Biologistics of lung cancer screening and management using field-effect of carcinogenesis and a novel biophotonics technique

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A. Research justification

According to the University of Memphis Biologistics Research Cluster, "biologistics is the management of the safe flow of high value, temperature sensitive and time-critical biological materials as they are delivered for patient care, analyzed for diagnostic purposes, processed to higher value products or stored to meet physical and data archival needs." In that context, the *biologistics* of early cancer detection and management is a preeminent challenge in modern medicine. In response, researchers are striving to understand biological structures and processes at the nanoscale, in particular the strong correlation between changes in intracellular nanoarchitecture related to basic cellular materials, such as DNA, RNA, lipids, and proteins, and the earliest stages of progressive carcinogenesis. Our group has recently developed an optical experimental technique based on a key principle of physics: mesoscopic light transport. This technique, termed partial wave spectroscopy, or PWS, is capable of detecting, for the first time, nanoscale alterations in a cell during early cancer progression. Preliminary results show that we can detect signs of ultra-early carcinogenesis in lung, colon, pancreas, esophagus and ovary cells by fast optical imaging. Moreover, we can quantify these nanoscale alterations, using an algorithm described later in this proposal. Importantly, based on the field effect of carcinogenesis, we can detect cancer from an easily accessible surrogate, far from the actual cancer site, but on the same epithelial track. For example, we were able to detect lung cancer from buccal cells, colon cancer from rectal cells, and ovarian cancer from cervical cells.

The PWS optical technique is cost-effective and can be used for fast population-based mass screening of several cancers. Important to this proposal, it has shown sensitivity and specificity of ~80% for field effect detection from their corresponding surrogate sites. However, for practical and optimal use, we require ultra-sensitivity of the technique, ~99% sensitivity and specificity, to absolutely meet *biologistics* requirements for early cancer screening and management for a large population. To accomplish this, we propose further engineering to modify/improve the present PWS optical system to enhance light signals scattered from nanoscale alterations by using a thin metallic cavity incorporated into the present PWS technology. Using this optimized (enhanced) PWS (EPWS) platform, we will be well positioned to concentrate on the *biologistics* of early lung cancer detection and management. Lung cancer has one of the highest mortality rates in the U.S., justifying the urgency of this screening technology. Our research objectives are the following:

***Objective 1*: Develop enhanced partial wave spectroscopy (EPWS) instrumentation for the fast screening of early lung cancer:** (i) design and incorporate a semi-transparent metallic cavity as a biological cell holder into the present PWS system and characterize this cavity-induced partial wave signal enhancement from nanoscale spatial refractive index fluctuations in the cell; (ii) optimize cavity, cell, and staining parameters for fast detection and screening.

***Objective 2*: Validate detection of lung cancer from buccal/cheek cells with high sensitivity and specificity to meet biologistics requirements for early cancer screening and cancer management:** As a pilot study, we will use EPWS to detect lung cancer in human subjects (n=50 lung cancer and n=100 normal) by collecting cells from (i) affected areas in lung biopsy (for cancer confirmation) and (ii) cheek /buccal cells, far from the affected areas, to test our method against the *"field effect"* of carcinogenesis.

B. Theoretical basis

Diffraction limit restricts the resolution of conventional light microscopy to, at best, ~ 200 nm, but this is larger than the sizes of the fundamental building blocks of the cell, such as membranes,

cytoskeleton, ribosome and nucleosome. *As a result, conventional light microscopy is insensitive to nanoscale changes in cell structure caused by genetic/epigenetic events.* However, when mesoscopic light transport theory is applied to this problem, we find that the partial wave back reflected signal is sensitive to any length scale of refractive index fluctuations, including those below the wavelength of light. This means that detection of changes in intracellular structural disorder can be easily detected down to ~20 nm. Based on this principle, we earlier designed an optical partial wave spectroscopy (PWS) technique [1-2] to measure and quantify the optical spectra of partial waves propagating in a biological cell. Our PWS studies of both human and animal cancer models show that intracellular nanoscale alterations (~10-100nm) are one of the earliest events in carcinogenesis [1-3]. Computationally, PWS measures the **degree of structural disorder** by the parameter $\boldsymbol{Ld = \Delta n^2 \times Lc}$, where Δn is strength of the refractive index fluctuation (proportional to the mass density fluctuations/nanoalteration), and Lc is its nanometer scale correlation length Lc.

