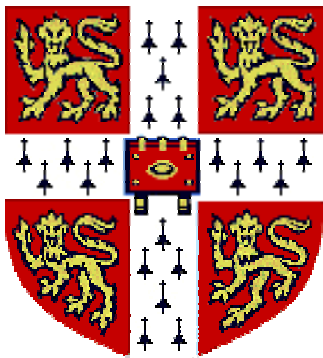


Probing cellular homeostasis *in vivo* using heavy water labelling of DNA

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K i n e M e d

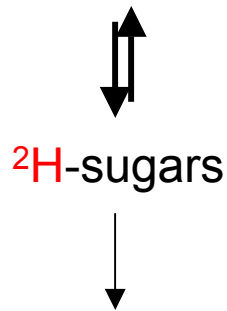
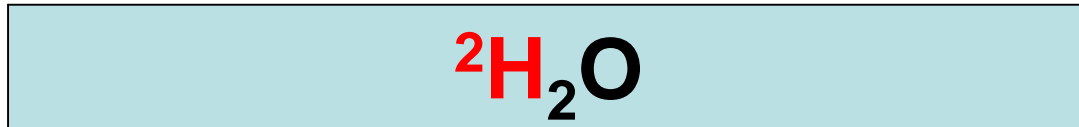
Why study lymphocyte kinetics?

- Basic biology
 - How are lymphocyte numbers/subsets regulated in healthy individuals?
 - Different answers for, e.g., memory, effector, regulatory T cells
 - Effect of normal variation: age, environment, genetics...
 - Effects of immunisation?
 - Vaccines
 - Infection
 - Correlates of protection
- Pathophysiology
 - Persistent viral infection: HIV1, EBV
 - Lymphopaenic autoimmune states: PSS, SLE
 - Haematologic malignancy
- Preclinical and clinical drug development
- “Snapshot” markers (DNA content, Ki67, PCNA) vs. biosynthetic labelling experiments
 - Choice of biosynthetic label

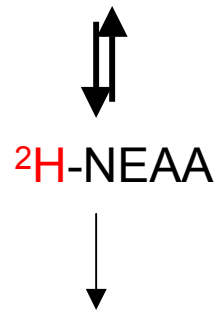
$^2\text{H}_2\text{O}$ labelling

- Methodology
- Applications

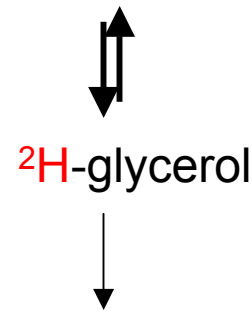
Heavy water as a universal label for slow biosynthetic processes



Neese et al.
2002
PNAS 99:
15345



Previs et al.
2004
AJPEndo 286:E665
Busch, Kim et al.
2002
BBA 1760:730



Turner et al.
2003
AJPEndo 285:E790

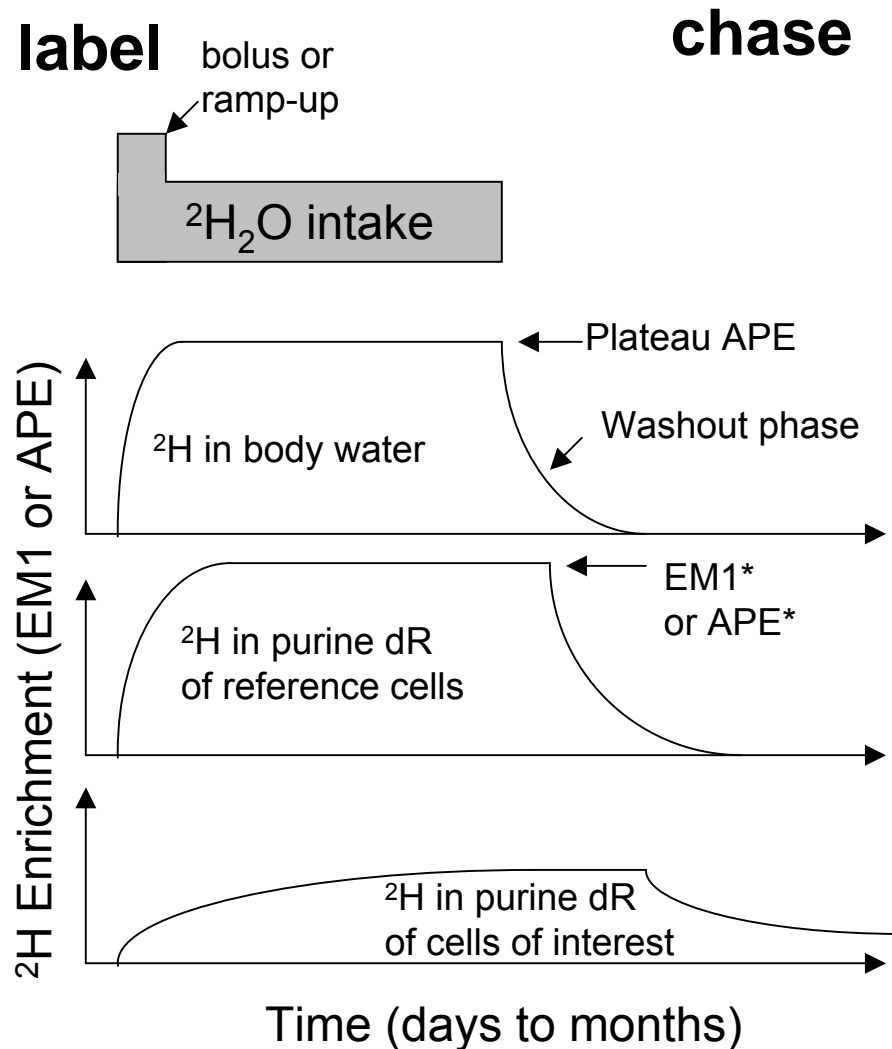
...

Liability or asset?

