignant exfoliated cells with that on subsequent tumor now permits the rational selection among the many potential new markers of carcinogenesis.

We had previously reported successful recognition of two immunoperoxidase-labeled monoclonal antibodies (Mabs) which have detected tumor-associated antigens expressed on sputum epithelial cells two years in advance of clinical lung cancer (sensitivity 91%, specificity 88%) from participants in the NCI-collaborative JHLP[1]. Two monoclonal antibodies originally developed against small cell and non-small cell lung cancer[2,3], were applied to archived specimens from the NCI-Johns Hopkins study (the JHLP)[4] and showed that in subjects with moderate atypical metaplasia, the uptake these antibodies could be quantified by enhanced video microscopy[5] and predict the later development of lung cancer at least two years prior to clinical recognition[1]. In related collaborative research, we have shown that a novel PCR-based technique has detected identical *k-ras* and *p53* gene mutations in both preclinical sputum specimens collected 1 to 13 months prior to diagnosis and in the patients' subsequently resected lung tumor[6].

In the present project we first established optimal conditions for cultured neoplastic and non-neoplastic (sputum) cells to take up H_2TCPP. This was accomplished using spectrofluorimetry and video-enhanced fluorescent microscopy to maximize H_2TCPP auto-fluorescence across a matrix of substrate conditions, including; reagent concentration, incubation time, temperature, and pH. The second aim was to validate H_2TCPP on clinical material obtained from subjects monitored in advance of clinical cancer and link those marker results with subsequent histologic confirmation of disease. This was accomplished by applying H_2TCPP to sputum specimens archived by the Frost Center at Johns Hopkins which maintains a record of the clinical course and long-term follow-up for the patients from whom the specimens were obtained.

We have used fluorescent immunostaining and flow cytometry to compare uptake of these cytoplasmic Mabs to that of a potential new marker of carcinogenesis, 5, 10, 15, 20 tetrakis (4 carboxyphenyl) porphene (H_2TCPP). The nuclear uptake of H_2TCPP was compared to a standard quantitative fluorescent DNA marker (7-AAD). Sample preparation for flow cytometry, has required identification, enrichment and separation of exfoliated epithelial cells undergoing carcinogenesis from the heterogenous cellular and mucoid sputum. We have met this formidable challenge and have reported a technique for mucus sulfhydryl bond disruption with dithiothreitol and microfiltration which successfully separates cellular elements from mucus glycoprotein. Nevertheless, our results suggest no advantage of H_2TCPP over existing nuclear (7-AAD) or cytoplasmic (703D4) Mabs for the early detection of pulmonary epithelial cells undergoing carcinogenesis.

II. Objectives, Significance and Previous Work

A. Objective

We have evaluated a synthetic porphyrin, 5,10,15,20 tetrakis (4-carboxyphenyl) porphene (H_2TCPP) as a marker of carcinogenesis. After establishment of the optimal conditions under which neoplastic and non-neoplastic cells take up H_2TCPP, we assessed the accuracy of the porphyrin as a biomarker of carcinogenesis. We determined that H_2TCPP did not fulfill its potential as a highly sensitive detector of carcinogenesis when compared to other carcinogenesis markers evaluated in our laboratory.

B. Rationale

No current techniques employing screening radiography, bronchioalveolar cytology, or direct biopsies have proven adequate for lung cancer detection at a curable stage[7,8]. Enhanced understanding of tumor biology has turned attention to markers of the preclinical process of carcinogenesis[9]. A focus on carcinogenesis shifts emphasis away from "early" detection of bulk clinical malignancy, which for many epithelial organs (especially lung, breast and colon) is often metastatic (incurable) at the time of diagnosis, toward detection of individual cellular markers of the pre-malignant process. New evidence indicates that sampling the epithelium at risk is the appropriate strategy for the detection of solid tissue (epithelial) carcinogenesis in colon/rectum and lung. While no blood (serum or plasma) markers have shown promise as markers of early (pre-invasive) solid tissue tumors[10], markers of carcinogenesis have been recognized in stool[11] and in sputum cells[6] desquamated from the epithelia at risk. Adding to our previously described immunohistochemical technique for detection of oncofetal (differentiation) and tumor-associated gene products, our collaborators recently have developed a PCR based technique which can identify one cell carrying a mutant gene among a large excess (greater than 10,000) of normal cells[12]. Using this technique *k-ras* mutations have been

detected in DNA purified from the stool of individuals with benign and malignant colorectal tumors prior to resection[11], as well as in sputum samples collected prior to clinical malignancy[6].

While detection of mutations may be the most specific class of carcinogenesis markers, the large number of potential mutations and the exacting technique required by these assays makes detection of individual mutations a poor choice for initial carcinogenesis screening. Similarly, the preparation and interpretion of immunocytological slides is intermediate in sensitivity, specificity and technical requirement. A preliminary sorting of cells by a sufficiently sensitive fluorescing porphyrin has the potential to rapidly identify those sputum specimens which should be evaluated by the more specific assays.

Neoplastic tissue has been detected by its increased fluorescence compared with surrounding normal tissue after systemic injection of the tumor-localizing porphyrin, porfimer sodium (Photofrin; Quadra Logic Technologies, Vancouver, BC, Canada), in murine[13] and hamster[14] models and in human cancers of the colon[15], bladder[16], and lung[17]. Frankly malignant cells have been detected in the sputum specimens from lung cancer patients following hematoporphyrin derivative injection[18]. Pre-malignant (moderate metaplasia and severe squamous dysplasia) changes in cervix[19] and in lung[20] also exhibit porphyrin fluorescence. In modeling the transformation from normal to malignant mucosa in the hamster cheek pouch (following exposure to 0.5% DMBA in acetone), Crean et al. injected the DMBA-exposed hamsters with 1.0 mg/kg of porfimer sodium at various stages of tumor development[14]. These investigators found a progressive increase in porfimer sodium uptake and fluorescence with progressive mucosal transformation as indicated by tyrosine kinase activity even prior to morphologic alteration. Fluorescence and tyrosine kinase activity showed linear increases in intensity as mucosal change progressed through dysplasia to frank malignancy. Antagonism of cancer promotion (application of bombesin antagonist or somatostatin analogue) resulted in mucosal improvement and decrease in fluorescence[21].

Yet, while the *in-vivo* association of malignant transformation and porphyrin fluorescence has been described, no one has yet reported porphyrin fluorescence in exfoliated epithelial cells prior to the detection of frank malignancy. A sensitive, easily applied marker of carcinogenesis would be of great importance to screening exfoliated cells for epithelial cancers of all types, potentially including lung, bladder and cervix.

C. Previous Work: Tissue Banking

Three specimen banks of prospectively collected exfoliated airways epithelial cells and subsequent tumor have been developed (or continue in progress) during longitudinal studies conducted by Dr. Tockman at Johns Hopkins. For each of these specimen banks, an associated prospective database includes demographic information, potential carcinogen exposure and other risk factors. Only by using these banks to directly link marker expression on premalignant exfoliated cells with marker expression

on subsequent tumor[1,6] are we now able to rationally select markers of carcinogenesis for validation from the myriad potential markers.

The importance of banking the carefully obtained serial pre-malignant specimens from a high risk population along with specimens of the subsequent tumors cannot be overstated. The genetic instability of cells undergoing malignant transformation leads to a plethora of mutational events. Those events which arise early in carcinogenesis and are preserved in the final tumor have the greatest potential as early detection biomarkers. If only the tumor were available to provide mutational clues, arbitrary selection of possibly late developing events could lead to misdirected efforts at screening. Similarly, if only sputum were available (as in comparisons of specimens from smokers and nonsmokers), markers of the genetic lesions/products later to be repaired during the *p53*-directed G_1 growth arrest[22] would identify only smokers, not cancer risk.

These three specimen banks were drawn from populations at 3 levels of lung cancer risk. The ELC drew specimens from community dwelling, middle-aged, male cigarette smokers whose average annual incidence of lung cancer was 5 per 1,000[23]. The LCEDWG has collected specimens from completely resected, stage I NSCLC patients. These former patients have an average annual incidence for a second primary of 3 per 100 (3%)[24], and represent the population at highest risk. At intermediate risk are the YTC miners, an industrially exposed, community dwelling population with an average annual lung cancer incidence of 1%[25].

The two banks of specimens from highest risk individuals (LCEDWG and YTC) are continuing to accrue specimens. These populations of highest risk individuals offer striking efficiencies for the preliminary testing of carcinogenesis markers and chemoprevention agents, requiring only 1/10 the accrual of a heavy smoking population (eg. the ELC) for a similar number of lung cancer cases.

D. Previous Work: Molecular/ Immunocytochemical/ Video-microscopy Studies and Pilot H_2TCPP Investigations

1. Development of PCR technique for sputum

Since the 1960's, the only clinical marker available to detect early pulmonary neoplastic changes was the recognition of atypical metaplasia in exfoliated epithelial cells by light microscopy[26]. We now know that cytomorphologic criteria alone are not sufficiently sensitive for lung cancer screening. Less than 10% of lung cancers in the NCI early lung cancer detection trials were detectable only by routine sputum cell morphology. More than half of the lung cancer cases presented clinically in the interval between annual screenings. Length-biased sampling, lead-time bias and misclassification, in addition to failures of detection and of therapy contributed to the lack of improvement in mortality rates [23,27,28,29].

Yet theoretically, even small early lung tumors can shed tumor cells into the airways. Presumably, these cells would be rare and degraded to some degree at

expectoration. These factors might be more pronounced if the tumor arose in the small distal airways, and may partly explain the low detection of these exfoliated epithelial cells by conventional methods. However, even degraded DNA could be amplified by the PCR a million- fold allowing detection of even a single cell. The remaining problem would be to sort out the rare cancer cell DNA from the surrounding normal cell DNA. This problem was overcome in other epithelial tumor types by our Johns Hopkins collaborators who have developed a novel PCR approach allowing identification and quantitation of mutant DNA contained within cancer cells[6,11]. As discussed above, these gene mutations within cancer cells are an integral part of tumor development. The very mutations that allow development of these neoplastic cells could be used as highly specific markers to identify the presence of tumor cells at an otherwise undetectable clinical stage.

Table 1.

ELC Resected NSCLC Patients with gene mutation analysis of sputum

Patient	Age/Sex	Tumor/Loc	Tumor Stage	Tumor Mutation	Sputum Mutation
L1	65-M	ADN-RUL	T1,N0,M0	Kras-12Ser	Same
L2	57-M	ADN-LUL	T2,N1,M0	Kras-12Asp	Same
L3	63-M	ADN-RUL	T1,N0,M0	Kras-12Val	Same
L4	51-M	ADN-LUL	T3,N0,M0	p53-273His	Same
L5	67-M	ADN-LUL	T1,N0,M0	Kras-12Cys	Same
L6	67-M	ADN-RUL	T3,N0,M0	p53-281Gly	Negative
L7	70-M	ADN-RUL	T1,N0,M0	Kras-12Cys	Same
L8	59-M	ADN-RUL	T1,N0,M0	Kras-12Cys	Same
L9	48-M	ULG-RUL	T1,N0,M0	Kras-12Val	Negative
L10	63-M	ADN-RLL	T1,N0,M0	Kras-12Cys	Same
L11	60-M	ADN-RUL	T3,N1,M0	None	Negative
L12	56-M	ADN-LLL	T1,N0,M0	None	Negative
L13	65-M	ADN-RUL	T1,N0,M0	None	Negative
L14	61-F	ADN-RUL	T2,N0,M0	None	Negative
L15	62-M	ADN-LUL	T2,N0,M0	None	Negative

Using the JHLP archive of sputum specimens we identified fifteen patients from that trial who later developed adenocarcinoma of the lung. The Sidransky method amplifies tumor target sequences through PCR, sub cloning into phage and plating on L-agar. Tumor DNA is then transferred to nylon filters and the plaques are probed with mutant-specific oligo-nucleotides which recognize the specific mutations The primary lung carcinomas from 10 of these 15 patients contained either a *ras* or a *p53* gene mutation (Table 1). Using this PCR-based assay, stored sputum samples obtained prior to clinical diagnosis then were examined for the presence of these same oncogene mutations. In eight out of 10 patients, the identical mutation identified in the primary tumor was also detected in at least one sputum sample. The earliest detection of a clonal population of cancer cells in sputum was found in a sample obtained more than one year prior to clinical diagnosis.

2. MONOCLONAL ANTIBODY/IMMUNOCHEMICAL DETECTION OF BIOMARKERS OF LUNG CARCINOGENESIS

Alternatively, we also have found that pre-neoplastic epithelial cells exfoliated into sputum express surface and cytoplasmic lung cancer-associated antigens. After extensive testing, we selected two monoclonal antibodies from those being developed by colleagues at the NCI for detection of antigen markers predictive of subsequent lung cancer on preserved sputum epithelial cells[1,30] from participants in the NCI-collaborative Early Lung Cancer detection trial at Johns Hopkins (the JHLP)[Error! Bookmark not defined.].

In our preliminary report[1], 69 preserved samples were selected from the 626 cases with atypical metaplasia. The last available sputum sample before the development of cancer (average of 26 months prior to cancer) was analyzed by staining the specimen with a biotinylated chromogen after incubation with primary antibody. Visualization of the bound monoclonal antibodies in fixed cytologic specimens was greatly enhanced by the development of a modified avidin-biotin complex immunostaining method as reported by Gupta et al[31]. Specimens from individuals who ultimately developed lung cancer stained with a sensitivity of 91%, 2 years before the earliest appearance of neoplasia, whether by cytology, chest radiograph or clinical criteria. Specificity was 88% among specimens from individuals who remained free of lung cancer for an overall clinical accuracy of 88.7% (See Table 2). In summary, it appears that these two monoclonal antibodies can, with reasonable accuracy, detect changes in sputum samples two or more years before routine clinical lung cancer detection. Of critical importance is the finding that not only squamous cell, but also adenocarcinoma, large cell carcinoma, and particularly small cell carcinoma can be detected before the onset of clinical cancer. In fact all five cases of small cell cancer included in our sample were detected by the small cell antibody.

Table 2

Development of Lung Cancer by Double-Bridge Immuno-Peroxidase Staining Applied to the Most Recent Sputum Specimen Showing Moderate (or more severe) Atypical Metaplasia Stored by the JHLP

	Cancer	No Cancer	Total
Satisfactory Specimen			
Stain (+)	20	5	25
Stain (-)	2	35	37
Subtotal	22	40	62
Unsatisfactory Specimen	4	3	7
TOTAL	26	43	69

Sensitivity = 91%
Specificity = 88%
Chi-square = 35.6
Accuracy = 88.7%
O.R.= 70
95% C.I.= 10.46 - 297.8
$p = < 1 \times 10^{-6}$

During the refinement of these biomarkers for validation in population trials, a moderate amount of overlap in binding of SCLC- and NSCLC-specific antibodies was observed. This overlap in specificities may represent in part, the common pathogenesis of the cell types of lung cancer [32,33,34] and may detect expression of

differentiation antigens rather than "lung cancer-specific" antigens. 624H12 has been shown to bind to a difucosylated Lewis X epitope[35]. Difucosylated Lewis X is known to be an immunodominant antigen which has enhanced expression in fetal organogenesis [36]. Re-expression of this carbohydrate, fetal differentiation marker may follow neoplastic transformation of airway epithelial cells, particularly to SCLC, representing an example of an oncofetal antigen. 703D4 recognizes a 31 KD protein, heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 expressed to some degree in all forms of lung cancer, although the expression is more frequent in NSCLC[37]. The range of cell types of lung cancer which express these antigens (91% of lung cancers) exceeds that of any single gene mutation possibly making these the most sensitive markers of carcinogenesis. In summary, a search for selected mutations or tumor associated antigens may accurately identify pulmonary carcinogenesis in advance of invasive malignancy. Recent data from these prospective studies have shown that almost 70% of those with hnRNP A2/B1 upregulation in their sputum would develop lung cancer in the first year of follow-up, compared with background lung cancer risks of 2.2 and 0.9% (35- and 76-fold increase, respectively)[38]. Future studies may show that carcinogenesis detected at this stage may be amenable to reversal and possibly cure after intervention (eg. retinoid[39] or endobronchial laser[40]), if not surgical resection.

