NON-INVASIVE, REAL-TIME EXAMINATION OF THYMIDINE UPTAKE EVENTS AND CELL SYNCHRONY USING CYTOSTAR-T™ SCINTILLATING MICROPLATES*

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Introduction

The uptake and incorporation of radiolabelled thymidine via the salvage pathway are widely used to examine cell cycle events and cell proliferation. The traditional method of measuring uptake involves trichoroacetic acid precipitation and liquid scintillation counting of the acid soluble and insoluble fractions. This study describes the use of Cytostar-T scintillating microplates in monitoring [^{14}C]thymidine incorporation and, in particular, how this method can be used to examine synchronously growing cells. The use of Cytostar-T plates in this way allows non-interventional, real-time monitoring of the rate of DNA synthesis in a microplate format.

Cytostar-T scintillating microplates are standard format, tissue culture treated 96 well plates. Figure 1 demonstrates the principle of the Cytostar-T scintillating microplate. The signal generated is proportional to the amount of radioisotope within the cell and can be detected and quantified using appropriate instrumentation.

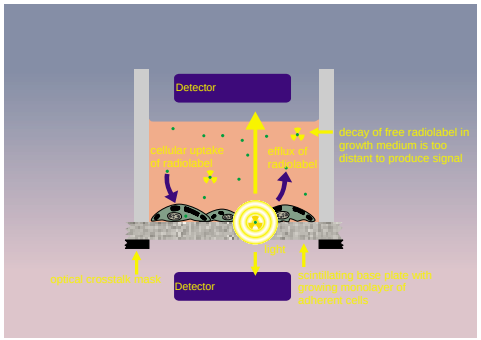

Figure 1. Cytostar-T scintillating microplate principle.

Results

Cytostar-T plates can be used to monitor thymidine incorporation.
V-79 Hamster lung fibroblasts were seeded into Cytostar-T plates at various densities, in a total volume of 0.2ml. [methyl-^{14}C]thymidine was added to a final concentration of 100nCi/ml. Control wells contained medium and [^{14}C]thymidine but no cells. Plates were incubated at 37°C and counted using a Wallac MicroBeta™ counter at a window setting between channels 50-450. Figure 2 shows the linear and cell-density dependent uptake of [^{14}C]thymidine. Linear uptake is consistent with a population of exponentially growing cells in which a constant proportion of the cells are traversing S-phase at each sampling time.

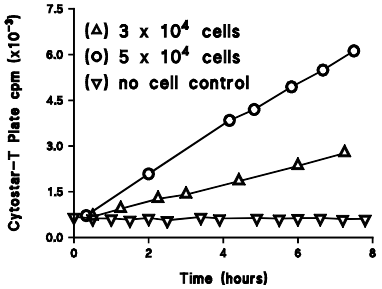

Figure 2. [^{14}C]Thymidine uptake in asynchronous V-79 cells.

Cell synchronization.
In this study, the use of sub-inhibitory concentrations of aphidicolin following mitotic shake-off was examined. Aphidicolin is a reversible inhibitor of DNA polymerase alpha[1]. 2 x 10^4 cells were seeded per well on 96 well Cytostar-T plates and incubated overnight with various concentrations of aphidicolin and [^{14}C]thymidine. Plates were incubated at 37°C and counted regularly. V-79 cells collected by mitotic shake off and preincubated with different concentrations of aphidicolin exhibit markedly different kinetics of thymidine uptake compared with untreated mitotic cells as demonstrated in Figure 3. Incubating the mitotic cells with aphidicolin delays entry into S-phase in a dose dependent manner. The degree of inhibition of progress through S-phase is dependent on aphidicolin concentration and is reversible. Following release from sub-inhibitory concentrations of aphidicolin, cells rapidly rejoin the cell cycle.

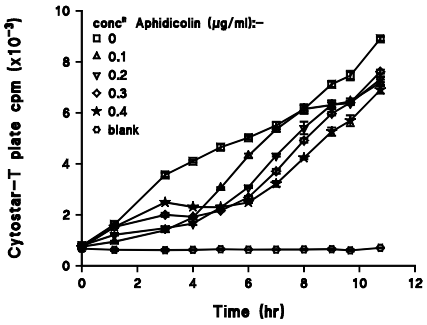

Figure 3. Dose-dependent delayed entry into S phase following over-night incubation with aphidicolin measured by [^{14}C] thymidine uptake.