Heavy water ($^2\text{H}_2\text{O}$) is a convenient tracer for long-term, continuous labelling studies

- Single oral dose distributes in body water within ≈ 15 min. (rodents) to an hour (humans)
- Easy to maintain (10%/d body water turnover in humans)
- Physiologically innocuous below about 15% ^2H in body water
- Monitored by sampling saliva, urine, or plasma
- Economical vs. stable isotope-labelled metabolites

Experimental design of $^2\text{H}_2\text{O}$ labelling experiments



Work flow of a $^2\text{H}_2\text{O}$ labelling experiment

Label cells or organisms with $^2\text{H}_2\text{O}$



Isolate desired cell population



Extract DNA



Hydrolyze



Derivatize

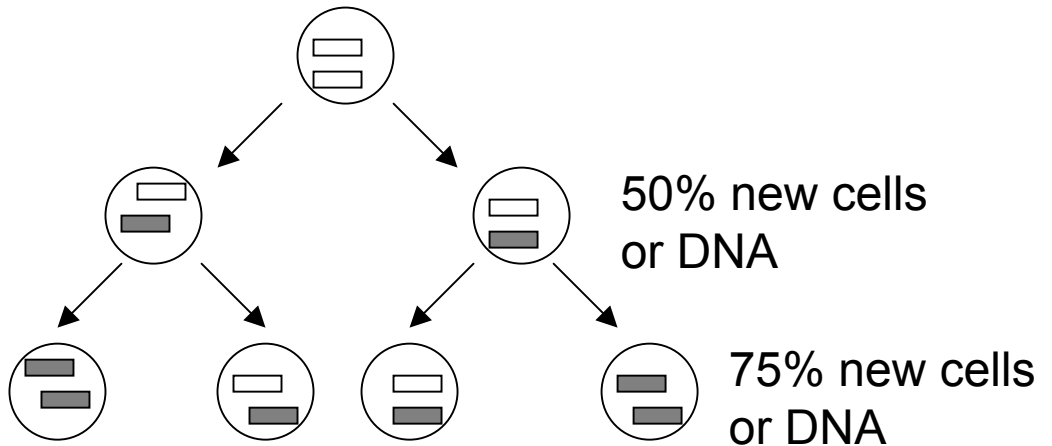
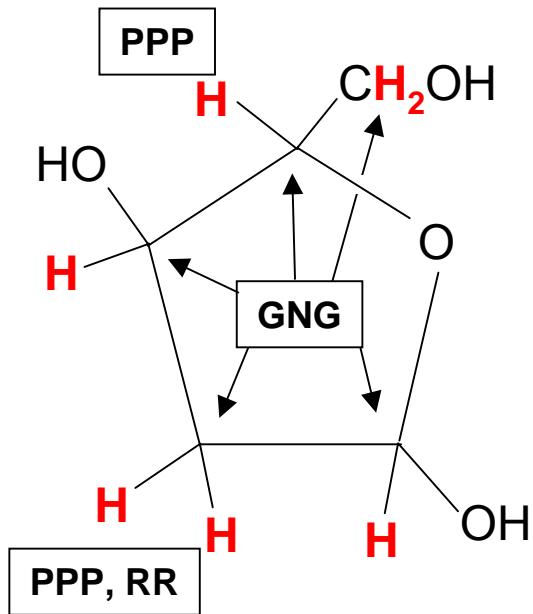
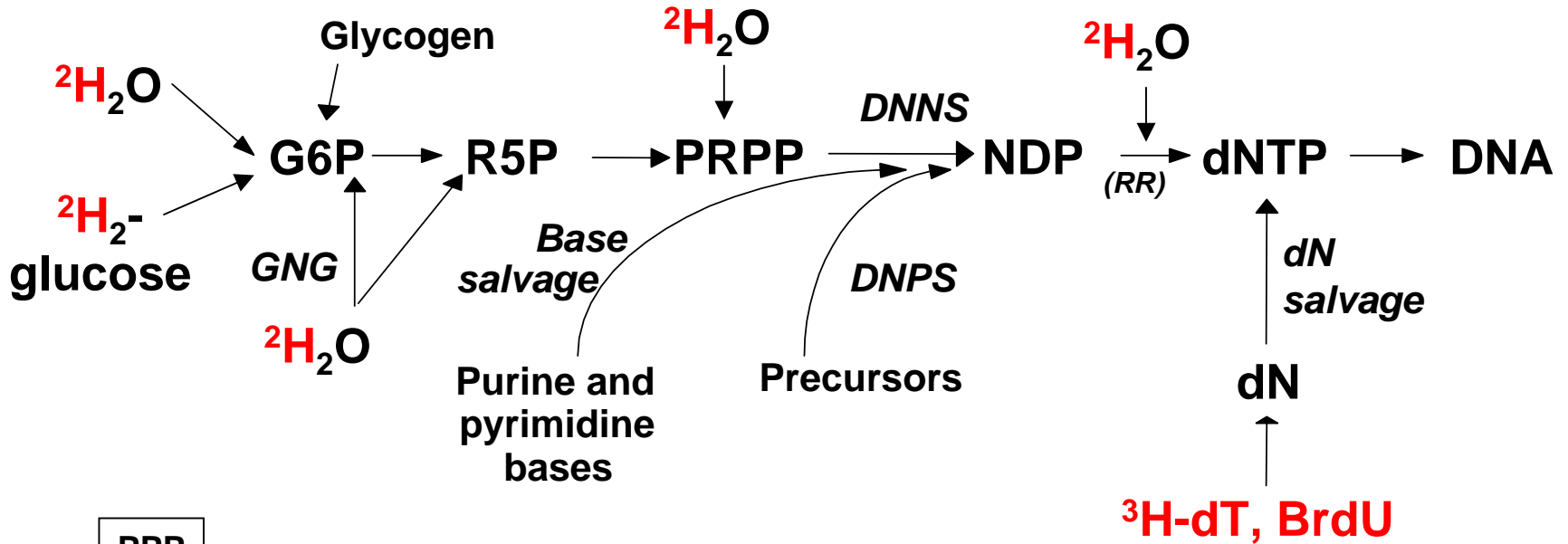