These observations are of potentially enormous significance. An increase in lead time of 2 or more years might be sufficient to make widespread screening feasible and warranted even if lung cancer patients without atypical metaplasia remained undetected by this technique. If our present studies demonstrate that as few as 36.9% of patients with moderate atypical metaplasia can reliably be recognized 2 years prior to sputum cell morphology or clinical evidence of cancer, and if any of the remaining patients without atypia can be diagnosed, it will be a powerful argument for re-considering formal trials of the efficacy of early detection and intervention for lung cancer mortality reduction. The availability of valid intermediate endpoints for lung cancer would be a significant resource for efficacy studies of chemo-prevention or early surgical intervention.

3. CYTOMETRIC VALIDATION OF IMMUNOCHEMICAL MARKER DETECTION

The lack of a unique chemical structure for tumor-associated antigens signifies that a **qualitative** (presence/absence) criterion of marker binding is not sufficiently specific to characterize antibody-epitope binding[41]. These circumstances require the development of rigorous **quantitative** criteria for marker binding based upon the number of probe adherence sites per cell and the frequency of labeled cells per specimen. We have been engaged in studies to quantify immuno-labeled cell detection by characterizing the source and magnitude of the optical/electronic probe signal compared to all other sources of variation (the noise)[42]. Noise may arise a) from technical variation in the specimen collection/preparation, b) from variation in the assay, c) from biologic variation in the host (e.g., in the degree of cytologic atypia) and d) in the quantitation of marker uptake. Careful standardization of procedures has minimized the first two sources of variability[30,43]. By design, only sputum specimens containing epithelial cells of moderate

or greater atypical metaplasia were preserved, minimizing the third source of variability. We have recently reported our procedures for quantitation of marker uptake[5,44].

Immunostained sputum specimens showing atypical metaplasia from JHLP patients who subsequently developed lung cancer and those who did not were similarly interrogated at 510 and 600 nm., the maxima for optical transmission of the methylene blue counterstain and DAB, respectively. Within each field, an isolated epithelial cell is defined as the region of interest. Following edge-enhancement using a LaPlace transform, a perimeter is automatically drawn around the cytoplasm. The size and shape features of the nucleus and cytoplasm are recorded. A second perimeter then excludes the nucleus, and the quantitative densitometry features of the cytoplasm are recorded. After normalization for cytoplasmic area, the ratio of the wavelength-specific cytoplasmic optical densities are recorded. Finally, indices of cytoplasmic texture are recorded. The results of these analyses are recorded in a table for each patient. Values averaged over the five fields as well as the most extreme values for each patient are entered into the discriminant function which provides the automated interpretation of immunostain uptake. This automated interpretation is entered into the patient database. Univariate, correlation, cluster, common factor and discriminant function analyses were performed using Systat software and confirmed using SPSS/PC+. Multivariate (e.g., stepwise linear discrimination analysis) statistical techniques are employed to produce discriminant functions that combine relatively independent (i.e., orthogonal) parameters. A learning set of the original JHLP slides was used to generate these discriminant functions.

Table 3

Accuracy and Goodness-of-fit Tests of the Video-Microscopy Discriminant Function

Classification Results		Video-Microscopy Algorithm Predicted Group			
		Never Cancer		Cancer	
Actual Group	No. of Cases	#	%	#	%
Never Cancer	24	21	87.5	3	12.5
Cancer	44	6	13.6	38	86.4

Percent correctly classified: 86.8%

	Multivariate Statistic	F-test	df	p-value
Overall Wilks _	0.487	6.783	9,58	0.00
Hotelling-Lowly	1.052	6.783	9,58	0.00

Significant differences were found between the moderately atypical cells from the JHLP study groups who developed squamous cell or small cell undifferentiated cancer compared to the moderately atypical cells from JHLP participants who remained cancer free. The moderately atypical cells from individuals who later developed lung cancer were more dense to transmitted light at the frequency of the DAB probe (avg. gray level), showed greater difference in light transmission at the frequency of DAB compared to the counterstain (gray ratio), and were more circular (shape factor), than were those whose atypical cells did not lead to respiratory cancer. Iterative principal axis (common factor) analysis showed that three factors; average gray,

gray ratio, and shape factor, together explained most of the variance. The coefficients of the canonical discriminant function were used to generate discriminant scores which demonstrate an excellent separation of cancer cases from controls. Finally, Table 3 shows the accuracy of the discriminant function in separating cells of the two groups of patients is 86.8%, comparable to the 88.7% accuracy of the original cytopathology interpretation of the same slides (Table 2). These results suggest that there was a rigorous, objective basis to immunochemical detection of carcinogenesis markers and that image analysis has potential for greatly refining the process of early lung cancer detection research.

4. PILOT H_2TCPP STUDIES WITH LOS ALAMOS

From 1991 through 1992, a collaboration between Dr. Dean Cole, Life Sciences Division, Los Alamos National Laboratory,and Dr. Tockman resulted in pilot studies[45] which determined that uptake and fluorescence of 5,10,15,20 tetrakis (4-carboxyphenyl) porphine (H_2TCPP) could distinguish pre-malignant exfoliated epithelial cells from clinical specimens.

Our pilot studies used the water soluble 5,10,15,20-tetrakis (4-carboxyphenyl) porphine (H_2TCPP) obtained through the Los Alamos National Laboratory (but now commercially available) to label exfoliated sputum cells. Sputum specimens were examined from 24 individuals who later developed lung cancer and from 35 individuals who did not develop cancer, maintained in Saccomanno's preservative (50% ethanol, 2% polyethylene glycol 1540) as part of the ELC specimen bank. These specimens were suspended and aliquots were removed for cytospin (800 rpm for 2 minutes) to obtain a concentration of 10^5 cells/cc. Glass slides were coated with poly-l-lysine to improve cell adherence. Slides were stained with the Papanicolaou (Pap) technique alone, H_2TCPP alone, and combined staining with H_2TCPP followed by Pap. After 24 hour incubation with 50μg/cc H_2TCPP in a humid chamber at 37°C.under reduced ambient light, slides were rinsed with PBS x 2, mounted in glycerol and sealed with a heated mixture of equal parts of vaseline, lanolin and paraffin[46] in preliminary studies, then sealed with colorless nail enamel in later studies. These studies demonstrated that pre-malignant epithelial cells (demonstrating moderate atypical metaplasia) from individuals who ultimately developed squamous cancer of the lung, took up the porphyrin and were distinguishable from background sputum cells under epifluorescent microscopy.

III. Approach

The purpose of this research is to evaluate a synthetic porphyrin, 5,10,15,20 tetrakis (4-carboxyphenyl) porphene (H_2TCPP) as a marker of carcinogenesis. After establishment of the optimal conditions under which neoplastic and non-neoplastic cells take up H_2TCPP, we will assess the accuracy of the porphyrin as a biomarker of carcinogenesis. We determined whether H_2TCPP fulfills its potential as a highly sensitive detector of carcinogenesis from its uptake by banked pre-neoplastic material when compared to other carcinogenesis markers evaluated in our laboratory.

While the mechanism which underlies the selective uptake of porphyrin by neoplastic tissue remains to be described, preferential uptake of porphyrin by frankly neoplastic tissue has been recognized for four decades[47]. Most earlier porphyrin studies have relied upon endoscopic installation of hematoporphyrin derivative (HPD) for localization of neoplastic invasion of bladder[16] and lung[4849]. More recently, frankly malignant cells have been detected in sputum specimens from lung cancer patients following injection of HPD[18]. Subcellular organelle localization of porphyrin has been reported in cultured malignant cells in mitochondria, the endoplasmic reticulum and in the perinuclear tubular structures (Golgi apparatus)[50]. Present studies explore the ability of H_2TCPP to detect pre-malignant epithelial cells in the process of carcinogenesis.

IV. Methods

1. Sputum Cells and cell culture

Sputum specimens induced after a 15 minute saline inhalation were obtained from Yunnan Tin Miners and preserved in Saccomanno's solution (50% EtOH, 2% polyethylene glycol [Carbowax] 1540) in the YTC Biologic Specimen Bank for Lung Cancer. Sputum cells without evidence of cancer by light microscopy were separated from mucus glycoprotein by enzymatic and mechanical techniques[51]. Briefly, sputum specimens were added to an equal volume liquefication buffer made of 1mM DTT (Sigma # D-9779) plus 20 Ku/ml DNAseI (Sigma # D-4263). Following a 15 min incubation at 37° C, the suspension was passed through a 40μ nylon mesh strainer (Falcon # 2340), centrifuged, washed with PBS and resuspended. The concentration of cells was adjusted to $1x10^6$/ml prior to use.

ATCC human bronchogenic cancer cell lines H520, HTB58 (Squamous Cell Cancer), H23, Calu-3 (Adenocarcinoma) and OH3 and H345 (Undifferentiated Small Cell Cancer) are maintained in our laboratory. H520 is incubated in RPMI-1640 supplemented with l-glutamine (Gibco-BRL, #320-1875-AJ) plus 10% FBS (Gibco-BRL, #230-6140-AJ, Grand Island NY). H23, Calu-3, H345 are incubated in RPMI-1640 supplemented with l-glutamine (Gibco-BRL, #320-1875-AJ) plus 5% FBS (Gibco-BRL, #230-6140-AJ, Grand Island, NY). OH3 is incubated in RPMI-1640 supplemented with l-glutamine (Gibco-BRL, #320-1875-AJ) plus 16% FBS (Gibco-BRL, #230-6140-AJ, Grand Island NY).. HTB58 is incubated in Eagle's MEM supplemented with EBSS, l-glutamine, and NEAA without sodium bicarbonate (Sigma #M-0643, St. Louis, MO) plus 10% FBS (Gibco-BRL, #230-6140-AJ, Grand Island, NY).

2. Fluorescent staining methods

Staining methods were compared for their ability to distinguish three groups of cells: normal sputum cells only, ATCC lung cancer cells only and 1:1 mixture of tumor and sputum cells.

7-amino Actinomycin-D (7-AAD)

7-AAD (Molecular Probes A-1310) is a membrane-impermeant fluorescent DNA intercalator which binds particularly well to GC-rich regions of DNA. Upon binding, 7-AAD undergoes a spectral shift resulting in an emission maximum at 655 nm after excitation at 555 nm. This chromatin marker has been used to distinguish cells held in the G_0 and G_1 phases of the cell cycle[52]. Earlier investigations have described the procedures for gentle permeabilization and fixation to simultaneously quantify DNA with 7-AAD and cell surface antigens with fluorescein-labeled antibodies[53]. Following a PBS wash, cells are resuspended in cold PBS and fixed by addition of freshly prepared 2% phosphate-buffered paraformaldehyde (Kodak, Rochester, NY) to a final concentration of 0.25%. Fixation at 4° C for 1 hour is followed by permeabilization with 0.2% tween-20 (Sigma # P-2287) in PBS for 15 minutes. After fixation and permeabilization, DNA was stained with 7-AAD by 30-minute incubation of cells in PBS containing 25 µg/ml of 7-AAD.

5,10,15,20-tetrakis (4-carboxyphenyl) porphine (H_2TCPP) fluorescent labeling

The synthetic H2TCPP is now obtained through Porphyrin Products Co. (P.O. Box 31, Logan, UT, Cat. No. T-790, as Mesotetra(4-carbo-xyphenol)porphine crystals not dried in acetone. Cell spreads and cytospin preparations are fixed in 95% ETOH for 10 min., then rinsed x2 with PBS. Under reduced ambient light, H_2TCPP is applied in concentrations from 50-200 µg/ml to the slides which are maintained in a dark humid chamber for 4 to 24 hours, at temperatures from 37 to 4°C. At the conclusion of incubation, excess porphyrin is removed by 2 quick PBS washes. Slides are then dehydrated, mounted in glycerol, cover slipped and sealed with colorless nail enamel.

Fluorescein-labeled tumor-associated monoclonal antibody

Monoclonal antibody 703D4 which binds to a cytoplasmic antigen associated with NSCLC was applied to cells which have been paraformaldehyde fixed and permeabilized as described above. Cells are suspended in a 1:50 dilution of Mab 703D4 and incubated overnight at 4° C. After washing with PBS x 2, the cells are resuspended in 1:50 dilution of biotinylated horse anti-mouse IgG (Vector Laboratories, BA-2000), incubated for 30 min at room temperature and again washed with PBS. Cell pellets are then resuspended in a 1:50 dilution of Fluorescein avidin D (Vector Laboratories A-2001), incubated for 30 min at 4° C in the dark and washed in PBS prior to measurement of FITC fluorescence.

3. Fluorescent microscopy and quantitation

Image acquisition is performed on a Zeiss Axiophot epifluorescence and phase contrast microscope equipped with a Zeiss automatic photomicrographic system, camera control panel and with Zeiss Plan-neofluar objectives: 10x/0.30 n.a., 20x/0.50 n.a., 40x/0.75 n.a., 40x/1.3 n.a. oil, and 63x/1.25 n.a. oil (Carl Zeiss, Inc., Microscope Division, West Germany). The microscope is mounted on a Vibraplane vibration isolation table (Kinetic Systems, Inc., 20 Arboretum Rd, Roslindale, MA, Model 1201-04-11). Standard 100 W mercury vapor illumination intensity is assured by using a Multispeck multispectral fluorescence microscopy standard (Molecular Probes, Inc.,

H_2TCPP uptake was determined upon sputum samples from individuals who did not develop lung cancer. In each cancer patient, H_2TCPP uptake was evaluated first upon sputum samples that were collected during the time the patient was known to have cancer and progressively work backward through the pre cancerous period. Sputum specimens were counter-stained with the standard Papanicolaou procedure to assess the morphology of the cells which take up the H_2TCPP.

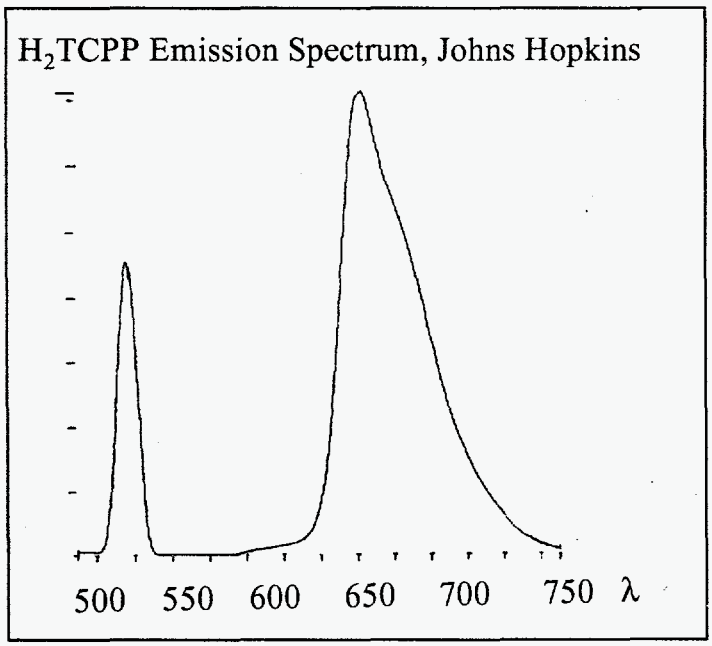
6. Flow Cytometry

$1x10^6$ cells suspended in 1 ml PBS were analyzed on an Elite flow cytometer. 7-AAD fluorescence was excited at 488 nm and measured through a 650 nm long pass filter. Porphyrin fluorescence was excited at 518 nm and collected through a 650 nm. long pass filter. FITC fluorescence was excited at 488 nm. and collected through a 510-530 nm narrow band filter.