Field effect of carcinogenesis may be defined as genetic/epigenetic alterations that occur in a lesion in one area of an organ, but can be detected throughout that organ. For example, lung cancer can be detected from cheek/buccal cells or colon cancer from rectal cells.

B.1. ***Successful implementation of partial wave spectroscopy (PWS) for early detection of cancer at the primary site or using field effect (PWS earlier results) far from primary sites:***

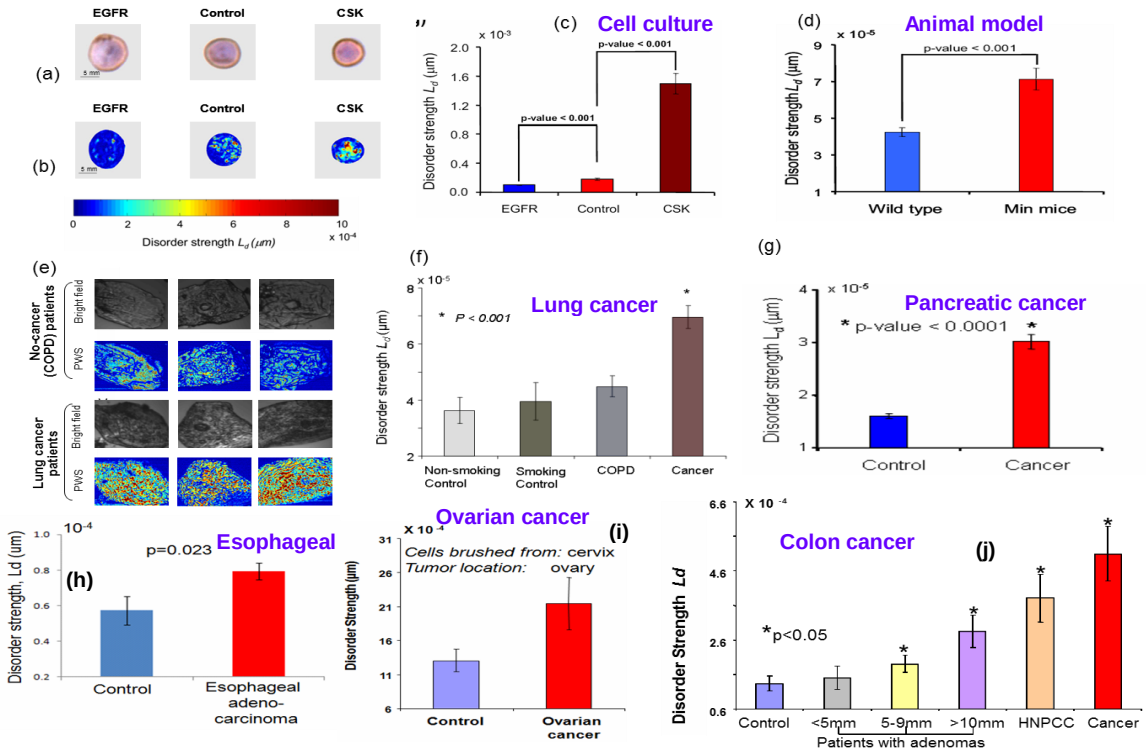

Figure 1(a-c): Histologically indistinguishable HT-29 cell lines (EGFR-knockdown, empty vector control, and CSK-knockdown) **(a)** and their PWS images **(b)**; Disorder strength of cell nanoarchitecture correlates with the neoplastic potential in these cells **(c).** **(d)** Disorder strength differences among histologically normal appearing intestinal cells in the MIN-mouse model of colon cancer. **(e-f)** Disorder strength is a marker of field effect associated with lung cancer, as detected by changes in buccal cells; bright-field image and PWS *Ld* image **(e)** and *Ld* values correlate with the degree of carcinogenesis **(f)**. **(g)** Pancreatic cancer**,** as detected in duodenal cells. **(h)** Ovarian cancer, as detected in cells collected from cervix. **(i)** Esophageal cancer, as detected from cells taken from throat. **(j)** Colon neoplasia, as detected in rectal cells.