Analyze by MS

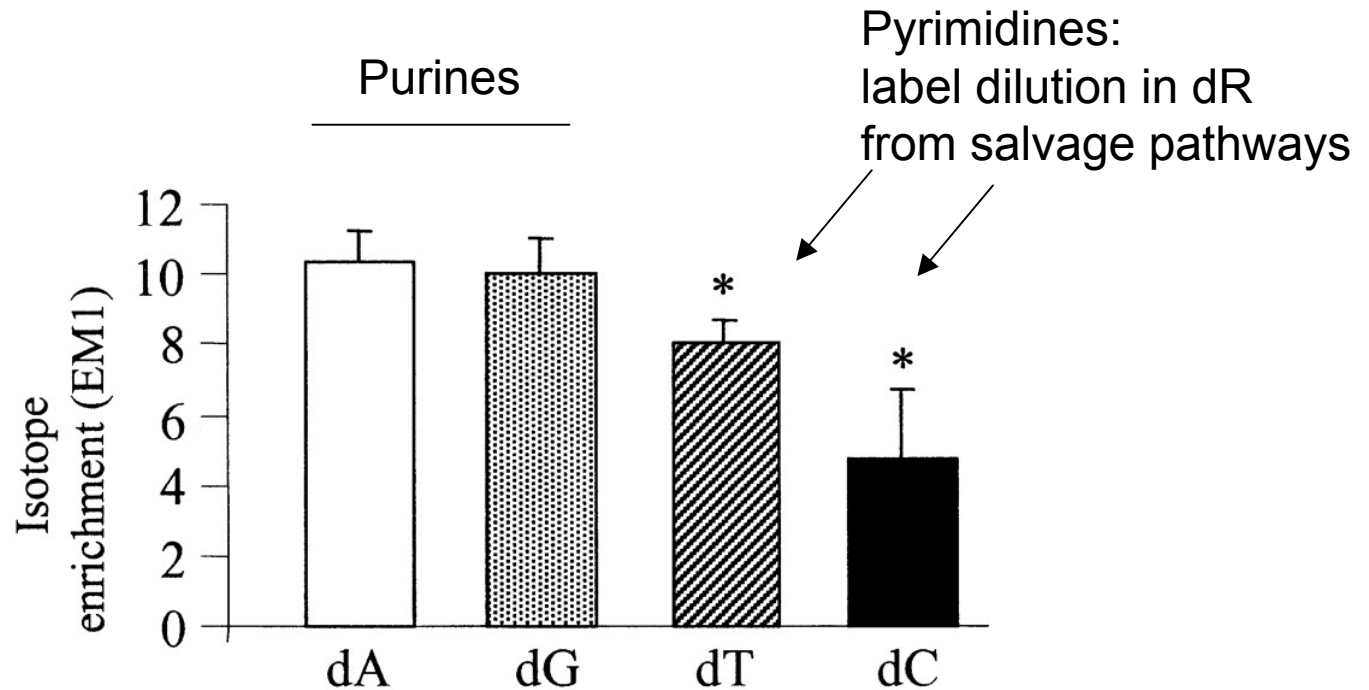


Calculate ^2H enrichment and f

Heavy water labelling of DNA



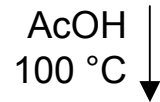
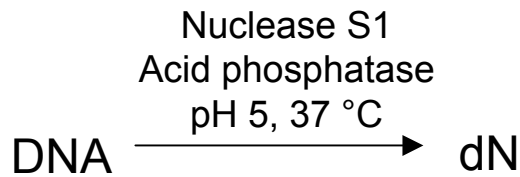
^2H incorporation from $^2\text{H}_2\text{O}$ into deoxyribose: purine vs. pyrimidine dN



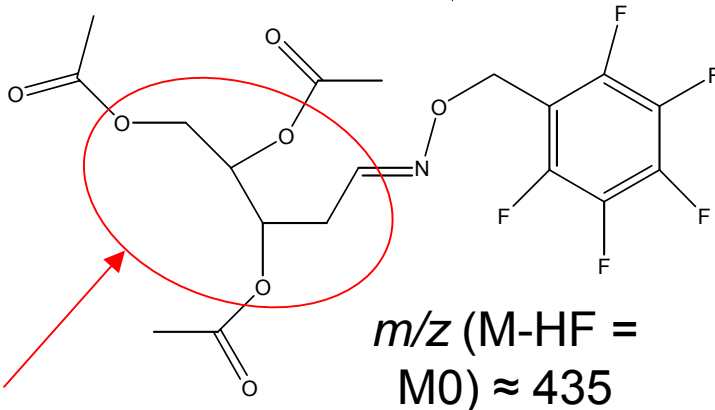
Mouse BM DNA, 4% $^2\text{H}_2\text{O}$ \times 3 weeks
Enzymatic hydrolysis at high pH, PTA derivative of dR