V. Results

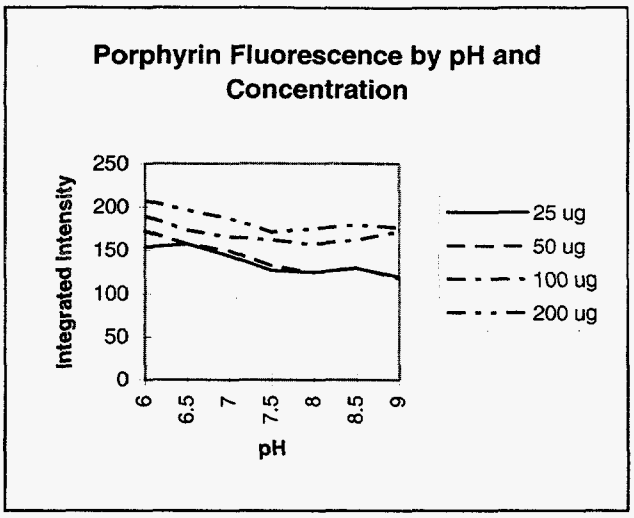
H_2TCPP FLUORESCENT EMISSION SPECTRA

Figure 2



Preliminary studies conducted at the Los Alamos National Laboratory had suggested that excitation of H_2TCPP by standard mercury vapor illumination filtered through a 495 λ fluorescein isothiocyanate (FITC) excitation filter caused the H_2TCPP to autofluoresce with a maximum emission at 560 λ. Following through after submission of the annual progress report, I have subsequently learned that the Los Alamos data showed an emission peak at 650 (not 560) λ, and was provided with copies of the emission spectrograph (Figure 1)[54].

Figure 3



To replicate this observation, we used a Perkin-Elmer Luminescence Spectrometer (Model LS50, Perkin Elmer Ltd., Beaconsfield, Buckinghamshire, England), to excite cuvettes of H_2TCPP at concentrations of 50 µg/ml. at the FITC wavelength and scan for the peak emission wavelength (Figure 2). Consistent with the Los Alamos data, the peak emission wavelength was 645λ (activity range 620-710 λ). Setting the emission wavelength at 645 λ, the H_2TCPP solution was scanned over the range 450 to 600 λ to determine the optimal excitation frequency. Optimal excitation occurred at 518 λ (activity range 490-545 λ), with subsidiary peaks at 557 and 584 λ. H_2TCPP excitation at 518 λ and emission at 645 λ is similar to the spectroscopic properties of propidium iodide, a common fluorochrome for measurement of DNA/RNA content.

Video-enhanced fluorescent microscopy was used to maximize cell-area normalized fluorescence across a matrix of substrate conditions. H_2TCPP concentration was increased (25, 50, 150 and 200 µg/ml.) while pH was varied from 6.0 through 9.0 (Figure 3). Increase in both parameters enhanced fluorescence, however, preliminary results suggest that low concentrations and neutral pH are optimal for intracellular localization of the porphyrin.

Quantitation of fluorescence is being conducted separately for freshly harvested, Saccomanno preserved human lung cancer tissue culture cell lines. Present studies have been limited to Squamous Cell Cancer (ATCC: HTB58).

Sputum Cell Separation

Sputum specimens treated with liquefying agents 0.5mM DTT plus 10Ku/ml DNAse I and filtered by 40 micron strainer shows that this method effectively reduces background mucus and clumps of leukocytes and epithelial cells[51]. Sputum cells were separated well and could be evaluated by flow cytometry.

Analysis of DNA Staining

The fluorescence histograms of 7-AAD are presented in the Appendix.

VI. Literature Cited

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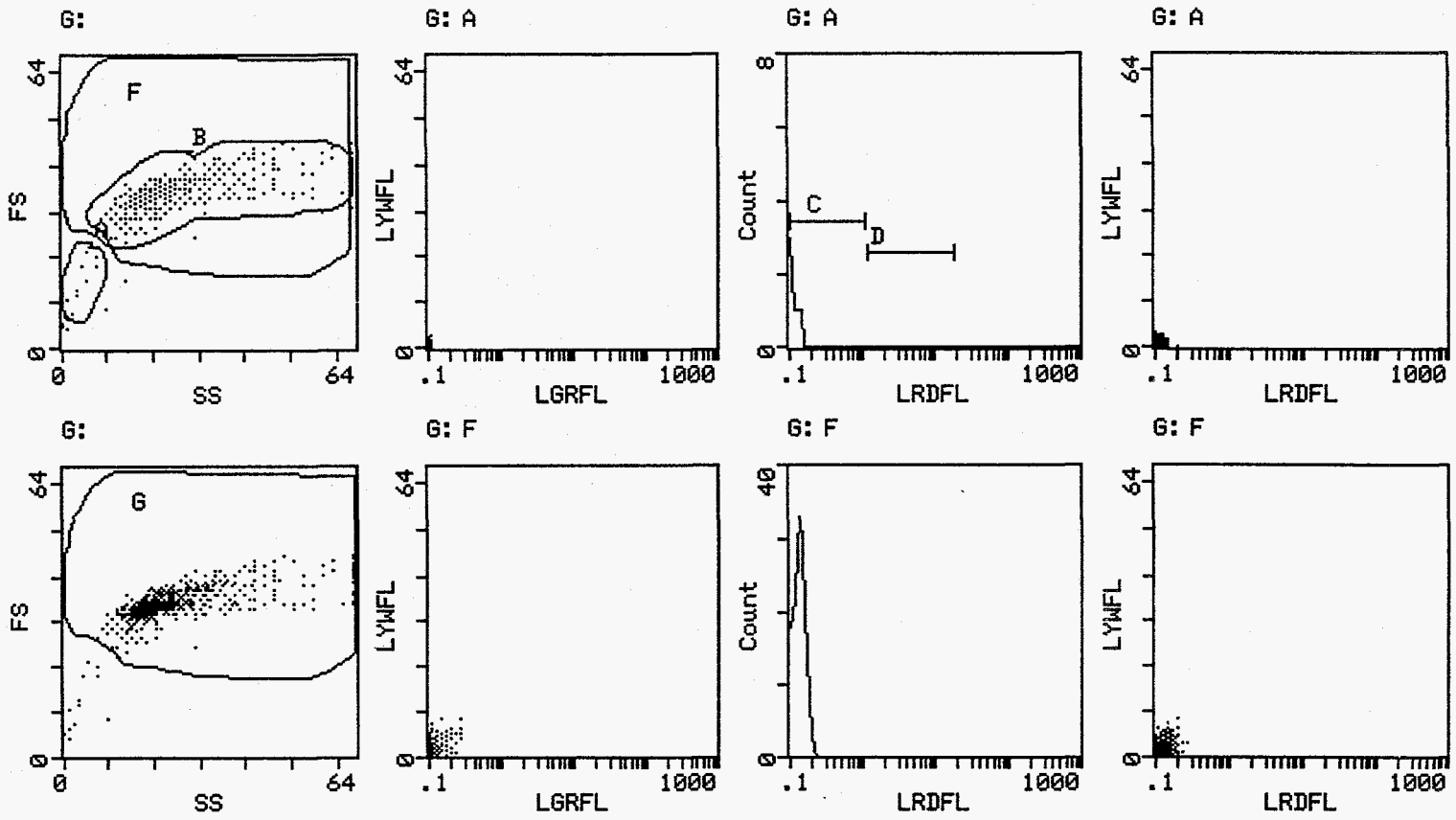
		Size		Granularity		Fluorescence			
	%	Mean	SD	Mean	SD	Color	Area	Mean	SD
Tumor Cells									
Unstained									
Small, Non Granular	3.1	11.4	4.2	2.9	2.1	red	55	0.12	0.02
Large, More Granular	89.0	34.2	3.6	25.6	10.7				
Small, Non Granular	39.9	5.3	2.7	0.5	1.4	green			
Large, More Granular	53.5	32.8	4.4	11.6	49.5				
Sputum Cells									
Unstained									
Small, Non Granular	60.0	10.1	4.8	3.5	2.7	red	4549	0.15	0.04
Large, More Granular	29.5	29.2	7.0	32.8	15.7				
Tumor Cells									
Porphyrin Stained									
Small, Non Granular	2.1	9.7	5.2	2.5	2.8				
Large, More Granular	90.7	34.3	3.5	26.6	12.0	red	8592	77.7	30.8
Sputum Cells									
Porphyrin Stained									
Small, Non Granular	61.4	11.2	4.9	3.4	2.6				
Large, More Granular	29.5	29.5	7.0	31.3	16.0	red	3517	68.9	28.9
							732	17.9	3.24
Tumor Cells									
7-AAD Stained									
Small, Non Granular	2.2	9.8	5.4	2.9	3.1				
Large, More Granular	87.7	34.6	3.7	26.0	12.1	red	3634	36.7	7.58
							5897	19.6	1.66
Sputum Cells									
7-AAD Stained									
Small, Non Granular	53.1	10.9	4.8	3.7	2.8				
Large, More Granular	34.9	29.3	7.0	33.6	15.6	red	5118	7.2	4.8
Tumor Cells									
FITC Stained									
Small, Non Granular	8.9	7.8	5.2	1.8	3.1				
Large, More Granular	84.7	32.8	4.4	24.3	11.6	green	8809	0.16	0.07
Sputum Cells									
FITC Stained									
Small, Non Granular	43.9	9.7	4.6	3.5	2.7				
Large, More Granular	42.0	29.8	7.2	32.9	14.4	green	6533	0.13	0.04

HISTOGRAM DISPLAY

SAMPLE NAME : UNSTAINED
SAMPLE NUMBER: TUMOR CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 11:38:18



SAMPLE INFO

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INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: UNSTAINED			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	TUMOR CELLS ONLY			SAMPLE TIME:	11:38:18
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

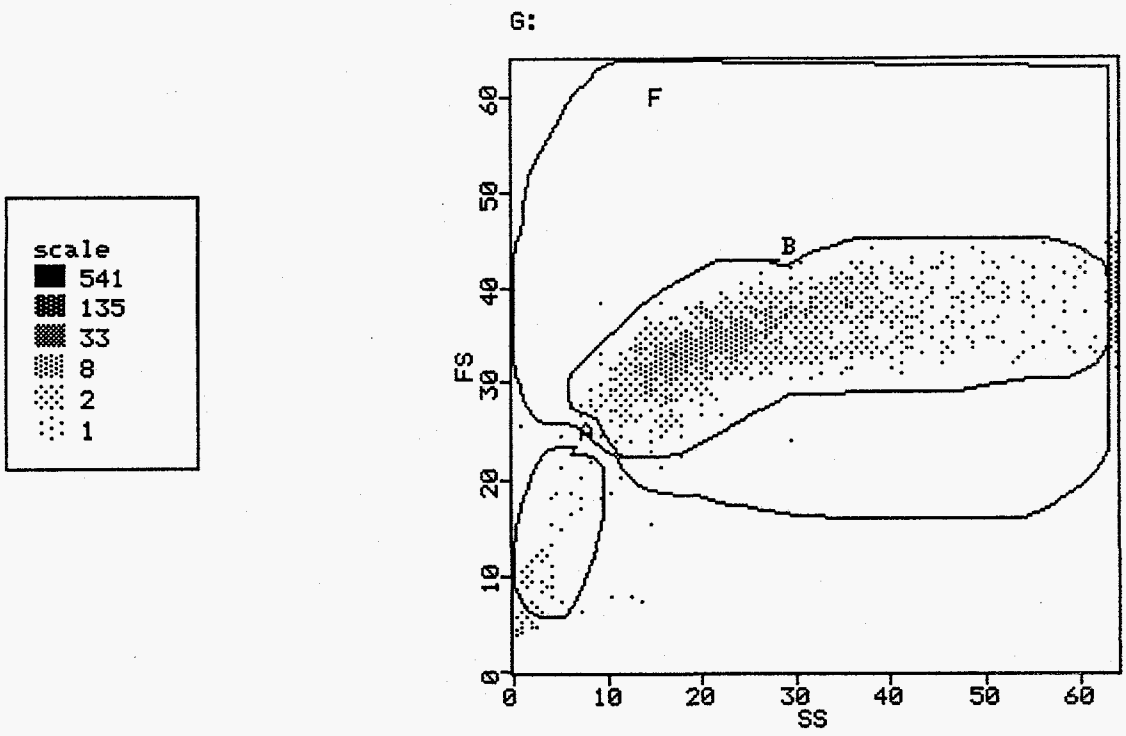
			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	100	55	0.10	10	0.119	0.016	13.6	0.464	0.10	1.0
D	0.0	0	****	*****	****	****	****	****	1.1	18

DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	3.1	55	1, 7	4	2.9	2.1	73.9	11.4	4.2	37.1
F	89.0	1603	18, 31	20	25.6	10.7	41.7	34.2	3.6	10.6

SAMPLE NAME : UNSTAINED
SAMPLE NUMBER: TUMOR CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 11:38:18



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN				
DATA RATE	: 31	HIST COUNT	: 1,802	LIST FILE	: 40829T01.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: UNSTAINED			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	TUMOR CELLS ONLY			SAMPLE TIME:	11:38:18
COMMENTS	:				

STATISTICS

DUAL PARAMETER STATISTICS

			.....Peak.....		....X Channel....			....Y Channel....		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	3.1	55	1, 7	4	2.9	2.1	73.9	11.4	4.2	37.1
B	92.9	1674	18, 31	20	27.3	13.0	47.6	34.3	3.6	10.5
F	89.0	1603	18, 31	20	25.6	10.7	41.7	34.2	3.6	10.6

A A AMORPHOUS
B B AMORPHOUS
F F AMORPHOUS

SAMPLE NAME : UNSTAINED
SAMPLE NUMBER: TUMOR CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 11:38:18

C Low Channel = 0.10, High Channel = 1.0 C
D Low Channel = 1.1, High Channel = 18 D
A A AMORPHOUS
F F AMORPHOUS

HISTOGRAM DISPLAY

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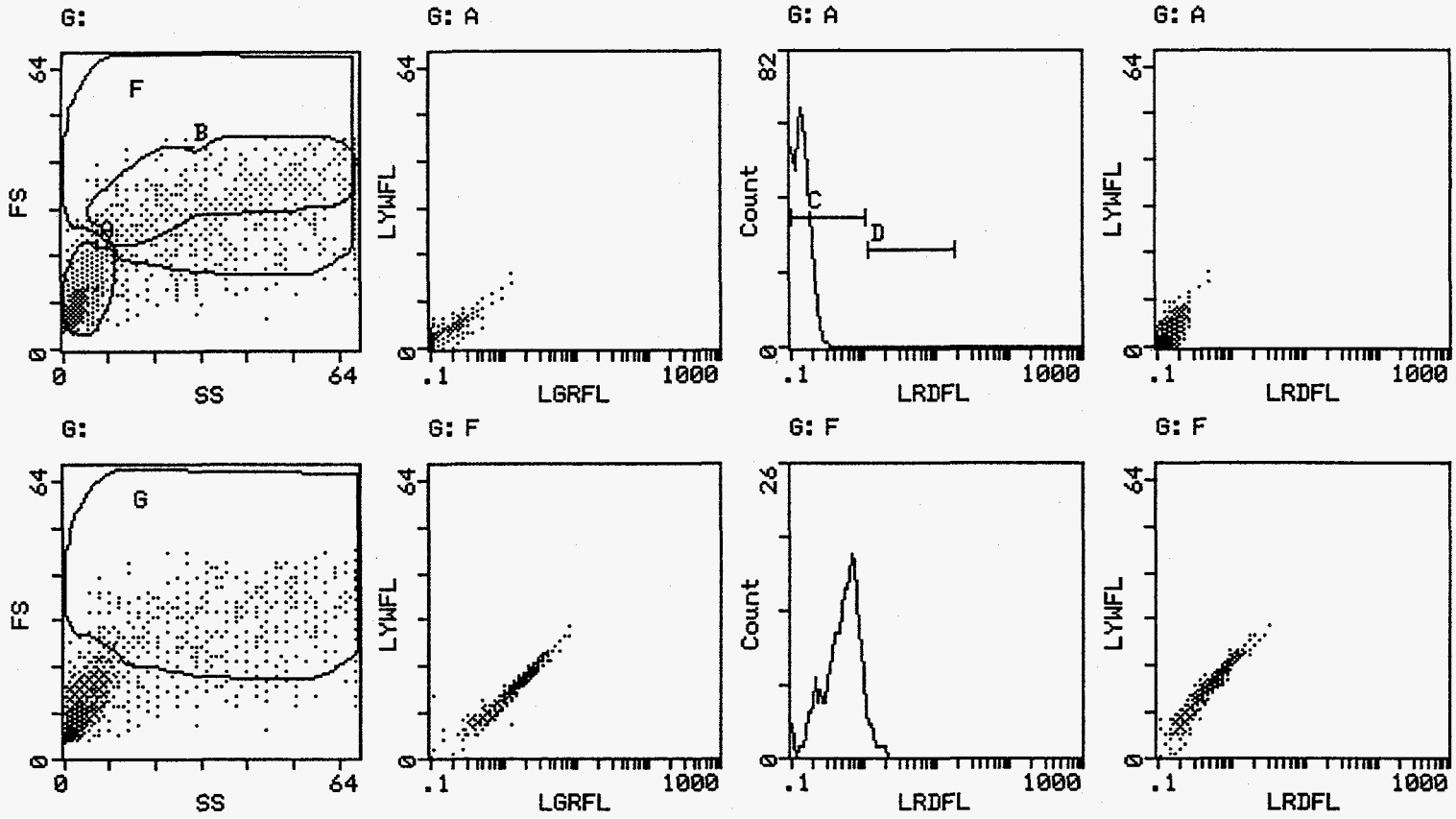
SAMPLE NUMBER: SPUTUM CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 11:42:16