Original Partial Wave Spectroscopy (PWS): Based on mesoscopic physics, a sub-discipline of condensed matter physics which involves materials of an intermediate length scale, and the application of optical imaging through PWS, we can quantify the degree of intracellular nanoscale structural disorder, or

$Ld=<\Delta n^2>Lc$, where Δn is the strength of the refractive index fluctuations at the nanometer scale, and Lc is its correlation length, which is beyond the diffraction limitation of ordinary microscopy.

PWS detects nanoarchitectural alterations in microscopically normal pre-neoplastic cells (1) Cell cultures (HT2 and its genetic variants EGFR, and CSK): [1-3] We used the small hairpin RNA (shRNA) (gene silencing) approach against a tumor suppressor gene, c-terminal src kinase (CSK), and the proto-oncogene epidermal growth factor receptor (EGFR) in human colon cancer cell line HT-29. Thus, the three cell lines were microscopically indistinguishable (Fig. 1(a)). However, the PWS parameter, *Ld*, as explained earlier, was markedly altered ($p<0.01$, Fig. 1(b,c)) such that the least aggressive (EGFR knockdowns) cells could be distinguished from intermediate (empty vector controls, HT29) cells and those, in turn, could be distinguished from the most aggressive cells (CSK knockdowns). ***(2) Animal studies (MIN-mouse model):*** *Ld* increase appears to be a common event in cells undergoing neoplastic transformation. Compared to wild-type mice before the actual development of neoplastic lesions, *Ld* was markedly increased in otherwise normal appearing intestinal cells in the MIN-mouse model of intestinal carcinogenesis (6-week-old mice, $p<0.01$, Fig. 1(d)). ***(3) Human studies (i. colon, ii. pancreas, iii. lung, iv. ovarian, and v. esophageal cancers):*** Results show that nanoarchitectural changes are not restricted to tumor cells, but rather, they are present outside of (pre) cancerous lesions, as well. Figure 1(e-j) shows results of the following human cancers*: lung, pancreatic, esophageal, ovarian, and colon cancer*. For lung cancer, we analyzed buccal cells from patients with and without lung cancer (n=127). The controls were smokers with COPD (Fig. 1(e)-(f)). *Ld* was also increased in duodenal periampullary cells in patients with pancreatic cancer (n=35, $p<10\text{-}4$, Fig. 1(g)). For esophageal cancer, the data taken from the throat also show an increase in *Ld* (Fig. 1(h)). For ovarian cancer, cells were collected from the cervix. *Ld* values increased from control to cancer (Fig. 1(i)). In colon cancer, *Ld* was increased in rectal cells in patients who harbored adenomas elsewhere in the colon when compared to neoplasia-free patients (n=35, $p<10\text{-}4$, Fig. 1(J)).

C. Research methodology

C.1. Objective 1: *Develop enhanced partial wave spectroscopy (EPWS) instrumentation for the fast screening of early lung cancer:*

C.1.1. Development of proposed EPWS instrumentation based on encouraging preliminary results: As shown in Figure 2, we have designed real-time EPWS instrumentation which can be directly and efficiently used in the clinical environment [1-2]. A 150 W Xenon lamp is combined with an intensified CCD camera to reduce acquisition time. White light from the Xenon lamp (150 w, Oriel) is collimated by a 4f-lens relay system and focused onto a sample by a low numerical aperture (NA) objective (NA = 0.4, Edmund Optics). The backscattered image is projected through the liquid crystal tunable filter (CRI,