Neese et al. 2002, PNAS 99:15345

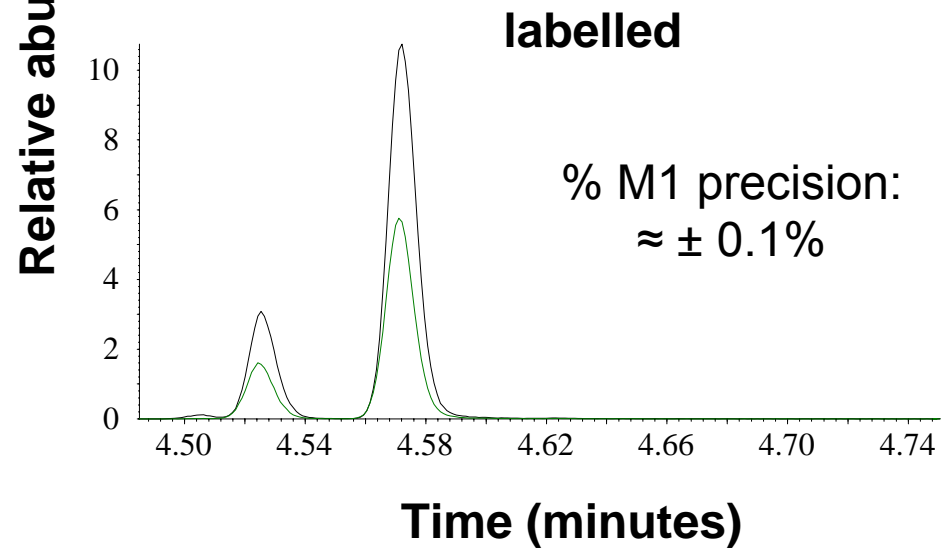
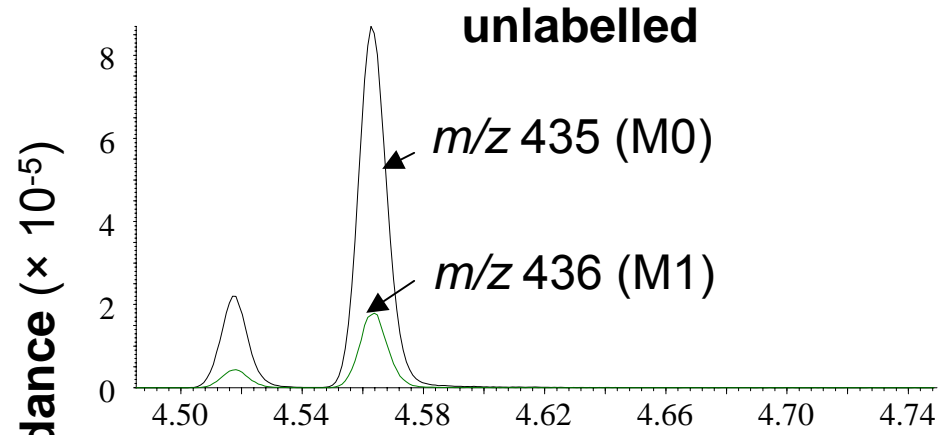
Measuring ^2H incorporation into purine deoxyribose moieties of DNA



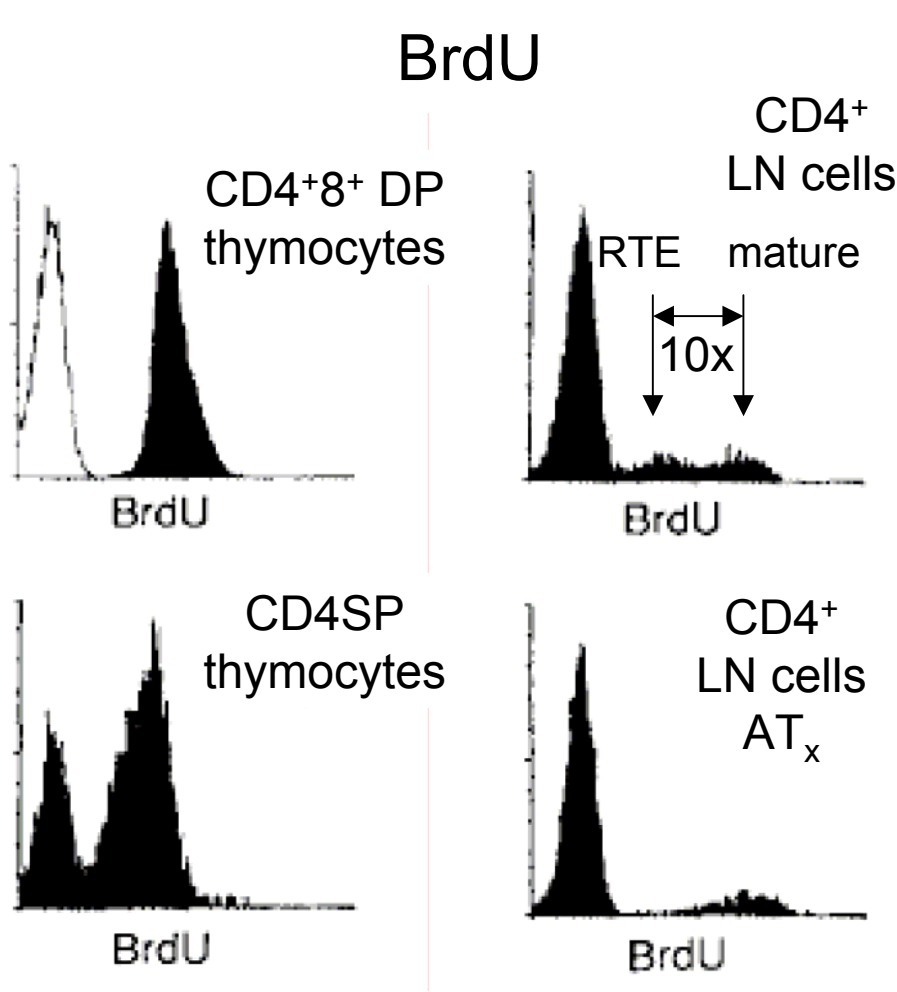
1. PFBHA
2. Ac_2O



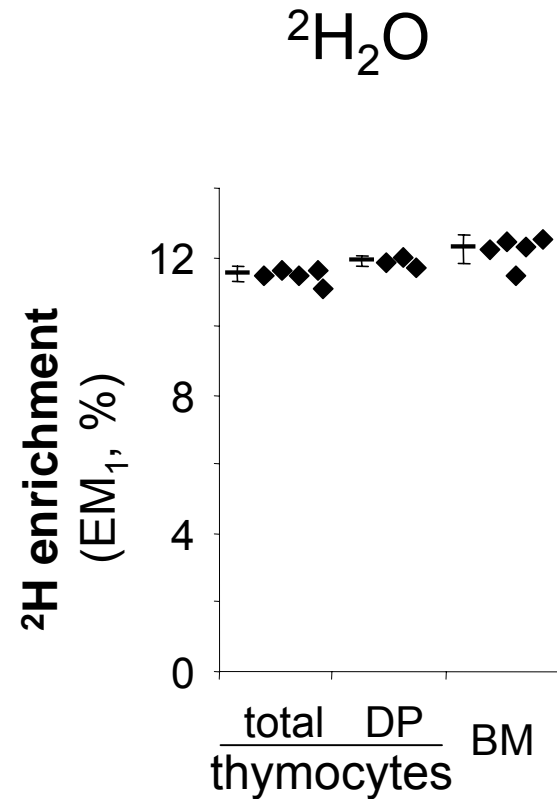
Purine dR



Thymic microenvironment inhibits BrdU incorporation but not $^2\text{H}_2\text{O}$ labeling