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
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INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: UNSTAINED			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	SPUTUM CELLS ONLY			SAMPLE TIME:	11:42:16
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	100	4549	0.10	244	0.149	0.036	24.5	0.441	0.10	1.0
D	0.0	1	1.2	1	1.25	0.000	0.000	0.000	1.1	18

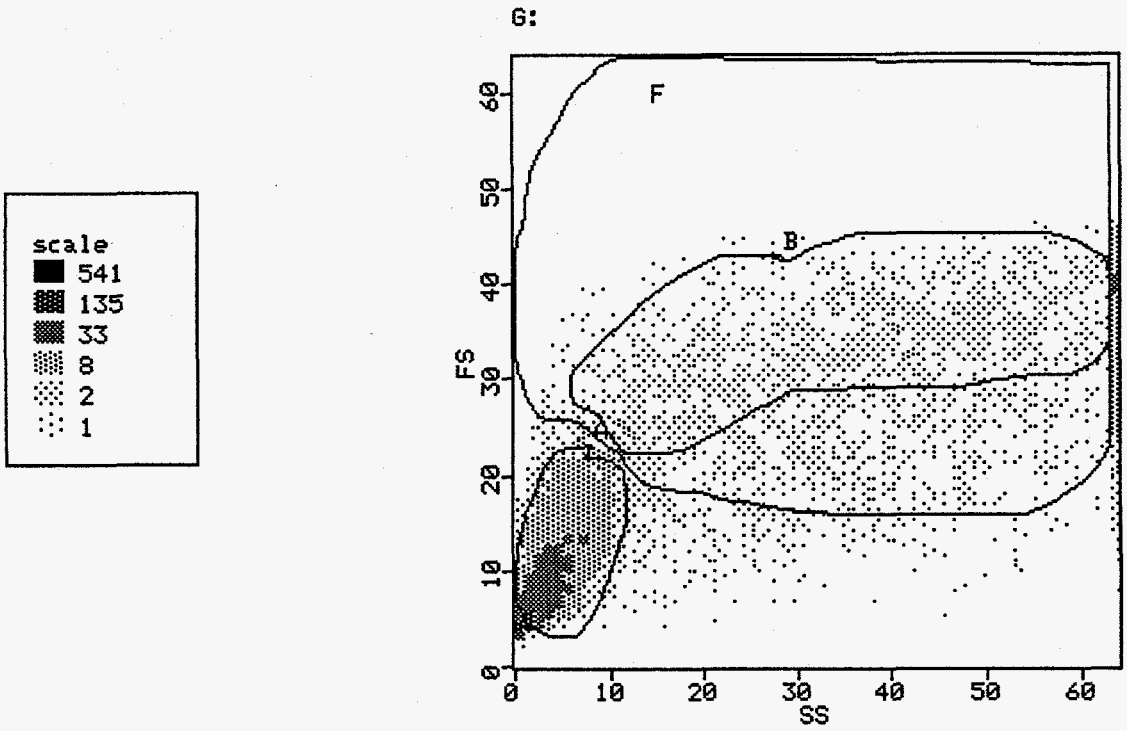
DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	60.0	4550	1, 5	189	3.5	2.7	76.9	10.1	4.8	47.6
F	29.5	2238	10, 21	12	32.8	15.7	48.0	29.2	7.0	23.9

GATES

SAMPLE NAME : UNSTAINED
SAMPLE NUMBER: SPUTUM CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 11:42:16



SAMPLE INFO

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INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: UNSTAINED			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	SPUTUM CELLS ONLY			SAMPLE TIME:	11:42:16
COMMENTS	:				

STATISTICS

DUAL PARAMETER STATISTICS

			.....Peak.....		....X Channel....			....Y Channel....		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	60.0	4550	1, 5	189	3.5	2.7	76.9	10.1	4.8	47.6
B	22.5	1706	63, 40	41	36.8	18.1	49.2	33.3	5.3	15.8
F	29.5	2238	10, 21	12	32.8	15.7	48.0	29.2	7.0	23.9

A A AMORPHOUS
B B AMORPHOUS
F F AMORPHOUS

SAMPLE NAME : UNSTAINED
SAMPLE NUMBER: SPUTUM CELLS ONLY

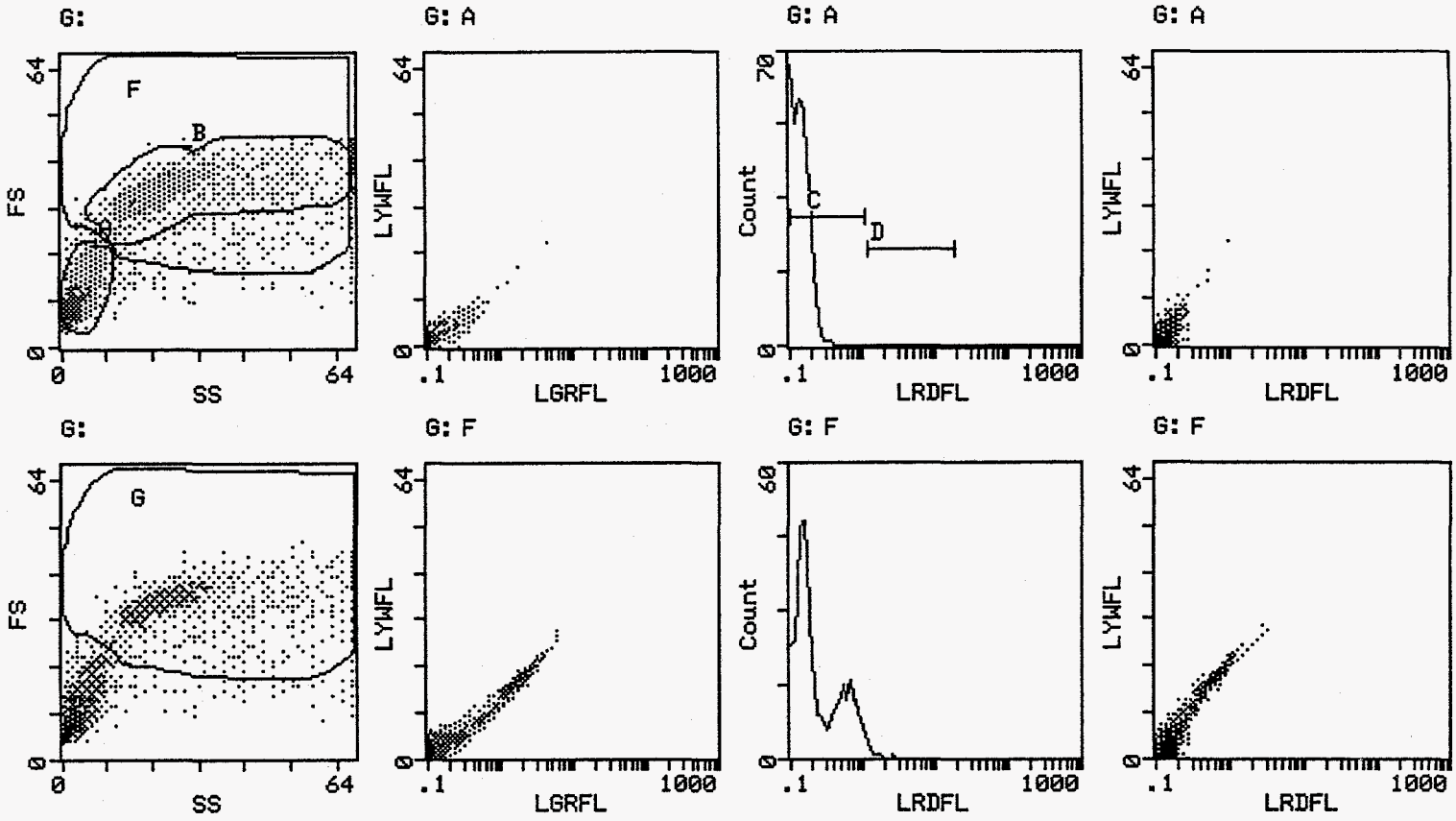
SAMPLE DATE: 29Aug94
SAMPLE TIME: 11:42:16

C Low Channel = 0.10, High Channel = 1.0 C
D Low Channel = 1.1, High Channel = 18 D
A A AMORPHOUS
F F AMORPHOUS

HISTOGRAM DISPLAY

SAMPLE NAME : UNSTAINED
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM CELLS

SAMPLE DATE: 29Aug94
SAMPLE TIME: 11:49:36



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
DATA RATE	: 285	TOTAL COUNT	: 10,279	LIST FILE	: 40829T03.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: UNSTAINED			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	MIX 50:50 TUMOR/SPUTUM CELLS			SAMPLE TIME:	11:49:36
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	100	4480	0.10	319	0.147	0.037	25.5	0.444	0.10	1.0
D	0.0	0	****	*****	****	****	****	****	1.1	18

DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	43.6	4480	0, 4	214	3.5	2.7	78.3	10.3	4.9	47.2
F	46.9	4819	15, 31	35	28.4	13.9	49.0	31.9	5.9	18.4

SAMPLE NAME : UNSTAINED
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM CELLS

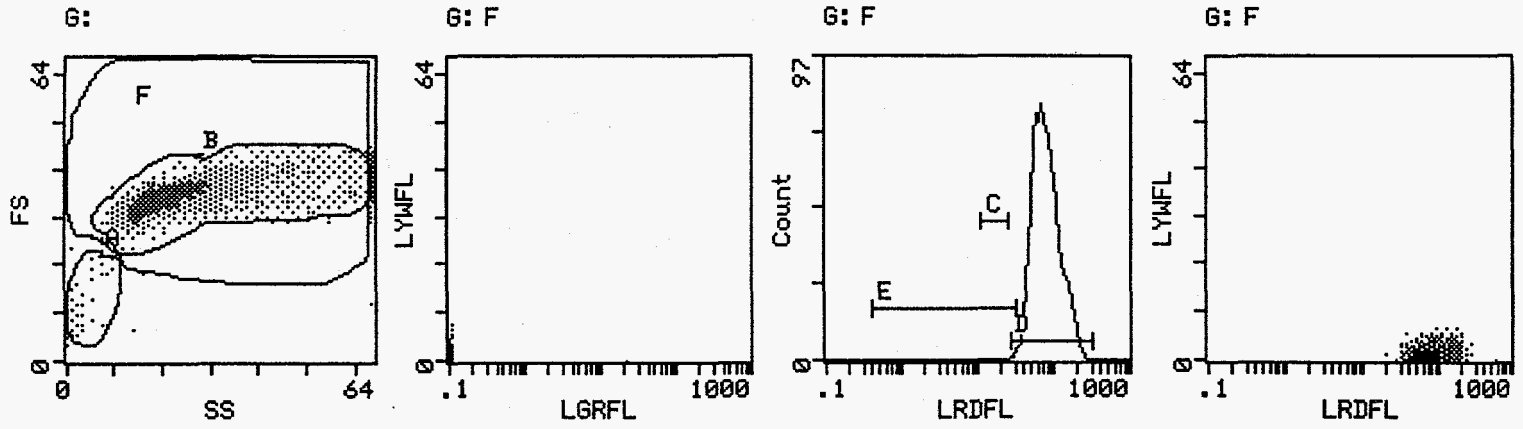
SAMPLE DATE: 29Aug94
SAMPLE TIME: 11:49:36

C Low Channel = 0.10, High Channel = 1.0 C
D Low Channel = 1.1, High Channel = 18 D
A A AMORPHOUS
F F AMORPHOUS

HISTOGRAM DISPLAY

SAMPLE NAME : STAINED PORPHIN
SAMPLE NUMBER: TUMOR CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:14:47



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
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INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED PORPHIN			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	TUMOR CELLS ONLY			SAMPLE TIME:	12:14:47
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	Half-CV	Min	Max
C	0.0	3	18	1	20.8	1.96	9.45	0.382	11	25
D	99.9	8592	73	97	77.7	30.8	39.7	28.0	26	310

DUAL PARAMETER STATISTICS

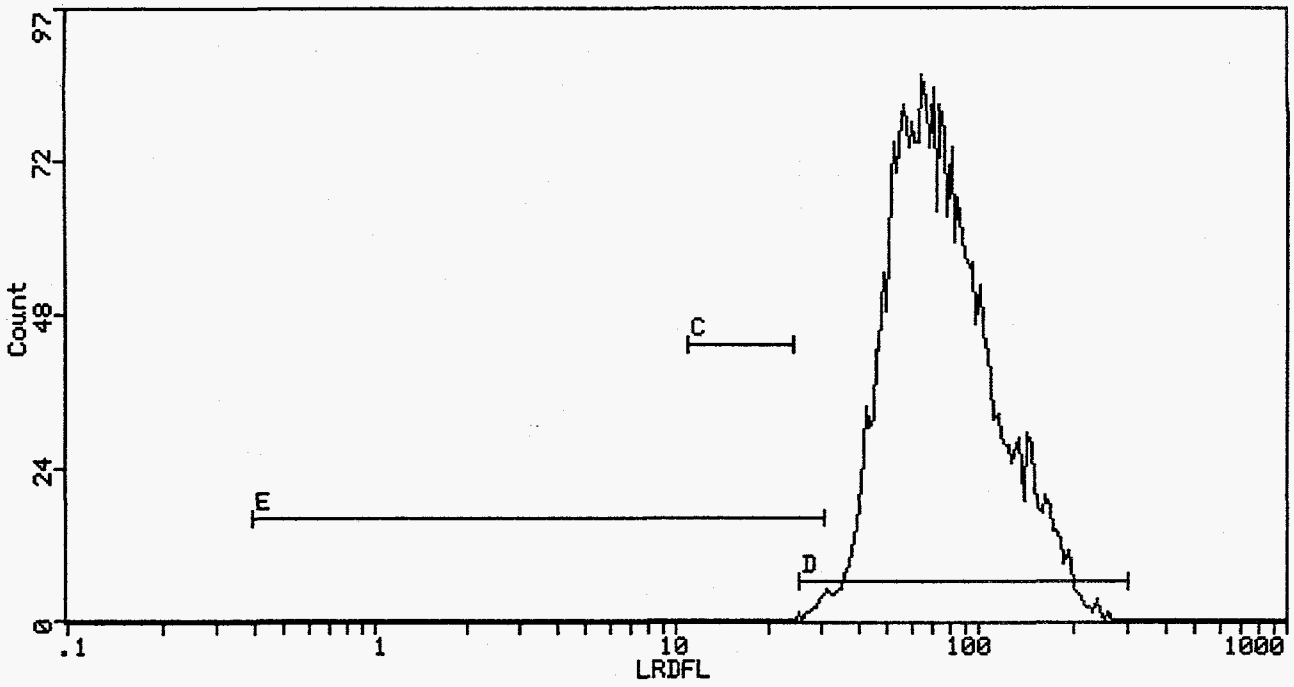
			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	2.1	196	0, 4	15	2.5	2.8	109.1	9.7	5.2	53.4
F	90.7	8603	16, 33	90	26.6	12.0	45.3	34.3	3.5	10.1