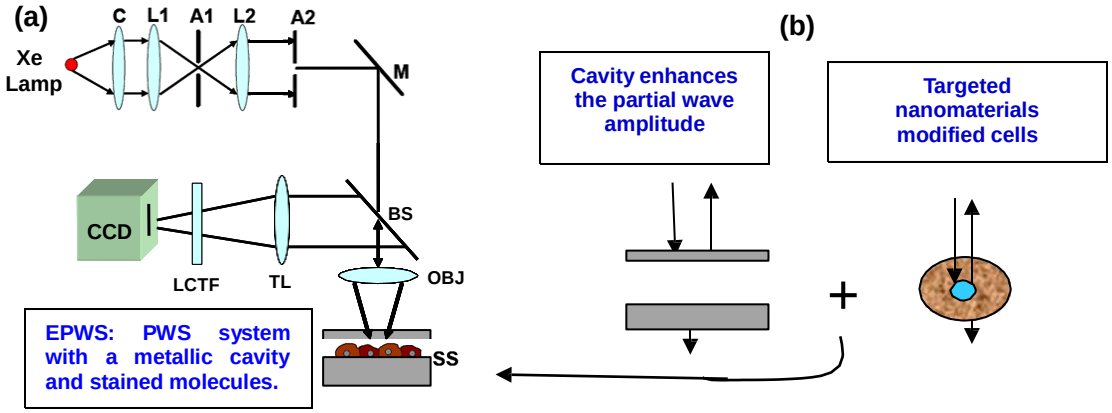

Figure 2. EPWS spectroscopy: **(a)** EPWS technique by inserting semi-transparent metallic cavity in PWS system as a biological cell holder. **(b)** Schematic illustration of the cells within a dielectric/metal cavity. Intracellular molecular materials will be targeted by using dye/nanoparticles staining protocols and then subsequently mounting the stained cell in the metal cavity.

Inc.) onto a CCD camera. The liquid crystal tunable filter (LCTF) replaces the spectrograph and scanning stage combination to obtain the image of a cell at a specified wavelength range (500-700 nm). This instrument scans (~ 5 sec) the entire cell by obtaining a snapshot of a single wavelength.

C.1.2. *Theoretical and experimental support of enhanced backscattering by EPWS:*

Enhancement in detection of Ld by using metal-cavity and staining protocol (experimental): In Figure 3(a), the disorder properties of unstained cells relative to stained cells can be seen. When the thin cell is stained by CytoStain, the dye induces an enhancement in nanoscale refractive index fluctuation, or the degree of disorder. Concurrently, the *Ld* map (Figure 3 (b)) clearly shows the increase in the disorder strength parameter *Ld* over an order of magnitude for the stained cells relative to the unstained cells. By inserting a metallic cavity as a sample holder, the degree of disorder of a stained cell can be further enhanced an order higher relative to the stained cell without a cavity.

Result summary: Overall, therefore, the experimental results show that the staining increases the refractive index fluctuations of a targeted organelle and that a stained cell in a cavity further enhances the signal from the nanoscale refractive index fluctuations. This significantly increases the structural disorder parameter *Ld*, in turn increasing specificity and sensitivity.

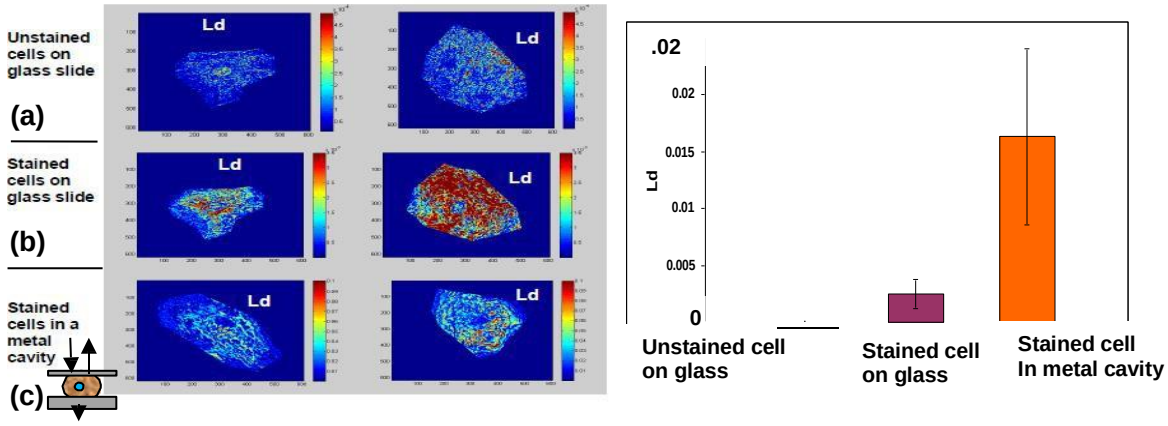

Figure 3. **(a)** The effect of staining on squamous (~1um thickness) cells. Unstained cells have a weak effective *Ld* value. **(b)** In contrast, the stained cells (same cells as **(a)**) show a signal such that disorder strength can be differentiated from the background. **(b)** The additional increase of the effective disorder parameter (*Ld*) with the stained cells kept in a metal cavity (EPWS**).**

