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Substrate evaluation for a microbeam endstation using unstained cell imaging

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ABSTRACT

A cellular imaging system, optimized for unstained cells seeded onto a thin substrate, is under development. This system will be a component of the endstation for the microbeam cell-irradiation facility at the University of Surrey. Previous irradiation experiments at the Gray Cancer Institute (GCI) have used Mylar™ film to support the cells [Folkard, M., Prise, K., Schettino, G., Shao, C., Gilchrist, S., Vojnovic, B., 2005. New insights into the cellular response to radiation using microbeams. Nucl. Instrum. Methods B 231, 189–194]. Although suitable for fluorescence microscopy, the Mylar™ often creates excessive optical noise when used with non-fluorescent microscopy. A variety of substrates are being investigated to provide appropriate optical clarity, cell adhesion, and radiation attenuation. This paper reports on our investigations to date.

1. Introduction

Cell-irradiation studies that use poorly penetrating radiations such as α-particles and low-energy X-rays frequently use sparse or confluent cell monolayers attached to polymer films (Folkard et al., 2005; Tartier et al., 2007). A new imaging system is being developed that will be optimized for imaging cellular and sub-cellular features for unstained cells seeded onto a thin substrate. This system will form a component of the endstation development for the new microbeam cell-irradiation facility at the University of Surrey (Folkard et al., 2008; Kirkby et al., 2007).

During irradiation experiments, the cells are seeded onto a thin substrate and radiation is applied from below. The first step in the development of this imaging system requires that an appropriate substrate on which to seed the cells is chosen. A variety of substrates are being investigated to meet the requirements of the imaging system.

2. Motivation

For current microbeam studies at GCI, a 0.9–3.0 μm thick Mylar™ film (polyethylene terephthalate) or 4.0 μm thick polypropylene film is used as a substrate to support the cells during irradiation. In some circumstances these substrates are pre-coated with agents to improve cell attachment. An epi-illuminating imaging system locates the position of the cells, viewing from above, while targeted irradiation is applied from below. DNA-binding dyes and epi-fluorescence microscopy are used to locate individual cell nuclei. Currently, the Hoechst DNA-binding dyes are used which require UV illumination. This combination of dye and UV exposure can introduce unwanted cell toxicity into experiments if not carefully controlled (Folkard et al., 1997; Schettino et al., 2001; Gault et al., 2007). For this reason, it is desirable to eliminate these factors through the development of an unstained cell imaging system.

When Mylar™ is imaged with non-fluorescence microscopy methods, excessive optical noise is present due to the granular structure of the Mylar™ film, and there are difficulties in imaging and delineating the cells from the Mylar™ foil. Also, with white-light imaging and in the absence of phase contrast, it is not possible to determine the presence of sub-cellular structures such as the nucleus. Fig. 1 shows the difference between an epifluorescent and a phase contrast image of HeLa cells seeded onto Mylar™. The Hela cells were grown in EMEM media (Cambrex, UK) supplemented with 2 mmol/L L-glutamine, 100 units/mL penicillin, and 10% fetal bovine serum (FBS). The cells were seeded onto the substrates and incubated for 4 h at 37 °C with 5% CO_2. Hoechst 33342 nuclei dye (0.2 μmol/L) was added to the cell dish and then incubated for an additional 15 min. The media was replaced with EMEM supplemented with the above stated quantities of L-glutamine and penicillin.

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A variety of substrates are being investigated to minimize the optical noise present in the images. Additionally, the energy loss through the substrates and the cell adhesion of the substrates are under investigation.

3. Substrate properties

Phase contrast microscopy has been used to determine the optical suitability of the substrates for our application. A variety of

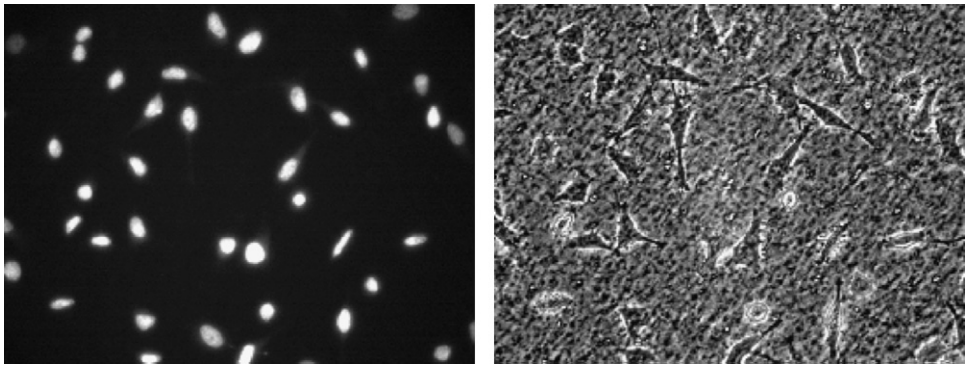

Fig. 1. Images acquired using wide-field epi-fluorescence microscopy and phase contrast microscopy; the left image shows the nuclei of HeLa cells, stained with Hoechst 33342, and seeded onto a 3 μm thick Mylar™ film. The right image shows the same cells imaged using phase contrast microscopy. A 20 × /0.4 NA objective was used for the imaging. The difficulty in distinguishing sub-cellular features from the cell dish substrate is apparent.