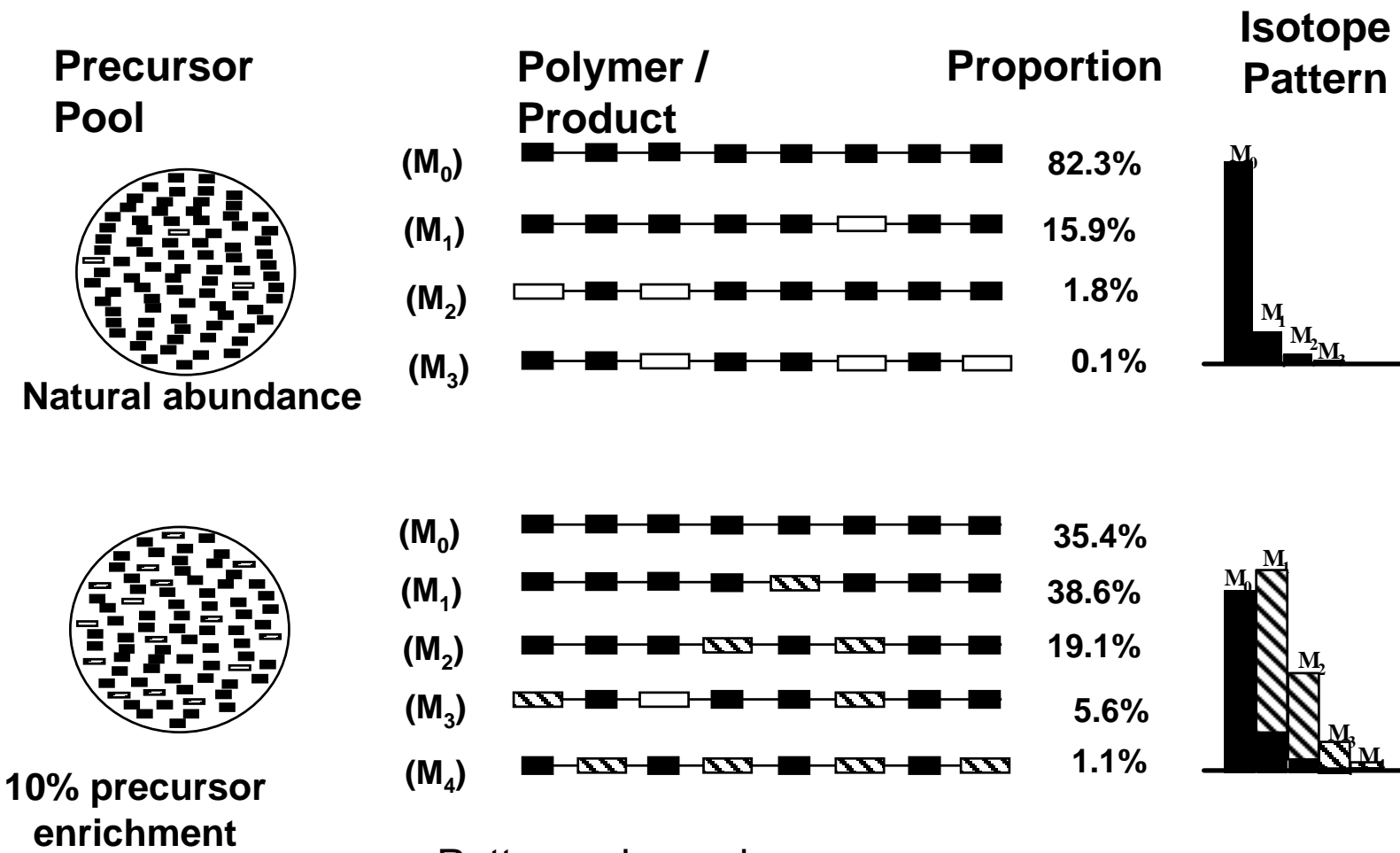
Tough & Sprent (1994)
J Exp Med 179:1127, 1994



6 wk old, female Balb/c
1 week $^2\text{H}_2\text{O}$ (4-5% in plasma)
Normal thymocyte counts

Busch, Neese et al. Nat. Protoc. 2:3058 (2007)

Mass isotopomer distribution analysis for analysis of precursor/product relationships