C.1.3. *Completion, Calibration, and Optimization of EPWS instrumentation of* ***Objective 1:***

We are in the process of designing and optimizing *enhanced partial wave spectroscopy* (EPWS), and initial results look quite promising for achieving the desired sensitivity. However, we want to further incorporate a metallic cavity designed for real-time application in clinical settings. *1) Cell mounting on metal-coated glass slide and covered with another metal-coated glass slide for cavity formation and optimization:* In preliminary EPWS experiments, cells were mounted on metal-coated glass slides and subsequently covered by a thin metallic coating using metal (Al) sputtering. In a clinical setting, we would use a cover slip with antireflective metal coating. To this end, we will try different types of metal-coated (Al, Au, Ag) glass slides or combinations thereof for maximal EPWS signal enhancement. *2) Method of calculation of Ld from EPWS experiments:* The EPWS instrument, as described, generates a data cube $R(k;x,y)$, where k is the wave number, (x,y) are the spatial coordinates of the pixel, and $< R >$ is the root mean square average of the fluctuating part of the backscattering spectrum. For a given pixel, $L_d = \frac{B < R >}{2k^2} \frac{(\Delta k^2)}{-\ln(C(\Delta K))}\Big|_{\Delta k \to 0}$, where B is the normalization constant and $C(\Delta k)$ is the autocorrelation function of R. The acquisition time for each cell is ~1 min. The data will be collected, and the mean and standard deviation of *Ld* will be calculated for the cell.

Optimization of EPWS: To achieve maximum nanoscale detection sensitivity from nanoscale

refractive index contrast, depending on cell sizes, we would optimize cavity dimension, as well as metallic thin film thickness.

Expected results from Objective 1: By combining the semi-transparent metallic cavity and staining protocol, as described above, with concomitant optimization of system parameters, we expect sensitive detection and quantification of nanoscale intracellular changes (i.e., degree of structural disorder), as reflected by *Ld* values, in buccal cells undergoing ultra-early progressive lung cancer.

C.2. Objective 2: *Validate detection of lung cancer from buccal/cheek cells with high sensitivity and specificity to meet biologistics requirements for early cancer screening and cancer management*

Having validated the performance of EPWS in *Objective 1*, we will proceed to detect lung cancer in human subjects with higher prediction of sensitivity and specificity with an ultimate goal of ~99%. *(i) Cell collection from patients:* We will use human samples collected by our collaborating pathologists, Drs. Azouz and Chauhan, (n=50 lung cancer patients in different stages and n=100 normal). Cells will represent (a) primary affected areas through lung biopsy and (b) field effect through cheek/buccal cells collected far from the primary affected areas [3]. All standard IRB procedures will be followed, as well as obtaining patients' informed consent for biopsy and cell sample collection. *(ii) "Field Effect" in lung carcinogenesis:* [3] Many screening techniques are designed to exploit the field effect of lung carcinogenesis. This proposition holds that the genetic/environmental milieu that results in neoplasia in one region of the lung should be detectable throughout the respiratory track, including buccal cells. *(iii) Cell collection and preparation for EPWS experiments: Degree of structural disorder Ld and cancer detection:* Buccal/cheek cells will be collected via a cytological brush and will be kept in the metallic cavity for EPWS experiments. *(iv) Structural disorder Ld measurement and early stage cancer detection:* We will calculate *Ld* from buccal cells using results of the optimized EPWS experimental setup, as described above, for normal and cancer patients.