GATES

SAMPLE NAME : STAINED PORPHIN
SAMPLE NUMBER: TUMOR CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:14:47

G: F



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN				
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INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED PORPHIN			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	TUMOR CELLS ONLY			SAMPLE TIME:	12:14:47
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	0.0	3	18	1	20.8	1.96	9.45	0.382	11	25
D	99.9	8592	73	97	77.7	30.8	39.7	28.0	26	310
E	0.5	45	31	5	25.0	13.5	54.0	0.796	0.40	32

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
E Low Channel = 0.40, High Channel = 32 E

SAMPLE NAME : STAINED PORPHIN
SAMPLE NUMBER: TUMOR CELLS ONLY

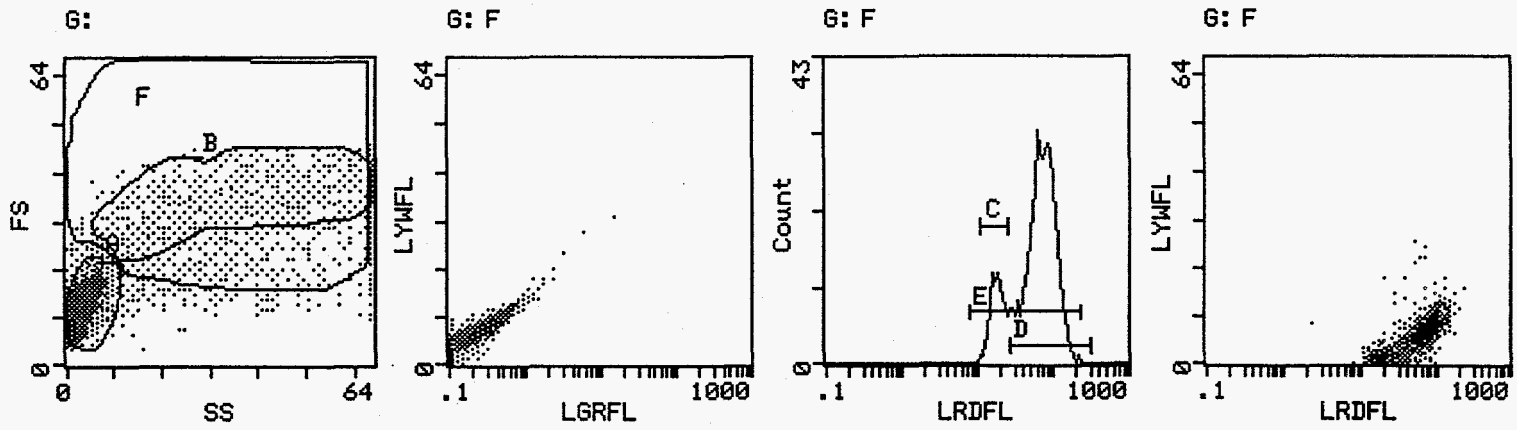
SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:14:47

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
A A AMORPHOUS
F F AMORPHOUS

HISTOGRAM DISPLAY

SAMPLE NAME : STAINED PORPHIN
SAMPLE NUMBER: SPUTUM CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:18:17



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
DATA RATE	: 607	TOTAL COUNT	: 14,579	LIST FILE	: 40829T08.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED PORPHIN			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	SPUTUM CELLS ONLY			SAMPLE TIME:	12:18:17
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	17.0	732	15	18	17.9	3.24	18.1	4.11	11	25
D	81.9	3517	78	43	68.9	28.9	42.0	5.23	26	310

DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	61.4	8945	0, 4	289	3.4	2.6	78.2	11.2	4.9	43.7
F	29.5	4296	9, 22	26	31.3	16.0	51.0	29.5	7.0	23.6

SAMPLE NAME : STAINED PORPHIN
SAMPLE NUMBER: SPUTUM CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:18:17

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
A A AMORPHOUS
F F AMORPHOUS

HISTOGRAM DISPLAY

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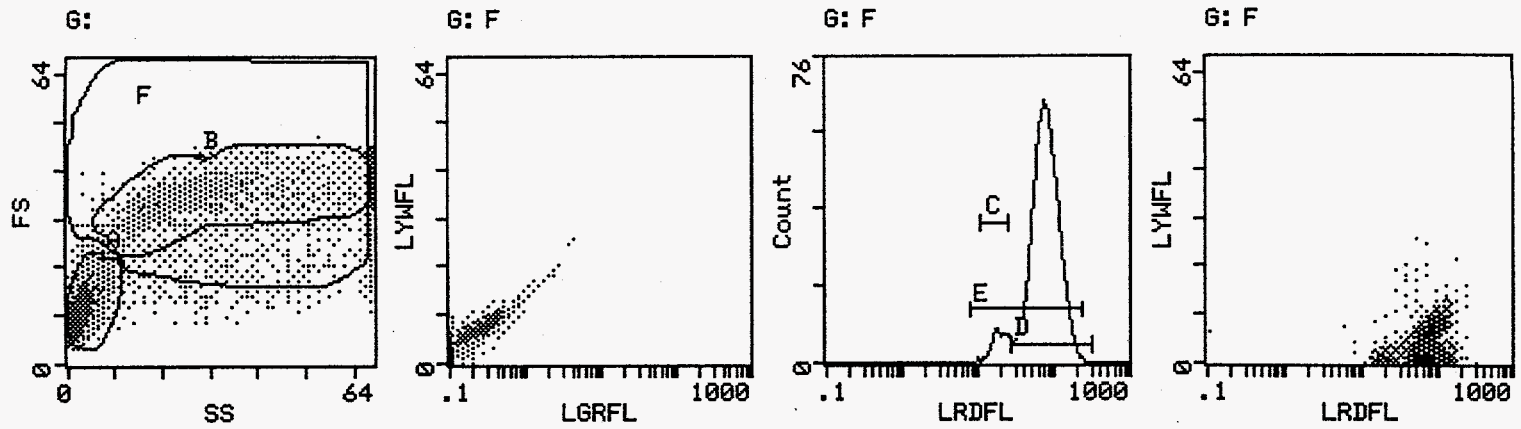
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:20:58



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
DATA RATE	: 632	TOTAL COUNT	: 13,914	LIST FILE	: 40829T09.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED PORPHIN			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	MIX 50:50 TUMOR/SPUTUM			SAMPLE TIME:	12:20:58
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	6.4	452	22	14	18.4	3.39	18.4	1.53	11	25
D	93.2	6620	73	76	75.3	29.5	39.2	21.3	26	310

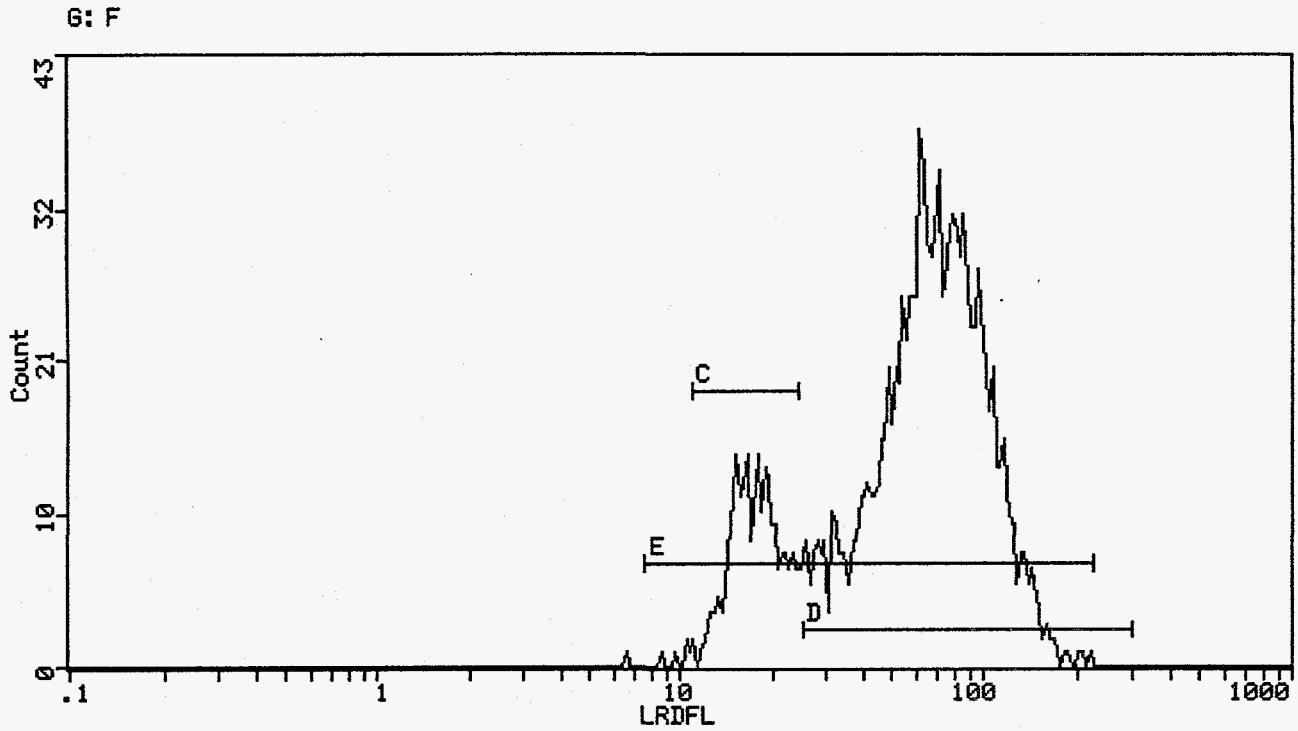
DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	37.2	5173	0, 4	174	3.5	2.6	75.2	11.4	5.0	43.4
F	51.1	7104	15, 32	33	30.6	14.5	47.4	31.6	6.3	19.9

GATES

SAMPLE,NAME : STAINED PORPHIN
SAMPLE NUMBER: SPUTUM CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:18:17



SAMPLE INFO

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INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED PORPHIN			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	SPUTUM CELLS ONLY			SAMPLE TIME:	12:18:17
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	Hal+CV	Min	Max
C	17.0	732	15	18	17.9	3.24	18.1	4.11	11	25
D	81.9	3517	78	43	68.9	28.9	42.0	5.23	26	310
E	99.7	4285	78	43	54.0	35.6	65.9	5.23	7.8	232

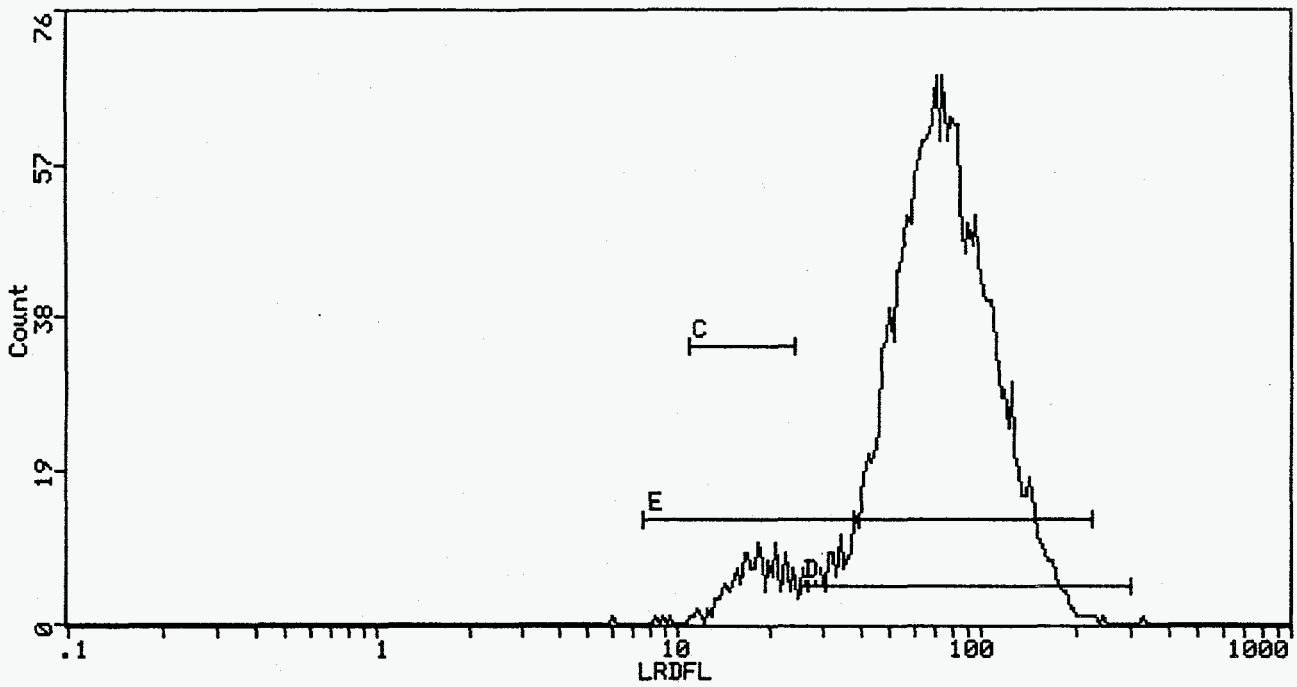
C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
E Low Channel = 7.8, High Channel = 232 E

GATES

SAMPLE NAME : STAINED PORPHIN
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:20:58

G: F



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN				
DATA RATE	: 632	HIST COUNT	: 7,104	LIST FILE	: 40829T09.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED PORPHIN			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	MIX 50:50 TUMOR/SPUTUM			SAMPLE TIME:	12:20:58
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	6.4	452	22	14	18.4	3.39	18.4	1.53	11	25
D	93.2	6620	73	76	75.3	29.5	39.2	21.3	26	310
E	99.7	7085	73	76	68.4	35.8	52.4	21.3	7.8	232

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
E Low Channel = 7.8, High Channel = 232 E

SAMPLE NAME : STAINED PORPHIN
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM

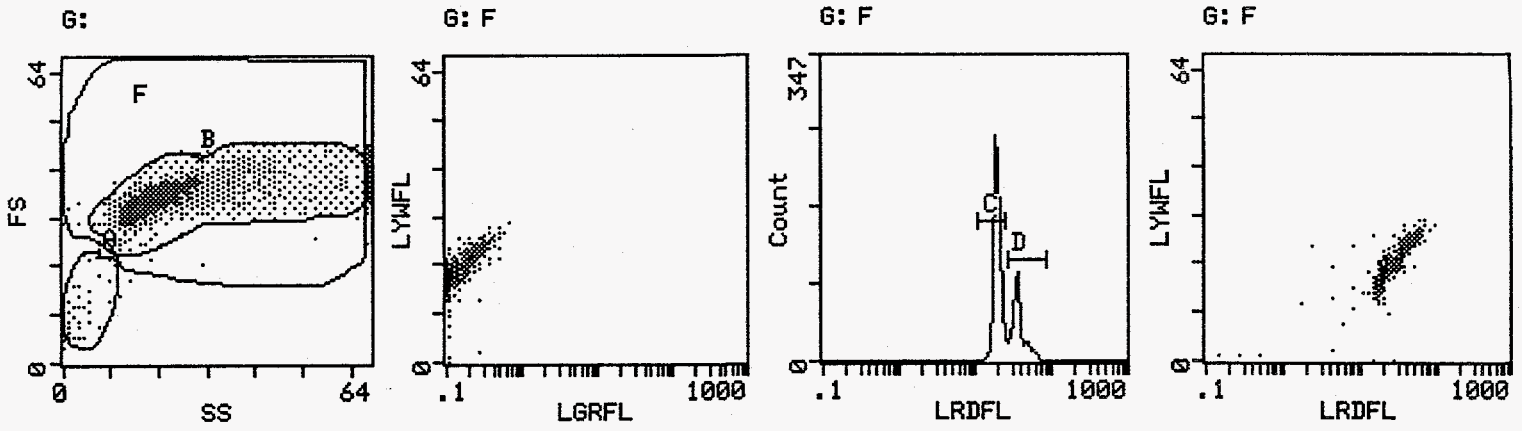
SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:20:58