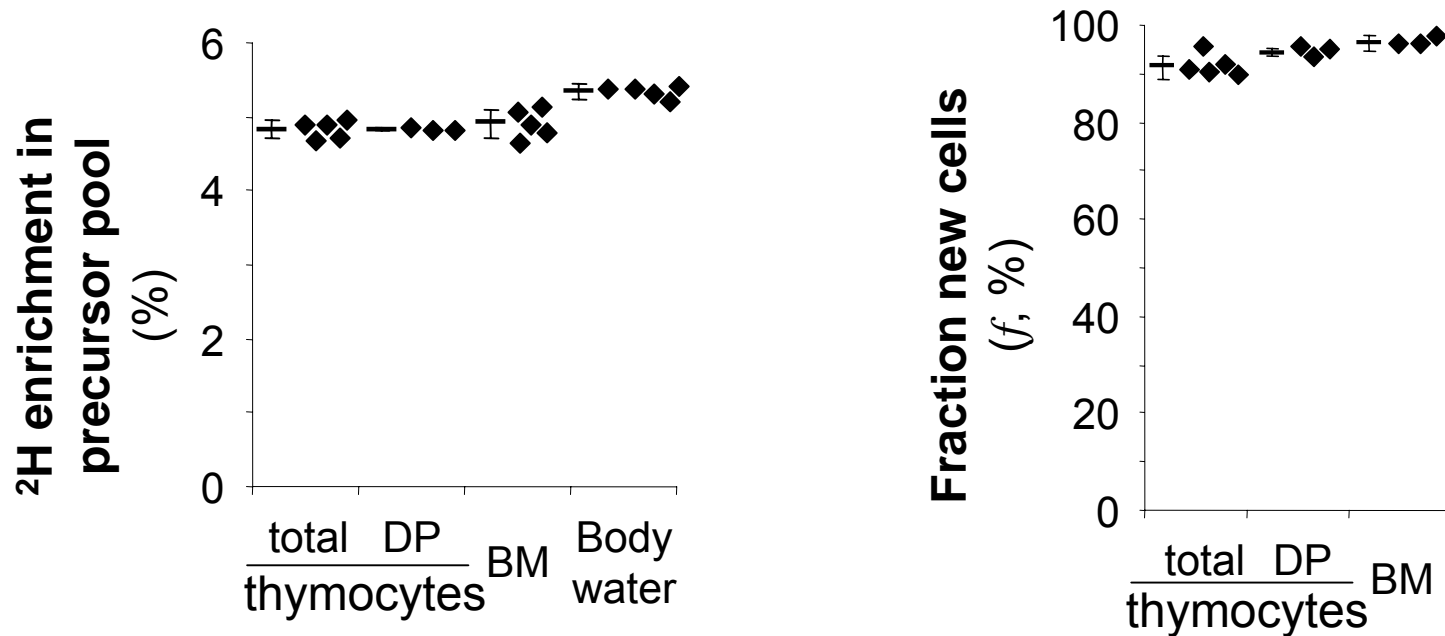
Patterns depend on:

(a) Number of labelled sites

(b) Label enrichment in precursor pool

Minimal label dilution in $^2\text{H}_2\text{O}$ -labelled purine dR precursor pools incorporated into DNA

Mass isotopomer distribution analysis



- Label incorporation is proportional to the percentage of divided cells, regardless of microenvironment
- An unlabelled cell has not divided

Technical optimisation of $^2\text{H}_2\text{O}$ labelling experiments

Label cells or organisms with $^2\text{H}_2\text{O}$

Compliance? Verify intake
Neese et al., 2002

↓
Isolate desired cell population

≥ 95% purity of cells of interest;
minimise monocyte contamination
Busch et al. 2004 JIM (CD4+ T)

↓
Extract DNA

↓
Hydrolyze

↓
Derivatize

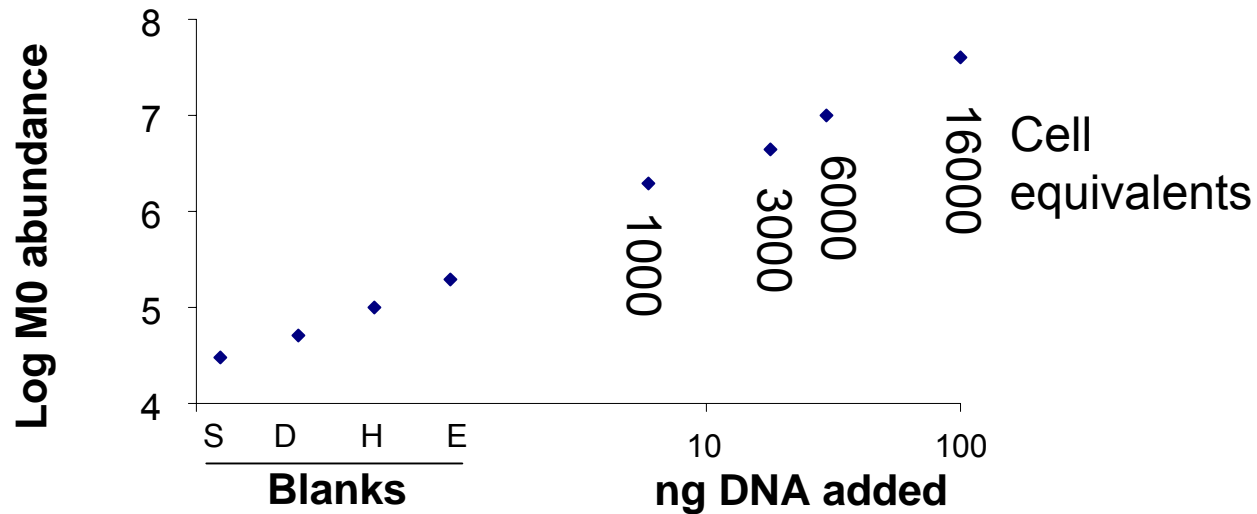
↓
Analyze by MS

- Use NCI-GC/MS to minimise cell requirements
- Minimise contamination
- Maximise recovery
Busch, Neese et al. 2007 Nat Protoc 2: 3045

- Use GC/P/IRMS to minimise label requirements
Voogt et al. 2007 Nat Protoc 2:3058

↓
Calculate ^2H enrichment and f

Sensitivity of purine dR analysis



Conclusions: Methodology

- $^2\text{H}_2\text{O}$ is a safe, versatile label for studies of macromolecular synthesis and turnover *in vivo*
- Enables long-term continuous labelling and pulse/chase designs
- Preferred label for slow-turnover cell populations
- Label incorporation into purine dR is efficient and independent of cellular microenvironment
- NCI-GC/MS enables analysis of small cell samples ($\approx 10^3$ cells)
- GC/P/IR-MS enables analysis of low-level label incorporation within abundant cell samples.
- Optimisation and validation of each step of the analytical protocol is critical