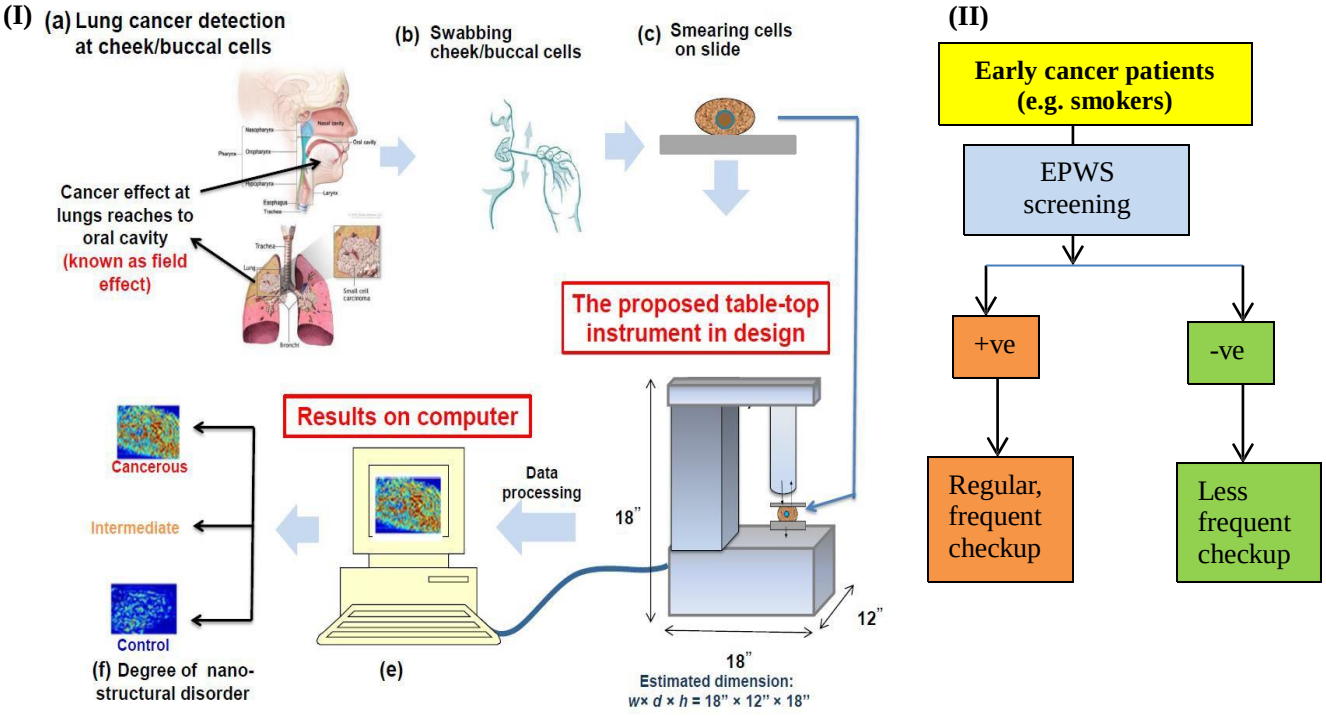

Figure 4: Schematic. Biologistics of (I) early lung cancer detection, and (II) management using epithelial buccal cells.

Expected results for Objective 2: This pilot study using buccal cells will confirm changes in structural disorder as carcinogenesis progresses in human lung cancer at both the primary site of lesion and distant site using buccal epithelial cells to test field effect. This will confirm the practicality of our method for

screening/detection and management of lung cancer in the context of biologistics, as shown in the schematic flow chart in Fig.4.

D. Anticipated contribution to theory and practice

We expect to develop our novel EPWS technology as a low-cost, ultra-sensitive, and clinically functional tool able to detect and screen nanoscale structural changes inside cells undergoing early carcinogenesis, in particular, lung cancer detection from cheek or buccal cells. We expect that EPWS will meet biologistics criteria for fast cancer screening and management. We have chosen lung cancer screening because it is statistically one of the highest reported cancers in the state of Tennessee and, indeed, worldwide. Currently, no well-defined lung cancer screening test is available. Therefore, it is anticipated that this proposed pilot research project will provide a new direction for the early detection and prevention of lung cancer in line with the mission of the University of Memphis Biologistics Research Cluster.

Project Management: Dr. Pradhan (PI) and his BioNanoPhotonics group at the Department of Physics, University of Memphis, will work on overall management of the project. The PI will perform all EPWS experiments in his recently renovated, state-of-the-art Biophotonics Lab. Dr. Pradhan is an expert in cancer detection using biophotonics. As explained in this proposal, EPWS is an engineering refinement of PWS, which was developed and introduced earlier by the PI in relation to early cancer detection. Lung cancer samples will be collected by our collaborating pathologists, Dr. Abdallah Azouz and Dr. Subhash Chauhan, University of Tennessee Health Science Centre (UTHSC), Memphis. Two graduate students and two undergraduate students will work on the project.

References:

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