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
A A AMORPHOUS
F F AMORPHOUS

HISTOGRAM DISPLAY

SAMPLE NAME : STAINED 7AAD
SAMPLE NUMBER: TUMOR ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 11:56:46



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
DATA RATE	: 56	TOTAL COUNT	: 10,991	LIST FILE	: 40829T04.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED 7AAD			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	TUMOR ONLY			SAMPLE TIME:	11:56:46
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

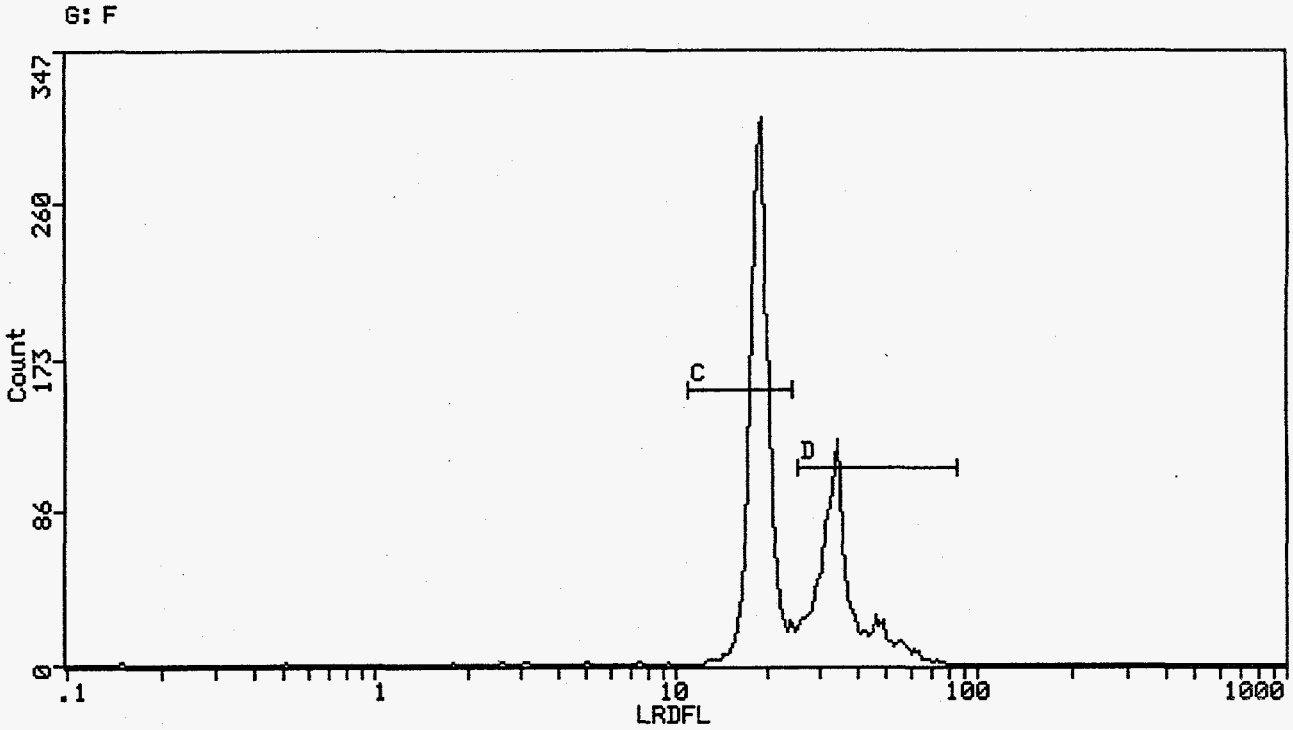
			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	61.2	5897	20	347	19.6	1.66	8.48	6.00	11	25
D	37.7	3634	35	138	36.7	7.58	20.7	6.15	26	88

DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	2.2	246	0, 4	20	2.9	3.1	107.3	9.8	5.4	55.5
F	87.7	9640	17, 33	104	26.0	12.1	46.5	34.6	3.7	10.7

SAMPLE NAME : STAINED 7AAD
SAMPLE NUMBER: TUMOR ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 11:56:46



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN				
DATA RATE	: 56	HIST COUNT	: 9,640	LIST FILE	: 40829T04.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED 7AAD			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	TUMOR ONLY			SAMPLE TIME:	11:56:46
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	61.2	5897	20	347	19.6	1.66	8.48	6.00	11	25
D	37.7	3634	35	138	36.7	7.58	20.7	6.15	26	88

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 88 D

SAMPLE NAME : STAINED 7AAD
SAMPLE.NUMBER: TUMOR ONLY

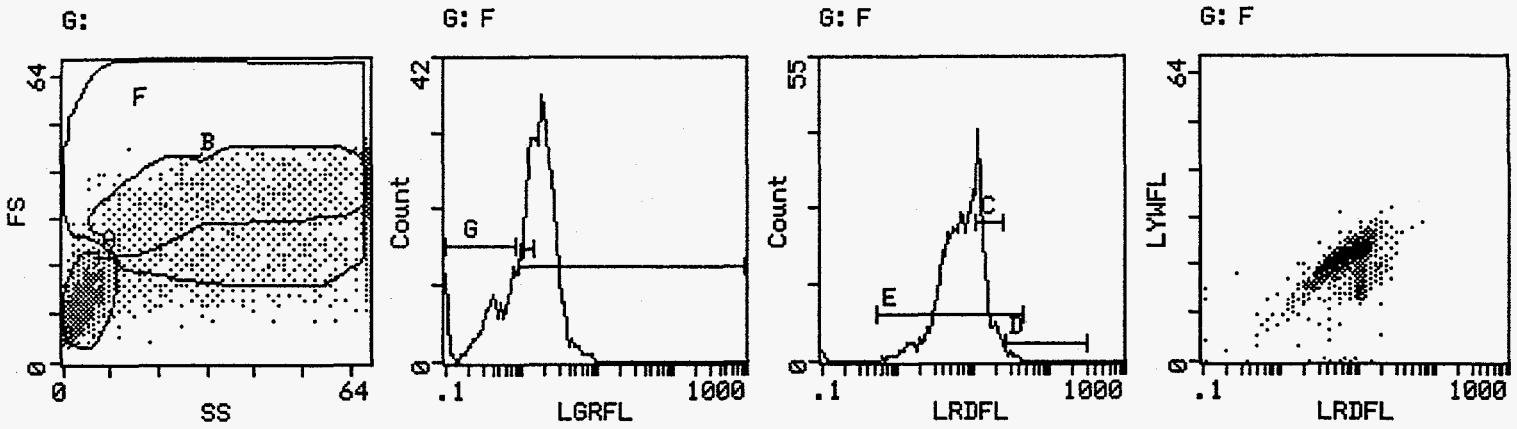
SAMPLE DATE: 29Aug94
SAMPLE TIME: 11:56:46

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 88 D
A A AMORPHOUS
F F AMORPHOUS

HISTOGRAM DISPLAY

SAMPLE NAME : STAINED 7AAD
SAMPLE NUMBER: SPUTUM CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:02:57



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
DATA RATE	: 527	TOTAL COUNT	: 14,782	LIST FILE	: 40829T05.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED 7AAD			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	SPUTUM CELLS ONLY			SAMPLE TIME:	12:02:57
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	27.0	1393	12	55	13.9	2.75	19.8	5.14	11	25
D	1.6	83	26	5	32.9	7.21	21.9	1.43	26	310

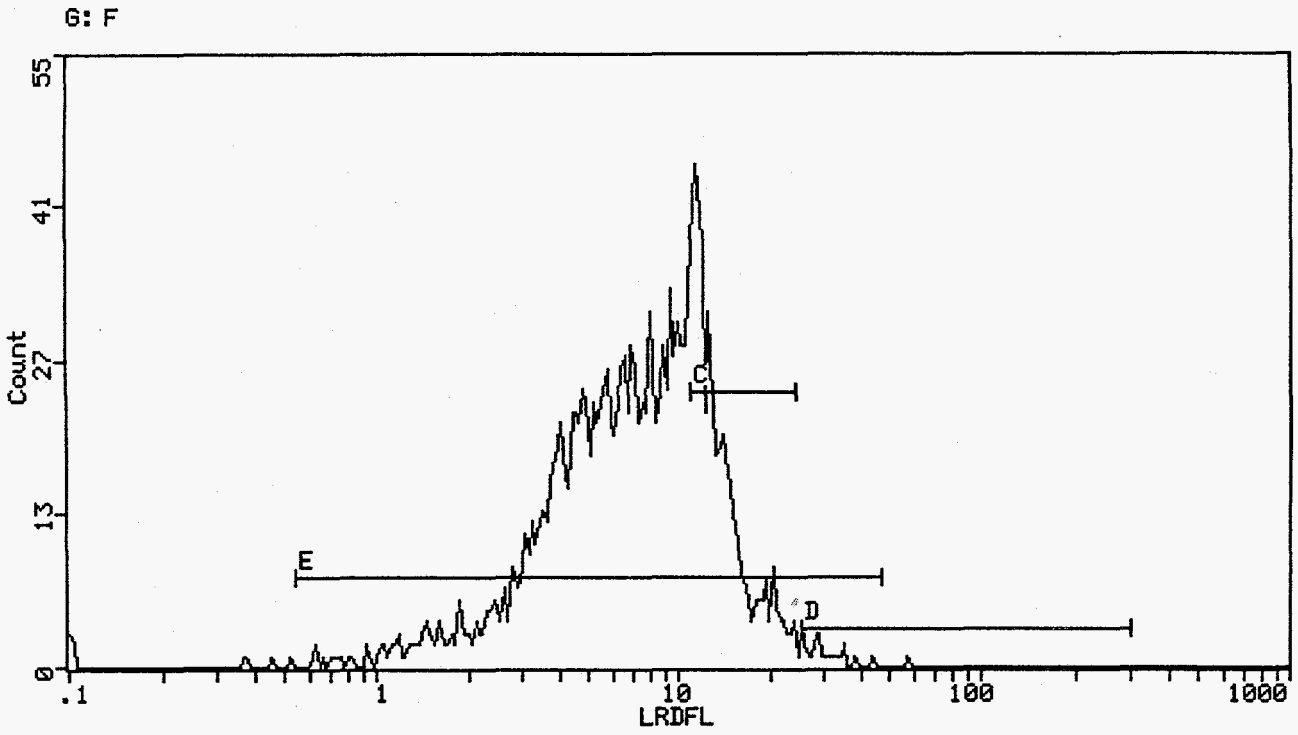
DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	53.1	7853	0, 4	294	3.7	2.8	75.2	10.9	4.8	44.4
F	34.9	5158	9, 22	20	33.6	15.6	46.4	29.3	7.0	23.8

GATES

SAMPLE NAME : STAINED 7AAD
SAMPLE NUMBER: SPUTUM CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:02:57



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN				
DATA RATE	: 527	HIST COUNT	: 5,158	LIST FILE	: 40829T05.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED 7AAD			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	SPUTUM CELLS ONLY			SAMPLE TIME:	12:02:57
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	27.0	1393	12	55	13.9	2.75	19.8	5.14	11	25
D	1.6	83	26	5	32.9	7.21	21.9	1.43	26	310
E	99.2	5118	12	55	7.20	4.80	66.7	5.14	0.55	48

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
E Low Channel = 0.55, High Channel = 48 E

SAMPLE NAME : STAINED 7AAD
SAMPLE NUMBER: SPUTUM CELLS ONLY

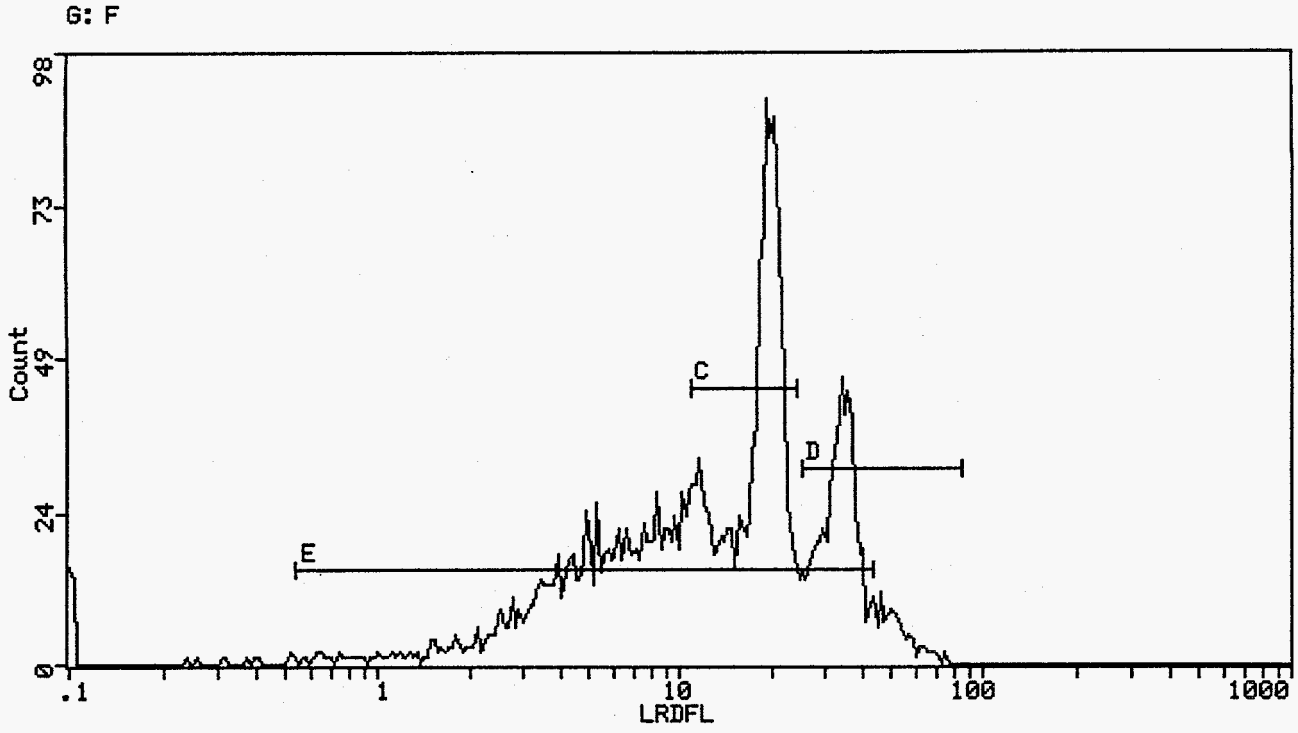
SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:02:57

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
A A AMORPHOUS
F F AMORPHOUS

GATES

SAMPLE NAME : STAINED 7AAD
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:07:01



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN				
DATA RATE	: 328	HIST COUNT	: 8,366	LIST FILE	: 40829T06.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED 7AAD			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	MIX 50:50 TUMOR/SPUTUM			SAMPLE TIME:	12:07:01
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