Outline

- Methodology
- Applications

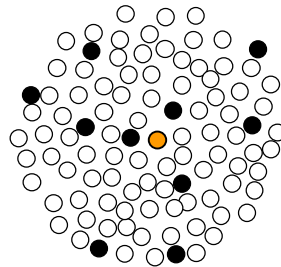
Cell dynamics in Ag-stimulated murine lymph nodes

- Baseline turnover (labeled)
- Ag-specific precursors
- Ag-specific proliferation (labeled)

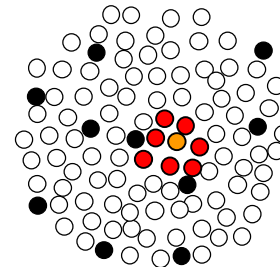
Clonal expansion

Recruitment

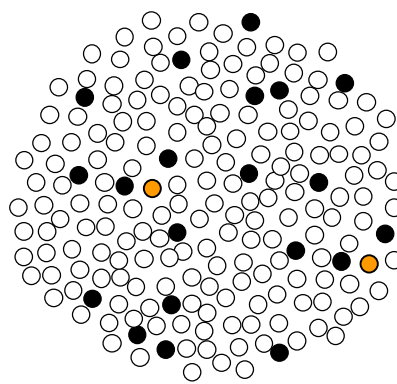
Cell count (c) = 100
 Fraction new (f) = 10%
 New cells (abs) = 10



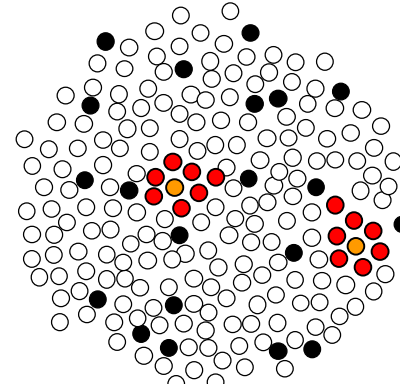
$c = 100 + 7 = 107$
 $f = (10+7)/107 = 16\%$
 $abs = 10 + 7 = 17$



$c = 200$
 $f = 10\%$
 $abs = 20$



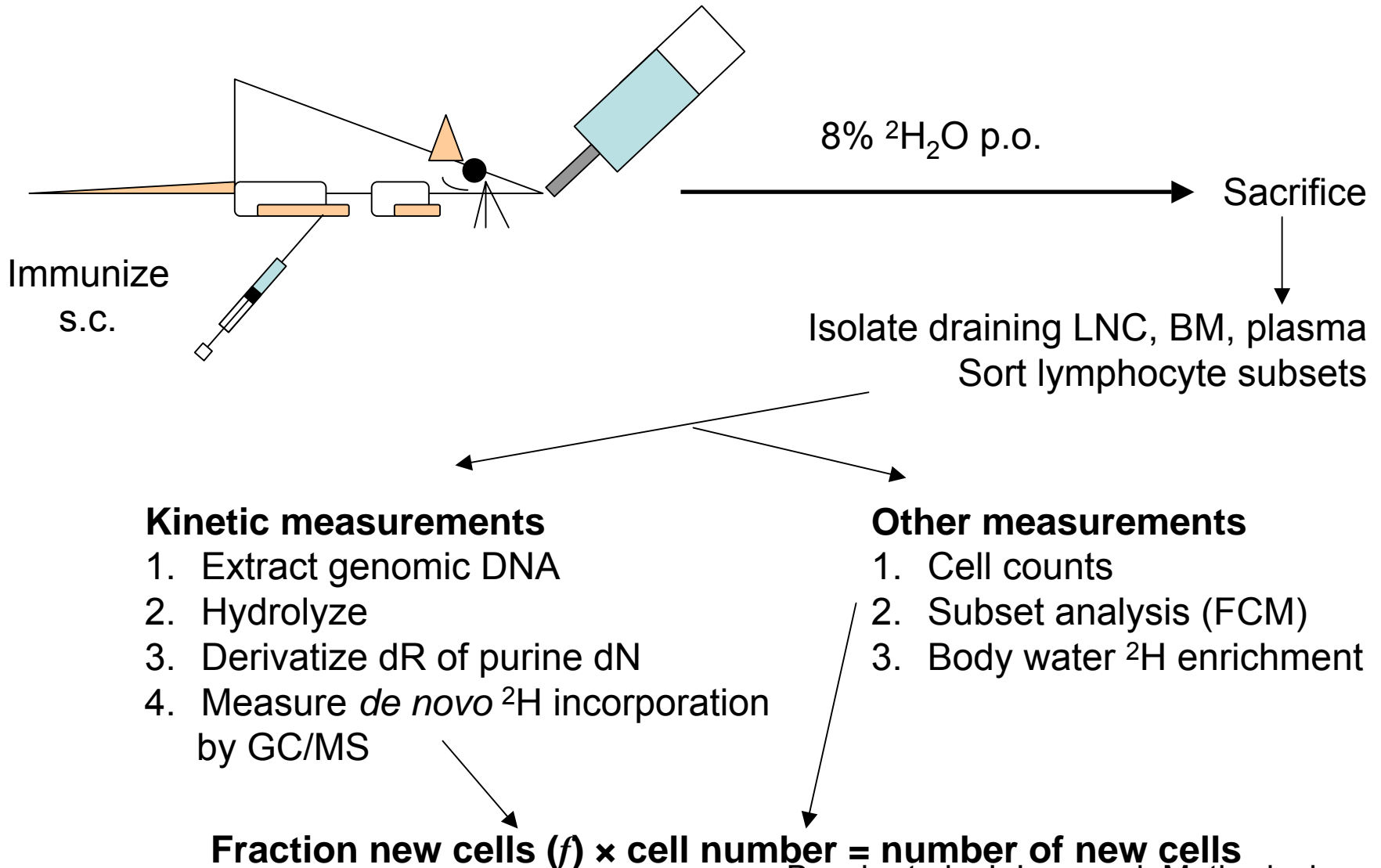
$c = 200 + 14 = 214$
 $f = (20+14)/214 = 16\%$
 $abs = 20 + 14 = 34$