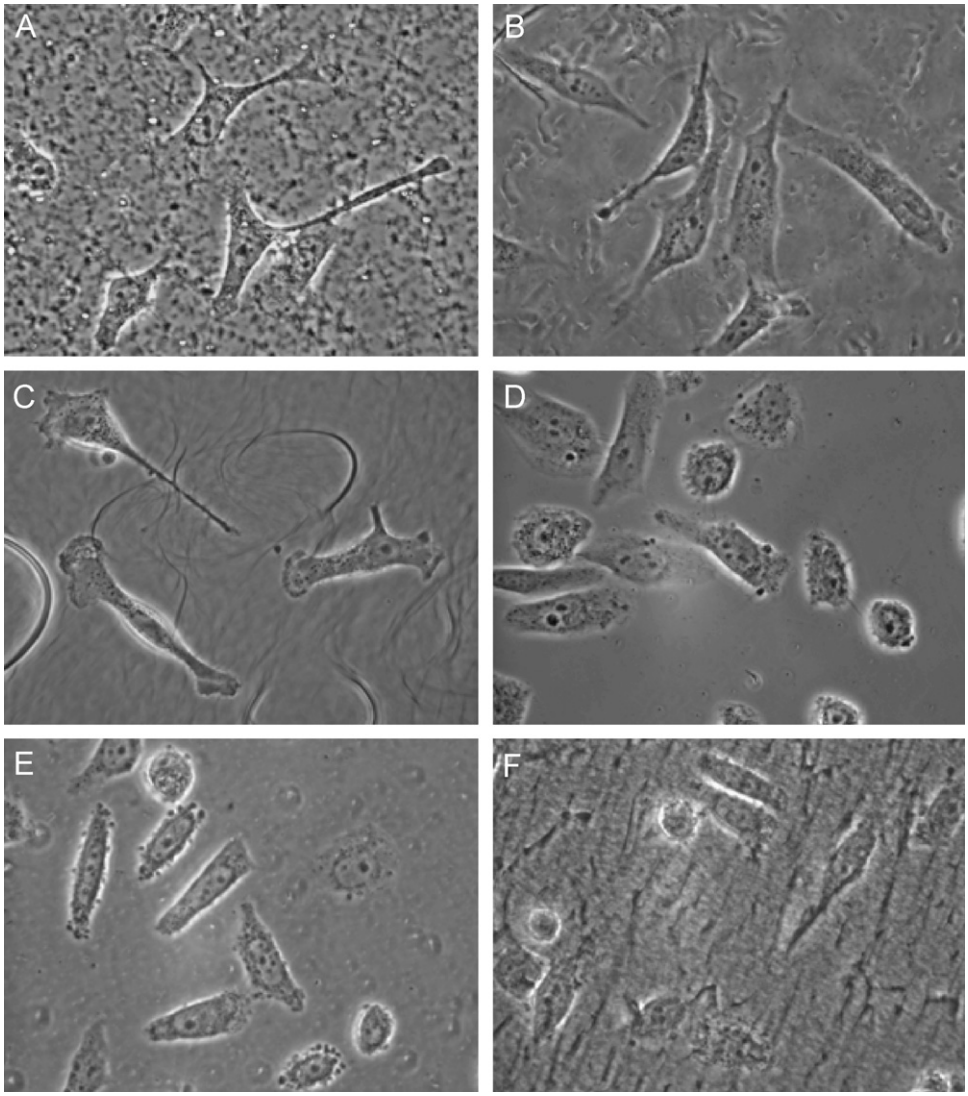

Fig. 2. Substrates seeded with HeLa cells and imaged with phase contrast microscopy with a 40 × /0.55 NA objective are shown: (A) 0.9 μm Mylar™ film, (B) 13 μm polyimide film, (C) 4 μm polypropylene film, (D) 6.25 μm Melinex film, (E) 12 μm Hostaphan RD film, and (F) 3 μm polycarbonate film.