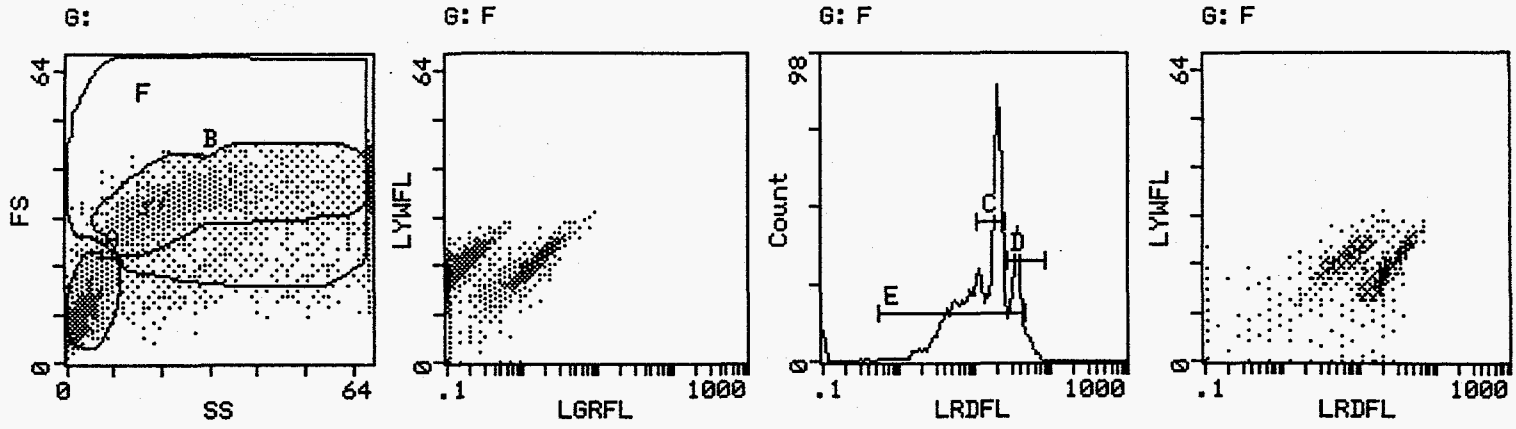
			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	40.2	3359	21	98	17.8	3.96	22.2	8.59	11	25
D	21.3	1786	35	57	36.8	7.84	21.3	7.41	26	88
E	94.8	7928	21	98	12.6	10.8	86.2	8.59	0.55	45

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 88 D
E Low Channel = 0.55, High Channel = 45 E

HISTOGRAM DISPLAY

SAMPLE NAME : STAINED 7AAD
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:07:01



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
DATA RATE	: 328	TOTAL COUNT	: 16,119	LIST FILE	: 40829T06.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED 7AAD			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	MIX 50:50 TUMOR/SPUTUM			SAMPLE TIME:	12:07:01
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	40.2	3359	21	98	17.8	3.96	22.2	8.59	11	25
D	21.3	1786	35	57	36.8	7.84	21.3	7.41	26	88

DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	36.3	5848	0, 4	206	3.6	2.8	76.6	10.7	4.9	45.4
F	51.9	8366	18, 33	41	28.4	14.2	49.9	31.2	6.2	20.0

SAMPLE NAME : STAINED 7AAD
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM

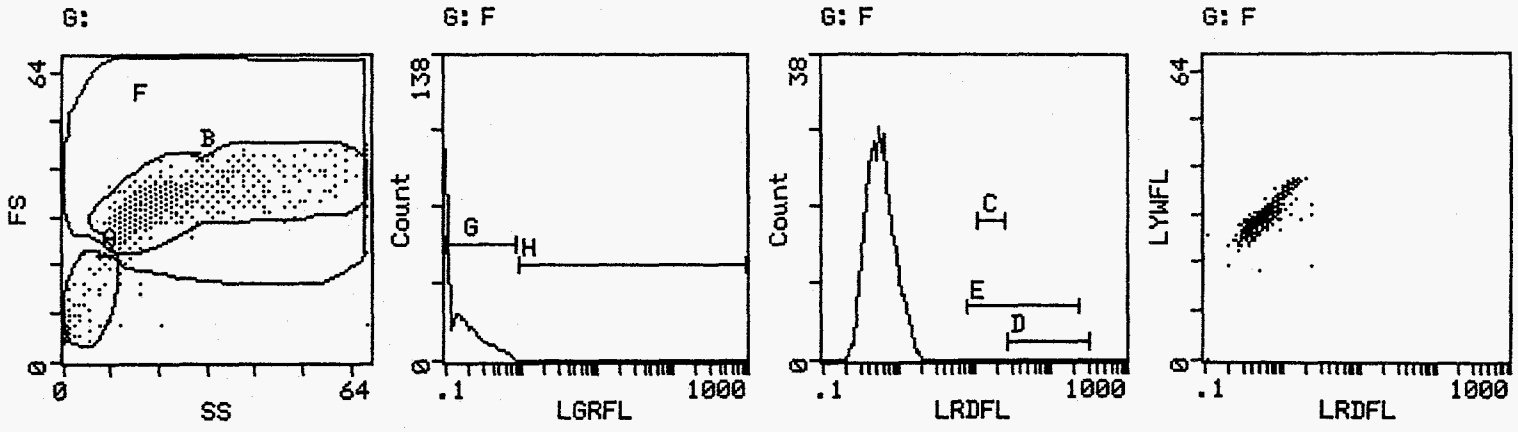
SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:07:01

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 88 D
A A AMORPHOUS
F F AMORPHOUS

HISTOGRAM DISPLAY

SAMPLE NAME : FITC NEG. CONTROL
SAMPLE NUMBER: TUMOR CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:28:13



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
DATA RATE	: 143	TOTAL COUNT	: 6,009	LIST FILE	: 40829T10.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: FITC NEG. CONTROL			SAMPLE DATE	: 29Aug94
SAMPLE NUMBER	: TUMOR CELLS ONLY			SAMPLE TIME	: 12:28:13
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

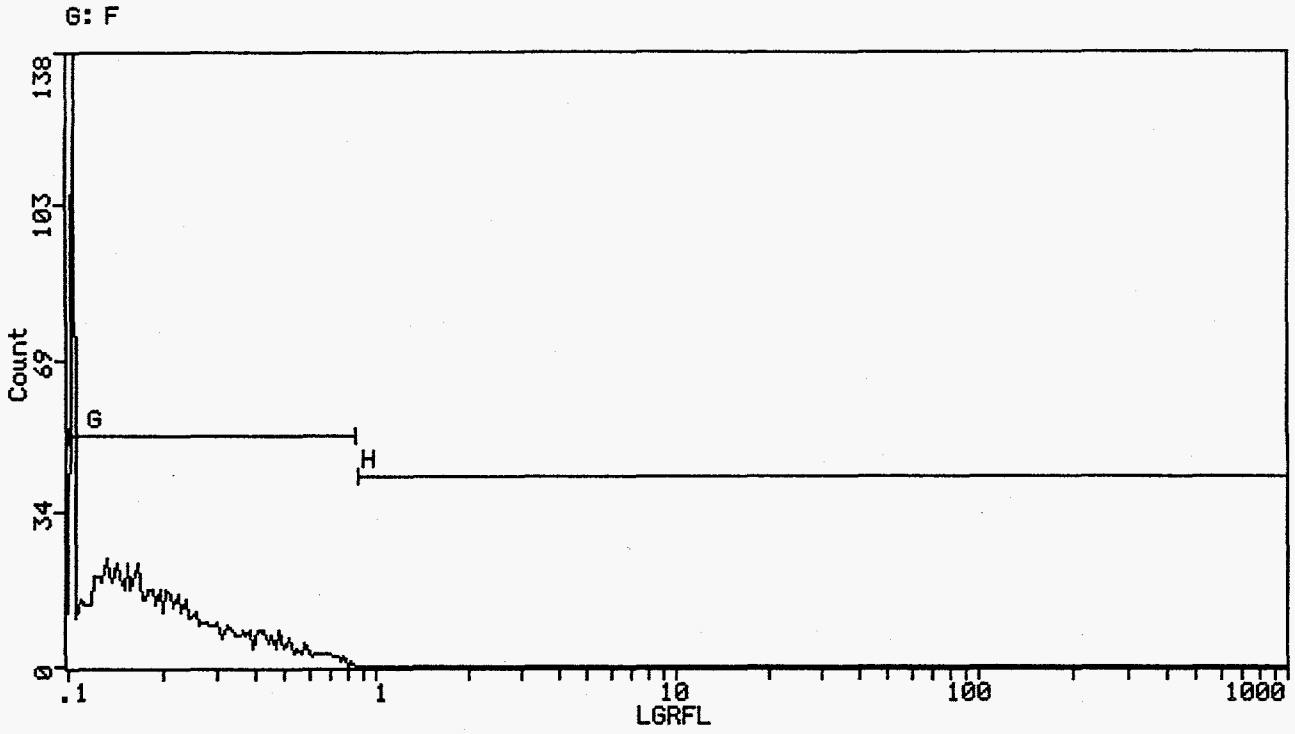
			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	0.0	0	****	*****	****	****	****	****	11	25
D	0.0	1	62	1	61.9	0.000	0.000	0.000	26	310

DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	39.9	2399	0, 4	1119	0.5	1.4	304.7	5.3	2.7	50.3
F	53.5	3214	16, 32	38	23.4	11.6	49.5	32.8	4.4	13.3

SAMPLE NAME : FITC NEG. CONTROL
SAMPLE NUMBER: TUMOR CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:28:13



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN				
DATA RATE	: 143	HIST COUNT	: 3,214	LIST FILE	: 40829T10.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: FITC NEG. CONTROL			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	TUMOR CELLS ONLY			SAMPLE TIME:	12:28:13
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
G	99.9	3212	0.11	627	0.180	0.096	53.0	0.447	0.10	0.87
H	0.1	2	0.89	1	1.03	0.144	14.0	0.382	0.89	1024

G Low Channel = 0.10, High Channel = 0.87 G
H Low Channel = 0.89, High Channel = 1024 H

SAMPLE NAME : FITC NEG. CONTROL
SAMPLE NUMBER: TUMOR CELLS ONLY

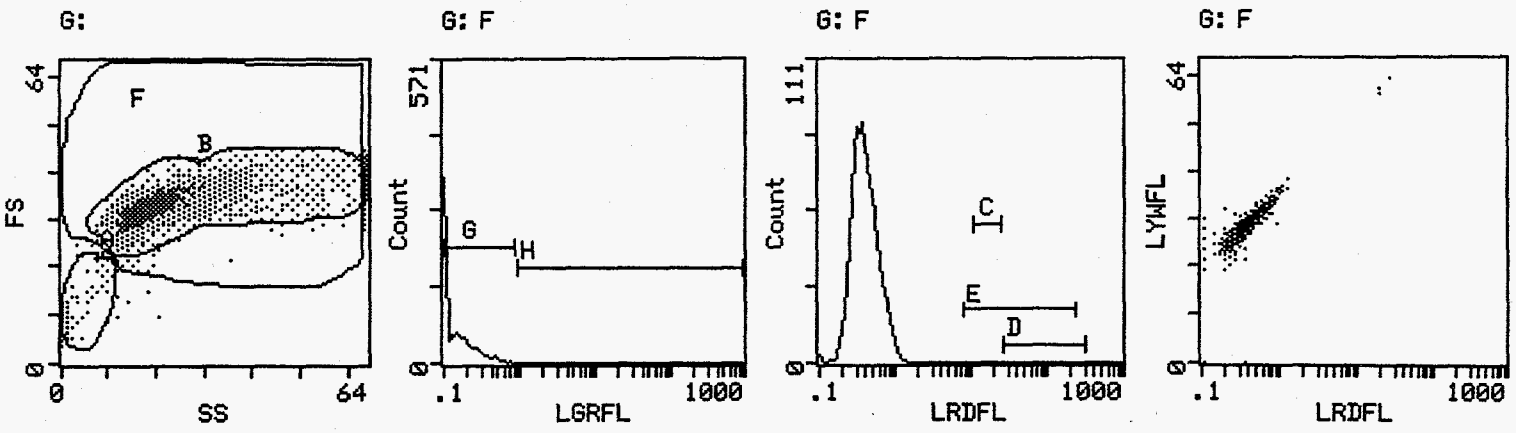
SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:28:13

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
A A AMORPHOUS
F F AMORPHOUS

HISTOGRAM DISPLAY

SAMPLE NAME : STAINED FITC
SAMPLE NUMBER: TUMOR CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:30:52



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
DATA RATE	: 496	TOTAL COUNT	: 10,420	LIST FILE	: 40829T11.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED FITC			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	TUMOR CELLS ONLY			SAMPLE TIME:	12:30:52
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	0.1	8	13	1	18.6	3.08	16.6	0.382	11	25
D	0.1	5	27	1	39.6	15.4	38.9	0.382	26	310

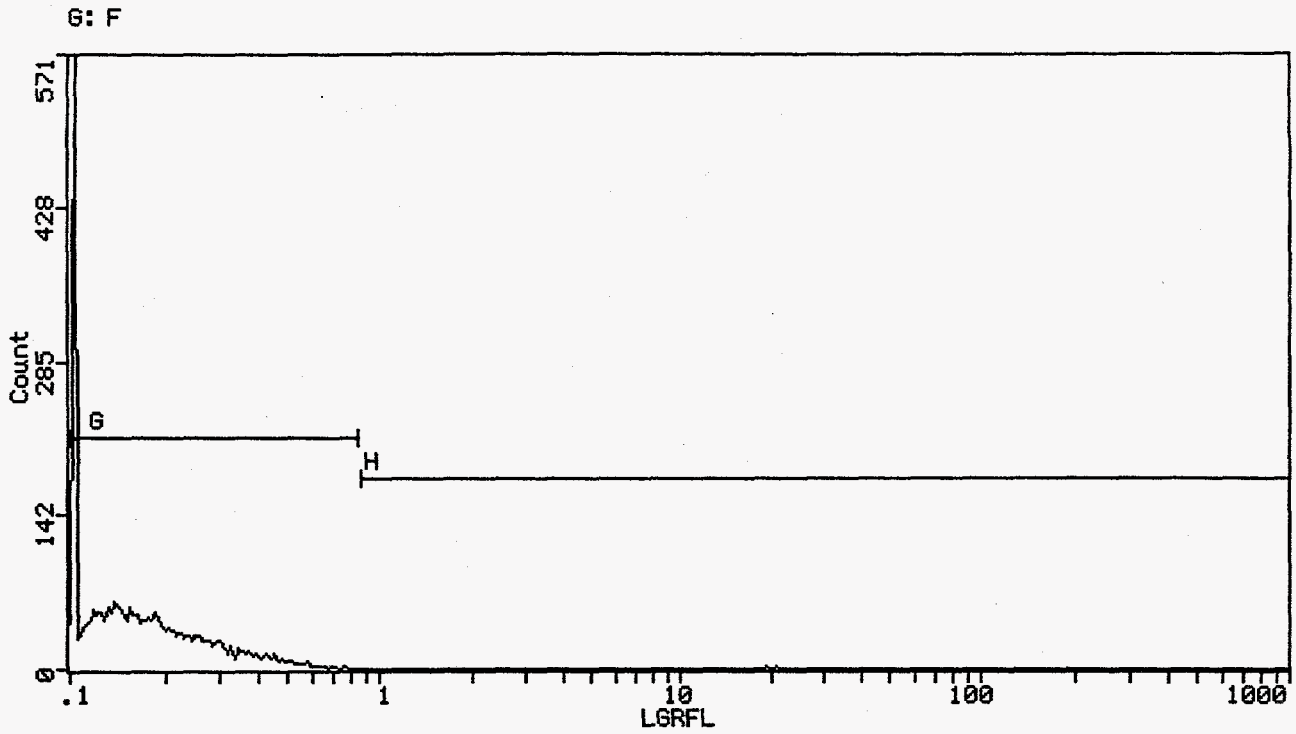
DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	8.9	926	0, 4	302	1.8	3.1	171.1	7.8	5.2	67.0
F	84.7	8824	16, 31	74	24.3	11.6	47.6	32.8	4.4	13.4