Figure 4 demonstrates the linear uptake of thymidine as previously seen with unsynchronized cells. In addition, the figure demonstrates the Cytostar-T thymidine uptake profile associated with V-79 mitotic cells released from a 0.2μg/ml aphidicolin overnight block. In this instance, it can be seen that the cells are nearing the end of S-phase at the beginning of this observation. The cells then progress through G_2/M (confirmed by microscopic examination) to G_1 and then to a new round of DNA replication in the second S-phase which can be seen by the sudden increase in Cytostar-T signal.

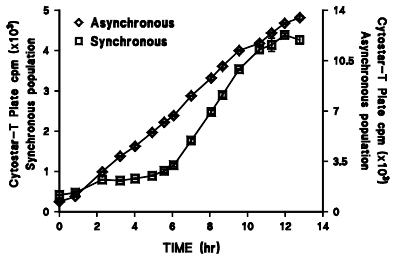

Figure 4. [^{14}C]Thymidine uptake in asynchronous and synchronous V-79 cells.

Changes in p34^{cdc2} activity in V-79 cells during the cell cycle.
p34^{cdc2} kinase is highly conserved among eukaryotes and controls the G_2 to M transition in the cell cycle[2]. As such, it can be considered a specific marker for progression into M-phase[3]. In parallel with Cytostar-T observations, samples were lysed and assayed for p34^{cdc2} activity using an Amersham™ Biotrak™ kit based on a peptide substrate derived from the SV49 large T antigen and specific for p34^{cdc2} kinase[4]. The kinase activity of the lysates was normalised for protein concentration. Figure 5 demonstrates the relationship between thymidine uptake as demonstrated by the Cytostar-T plate signal and p34^{cdc2} activity. A characteristic peak of activity is apparent at the point in the cycle corresponding to G_2/M.

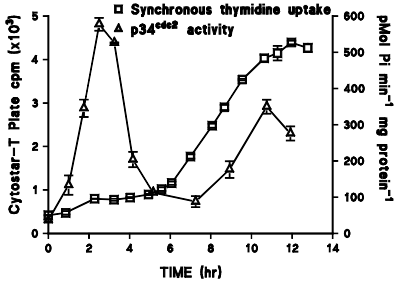

Figure 5. [^{14}C]Thymidine uptake in synchronous V-79 cells related to p34^{cdc2} activity.

Studies of inhibitors of cell cycle progression.
Figure 6 demonstrates the effect of the mitotic spindle inhibitor, colcemid, on synchronized V-79 fibroblasts. Following synchronization and [^{14}C]thymidine addition, colcemid (0.1μg/ml) was added at various times to the incubating cells and the plate signal monitored. Addition of colcemid at +1.5hr completely prevented the uptake of thymidine, confirming that these cells enter mitosis after aphidicolin release and are subsequently held in that state by the drug. Later addition of colcemid did not inhibit the entry into or completion of S-phase. The conclusions from these studies were supported by microscopic examination which showed the colcemid arrested cells remained rounded-up in appearance, consistent with cells in S-phase. The experiment demonstrates the utility of the system in examining points of intervention of cell cycle intervention of inhibitors.

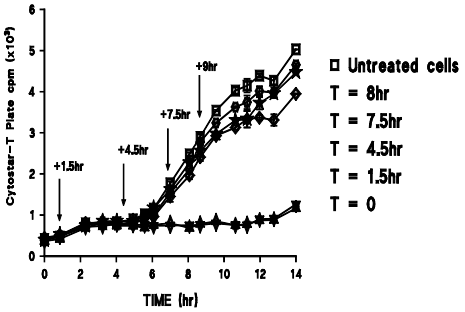

Figure 6. The effect of colcemid addition on synchronized V-79 cells.

Discussion

Cytostar-T scintillating microplates have been used to examine synchronized mammalian cell growth in a non-invasive, real time manner. Observation of synchronous populations of cells and other cell cycle events will facilitate advances in research and drug discovery in the field of tumour progression and other disease states. These experiments suggest a role for Cytostar-T scintillating microplates in research and drug discovery.

References

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