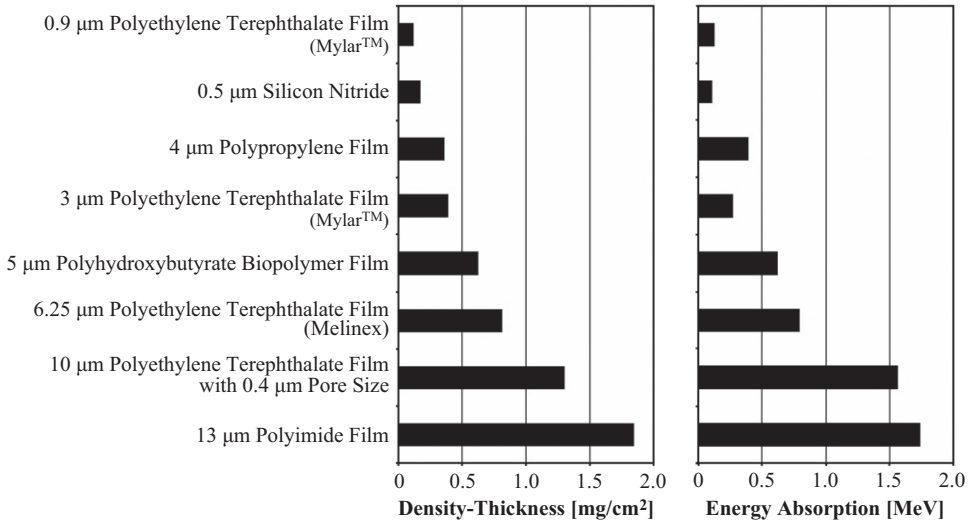

Fig. 3. The left chart shows the density-thickness of a selection of substrates. The right chart shows the energy absorption of substrates measured by placing various materials at the output of the microbeam. The input energy of $^{3}He^{2+}$ particles to the substrates was 3.85 MeV.

substrates were mounted into 361L stainless steel cell dishes. Next, cells were seeded onto the surface of the substrates. Fig. 2 shows phase contrast images of six of the substrates that were examined. The difference in visibility of the cells on various substrates can be observed from these images. The ability to segment the nucleus and the cytoplasm within the cells is important for targeted radiation studies. Ultimately, image processing will be used to automate cellular and sub-cellular feature detection. It is therefore desirable, given the fact that no substrate is optically perfect, that substrate imperfections at least appear substantially different from cellular features. In these images, it can be seen that the cells are the least visible on the Mylar™ substrate. Although features within the polypropylene and polyimide substrates are visible in the images, these features have the advantage of differing in shape from the cellular organelles, increasing the possibility of identifying the cells in the images. The features within the Melinex and Hostaphan films are the least noticeable. However, minimal amount of features in these films may be due to the thickness of the substrates.

During a microbeam-irradiation experiment, particles penetrate the substrate and enter the cell seeded on the top surface of the substrate. As the particles travel through the substrate and cells, some energy is lost. A low substrate energy loss allows a high cell penetration by the irradiation and is therefore desirable. The charts in Fig. 3 show the density-thickness and particle energy absorption of various substrates that have been tested. The density thickness values are calculated based on published material properties (DeLassus and Whiteman, 1999). These values are compared to experimental data in Fig. 3. Each of the substrates was placed at the output of the GCI microbeam vacuum window. Above the substrates, a surface barrier detector (SBD) (♯BR-015-050-100, E G & G Ortec, USA) was used to measure the energy of the particles. The SBD was previously calibrated against a known emission of Am-241 in an evacuated chamber. A beam of $^{3}He^{2+}$ particles with mean input energies of 2.79, 3.85, and 4.24 MeV were emitted at the vacuum window. The SBD output was recorded with a multichannel analyzer (MCA) (♯541, E. G. & G Ortec, USA) and Maestro MCA emulator software (♯A65-B32, Ortec, USA) and the mean energies were recorded. The energy loss was measured as the particles penetrated each of the substrates. The results were normalized based on the loss of energy from the air gap between the substrates and the detector input.

4. Discussion and conclusion

From the results obtained to date, polypropylene, polyimide, and Melinex are all possible substrates for our application. Each of these materials have good optical clarity when imaged with phase contrast as the features within the substrates are significantly dissimilar to the appearance of the nuclei, and therefore are included in the list of potential substrates.

Phase contrast imaging is just one of the imaging modalities that will be used in the endstation design. Whilst optically clear substrates are desirable and possibly essential, the performance of different substrates when biological samples are observed with fluorescence excitation still needs to be evaluated. Although images presented here show clear differences between substrates, it is inevitable that manufacturing processes or tolerances may further influence the results.

The 4 μm thick polypropylene and the 0.5 μm thick silicon nitride have energy absorption values that are similar to the 3 μm thick Mylar™ that has previously been used. The 13 μm thick polyimide and 6.25 μm Melinex attenuate the energy significantly more than the other two substrates, but show promise as suitable substrates, provided thinner films can be readily sourced.

Further experiments are in progress to quantify the ability of the cells to attach to the substrates and to measure the motility of cells over periods of a few days using time-lapse microscopy. In addition to imaging the cells using phase contrast microscopy, nuclear, and cytoplasmic staining techniques will be used to identify the intracellular and cell-substrate boundaries, as well as other widefield microscopy imaging methods using transillumination.

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