GATES

SAMPLE NAME : STAINED FITC
SAMPLE NUMBER: TUMOR CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:30:52



SAMPLE INFO

PROTOCOL	:	POR 1 - TOCKMAN			
DATA RATE	:	496	HIST COUNT :	8,824	LIST FILE : 40829T11.LMD
INSTRUMENT	:	Elite			PROT FILE : P0000034.PRO
SAMPLE NAME	:	STAINED FITC			SAMPLE DATE: 29Aug94
SAMPLE NUMBER:		TUMOR CELLS ONLY			SAMPLE TIME: 12:30:52
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
G	99.8	8809	0.11	2257	0.159	0.073	45.8	0.458	0.10	0.87
H	0.2	15	20	2	11.3	13.6	120.7	0.382	0.89	1024

G Low Channel = 0.10, High Channel = 0.87 G
H Low Channel = 0.89, High Channel = 1024 H

SAMPLE NAME : STAINED FITC
SAMPLE NUMBER: TUMOR CELLS ONLY

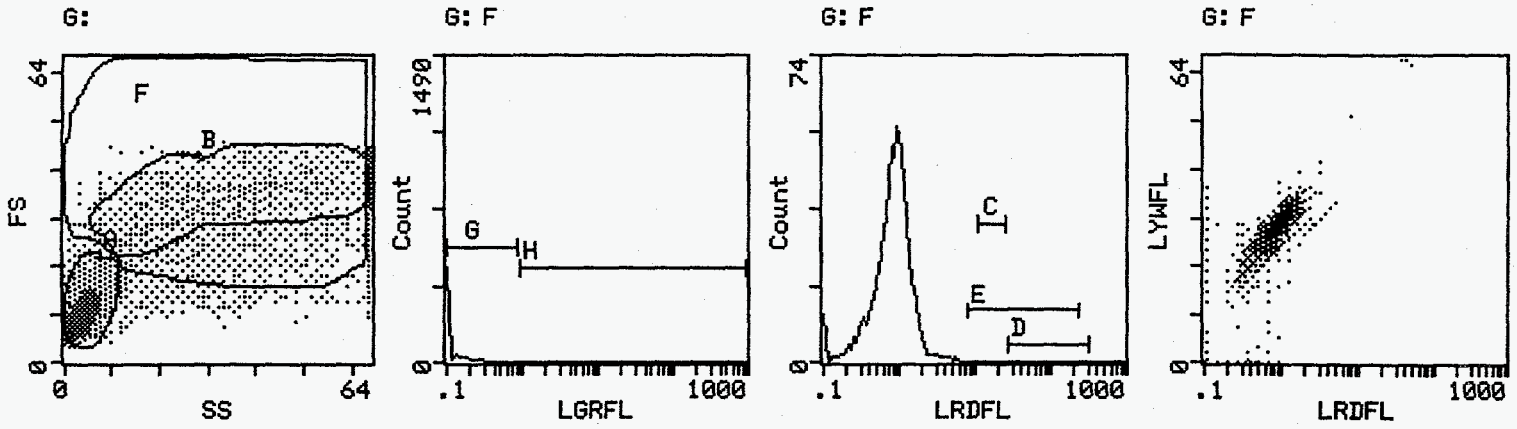
SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:30:52

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
A A AMORPHOUS
F F AMORPHOUS

HISTOGRAM DISPLAY

SAMPLE NAME : STAINED FITC
SAMPLE NUMBER: SPUTUM CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:32:37



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
DATA RATE	: 487	TOTAL COUNT	: 15,600	LIST FILE	: 40829T12.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED FITC			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	SPUTUM CELLS ONLY			SAMPLE TIME:	12:32:37
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	Half*CV	Min	Max
C	0.1	6	12	1	18.0	3.83	21.2	0.382	11	25
D	0.1	9	30	1	42.7	7.92	18.6	0.382	26	310

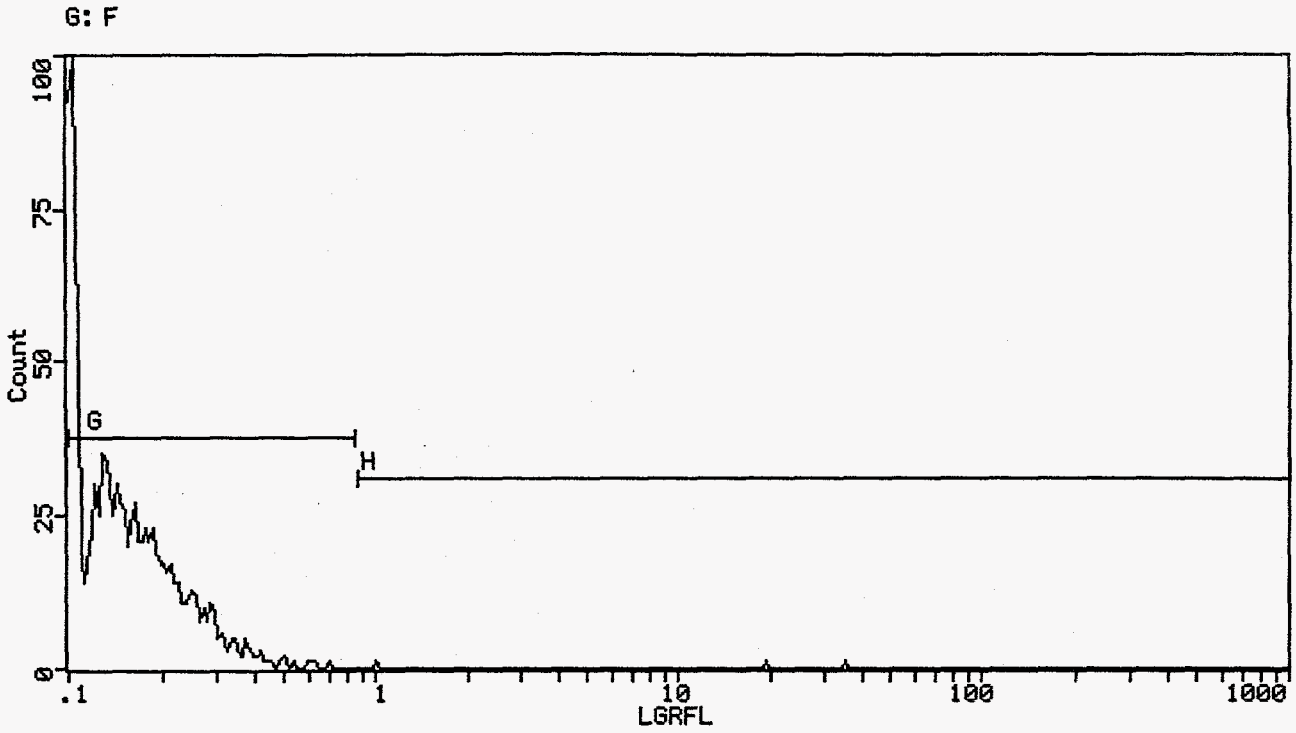
DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	43.9	6850	0, 4	352	3.5	2.7	76.3	9.7	4.6	47.5
F	42.0	6559	44, 37	17	32.9	14.4	43.8	29.8	7.2	24.2

GATES

SAMPLE NAME : STAINED FITC
SAMPLE NUMBER: SPUTUM CELLS ONLY

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:32:37



SAMPLE INFO

PROTOCOL	:	POR 1 - TOCKMAN			
DATA RATE	:	487	HIST COUNT :	6,559	LIST FILE : 40829T12.LMD
INSTRUMENT	:	Elite			PROT FILE : P0000034.PRO
SAMPLE NAME	:	STAINED FITC			SAMPLE DATE: 29Aug94
SAMPLE NUMBER:		SPUTUM CELLS ONLY			SAMPLE TIME: 12:32:37
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	Half CV	Min	Max
G	99.6	6533	0.11	1490	0.129	0.043	33.2	1.01	0.10	0.87
H	0.4	26	0.96	1	10.1	16.3	161.2	0.382	0.89	1024

G Low Channel = 0.10, High Channel = 0.87 G
H Low Channel = 0.89, High Channel = 1024 H

SAMPLE NAME : STAINED FITC
SAMPLE NUMBER: SPUTUM CELLS ONLY

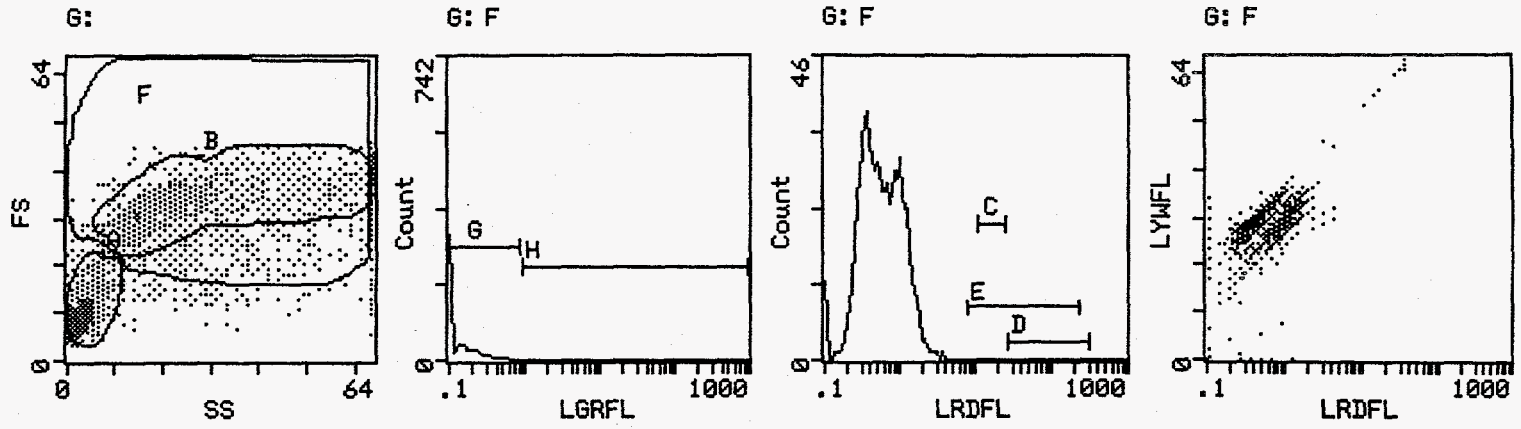
SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:32:37

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
A A AMORPHOUS
F F AMORPHOUS

HISTOGRAM DISPLAY

SAMPLE NAME : STAINED FITC
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:34:59



SAMPLE INFO

PROTOCOL	: POR 1 - TOCKMAN			LAST HIST	:
DATA RATE	: 836	TOTAL COUNT	: 12,543	LIST FILE	: 40829T13.LMD
INSTRUMENT	: Elite			PROT FILE	: P0000034.PRO
SAMPLE NAME	: STAINED FITC			SAMPLE DATE:	29Aug94
SAMPLE NUMBER:	MIX 50:50 TUMOR/SPUTUM			SAMPLE TIME:	12:34:59
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	0.2	10	11	1	17.3	4.44	25.7	0.764	11	25
D	0.2	14	33	2	38.9	8.79	22.6	0.382	26	310

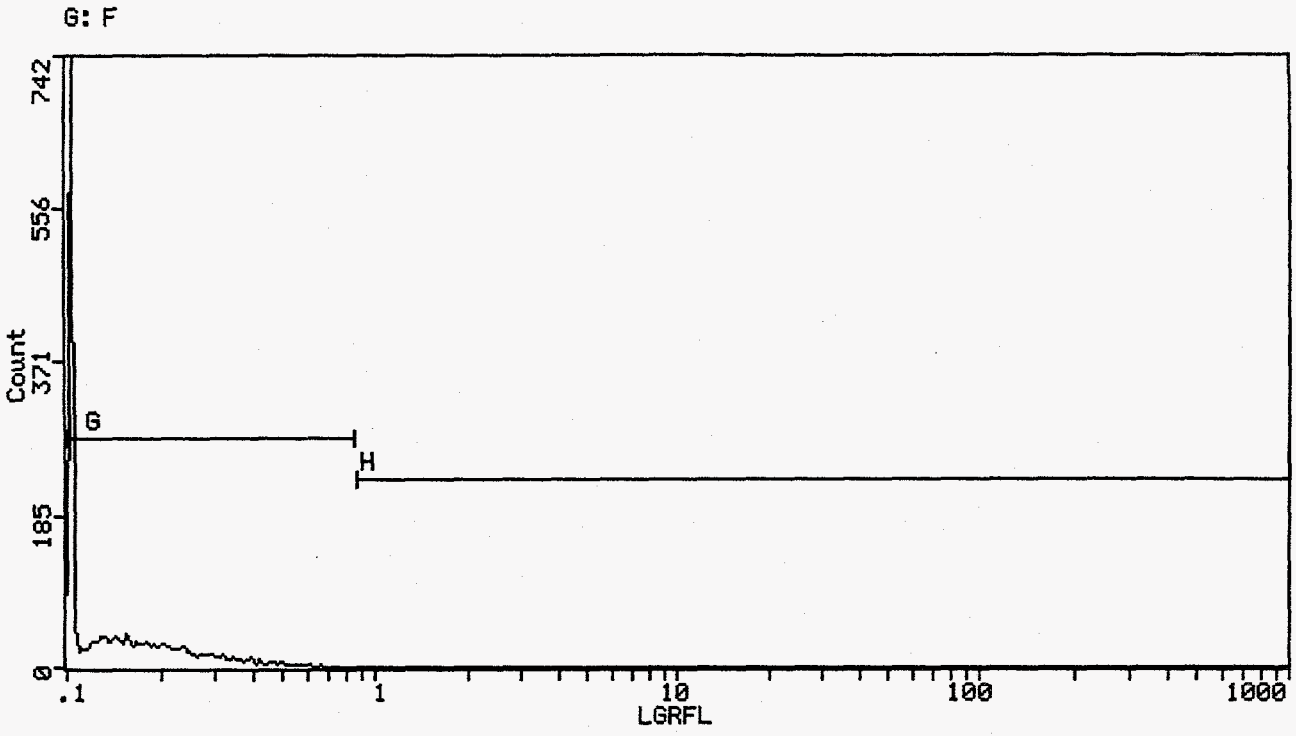
DUAL PARAMETER STATISTICS

			Peak		X Channel			Y Channel		
ID	Pcnt	Area	Position	Height	Mean	SD	CV	Mean	SD	CV
A	40.9	5133	0, 4	410	3.2	2.7	85.5	9.4	4.7	49.7
F	50.0	6275	14, 31	35	27.0	14.2	52.3	31.0	6.1	19.7

GATES

SAMPLE NAME : STAINED FITC
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:34:59



SAMPLE INFO

PROTOCOL	:	POR 1 - TOCKMAN			
DATA RATE	:	836	HIST COUNT : 6,275	LIST FILE :	40829T13.LMD
INSTRUMENT	:	Elite		PROT FILE :	P0000034.PRO
SAMPLE NAME	:	STAINED FITC		SAMPLE DATE:	29Aug94
SAMPLE NUMBER:		MIX 50:50 TUMOR/SPUTUM		SAMPLE TIME:	12:34:59
COMMENTS	:				

STATISTICS

SINGLE PARAMETER STATISTICS

			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
G	99.5	6243	0.11	1318	0.150	0.066	44.2	0.700	0.10	0.87
H	0.5	32	0.96	1	18.1	20.4	112.8	0.382	0.89	1024

G Low Channel = 0.10, High Channel = 0.87 G
H Low Channel = 0.89, High Channel = 1024 H

SAMPLE NAME : STAINED FITC
SAMPLE NUMBER: MIX 50:50 TUMOR/SPUTUM

SAMPLE DATE: 29Aug94
SAMPLE TIME: 12:34:59

C Low Channel = 11, High Channel = 25 C
D Low Channel = 26, High Channel = 310 D
A A AMORPHOUS
F F AMORPHOUS