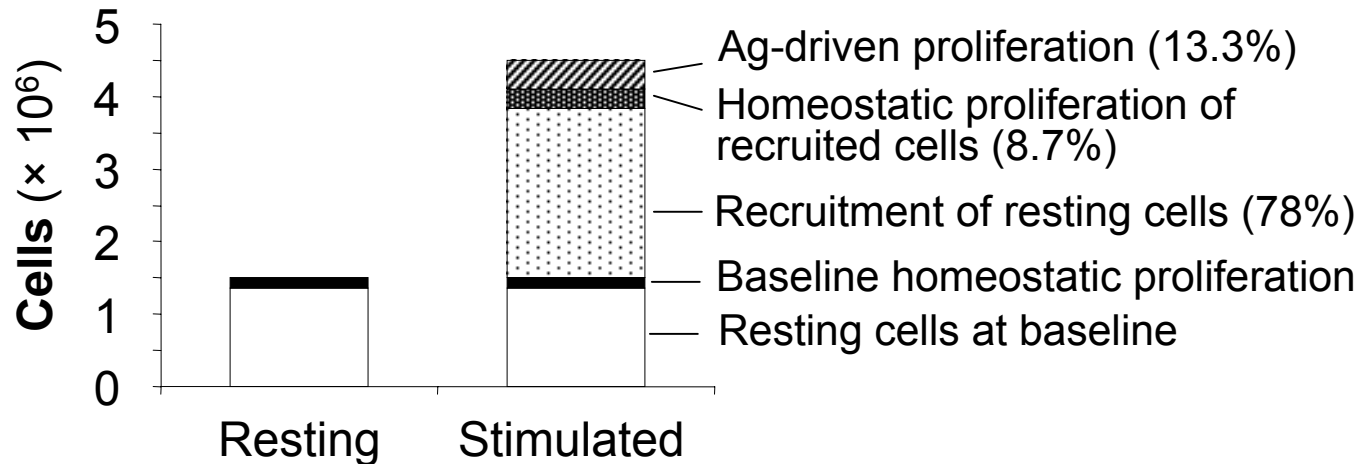
Dissecting these contributions requires unbiased measurement of f .

Busch et al., J. Immunol. Methods, in press.

Measuring lymphoproliferation during a local immune response (modified PLNA)



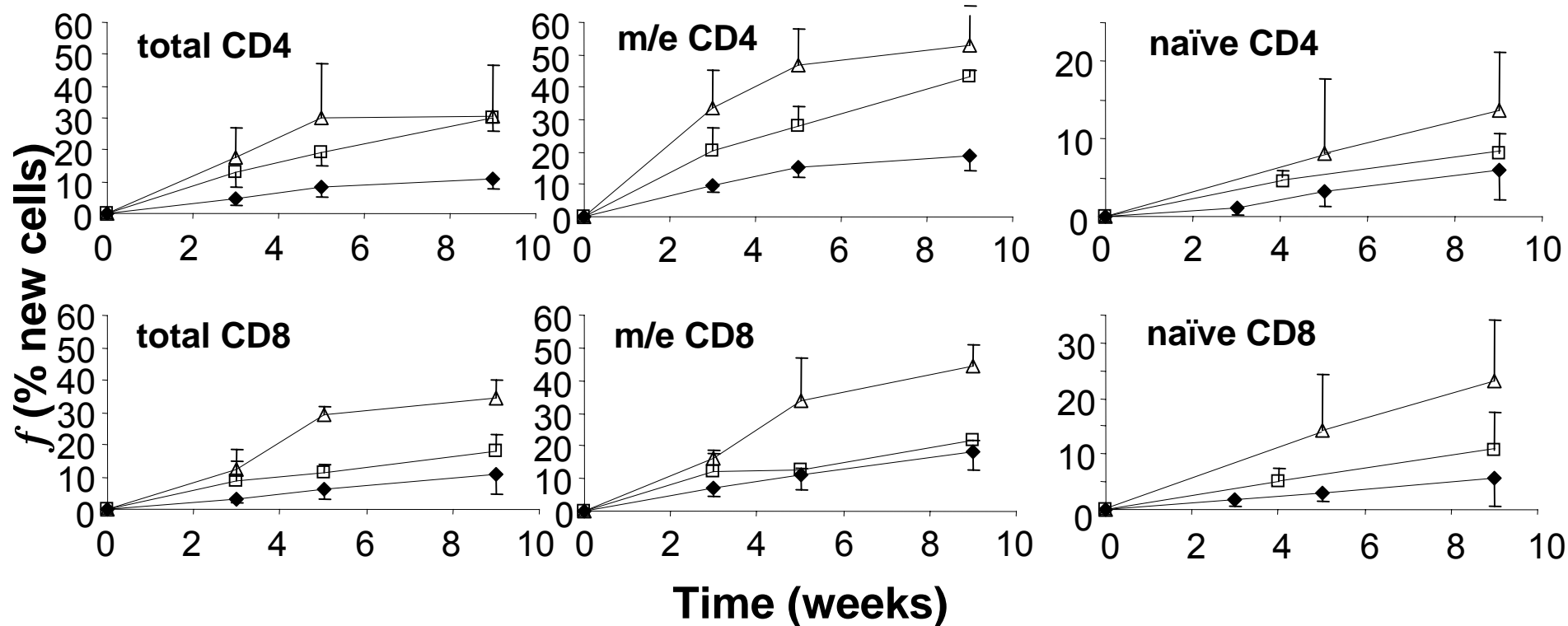
KLH-stimulated lymphadenopathy in Balb/c mice: primarily driven by lymphocyte recruitment



- f was increased by antigens, but not by nonspecific irritants
- Cell recruitment (cell counts) and clonal expansion (f) were differentially regulated by antigen dose, adjuvant, and immunosuppressants

Increased T-cell turnover in HIV1 infection revealed by continuous $^2\text{H}_2\text{O}$ labeling

- ◆ HIV-negative controls
- △ HIV/AIDS
- LT ARV

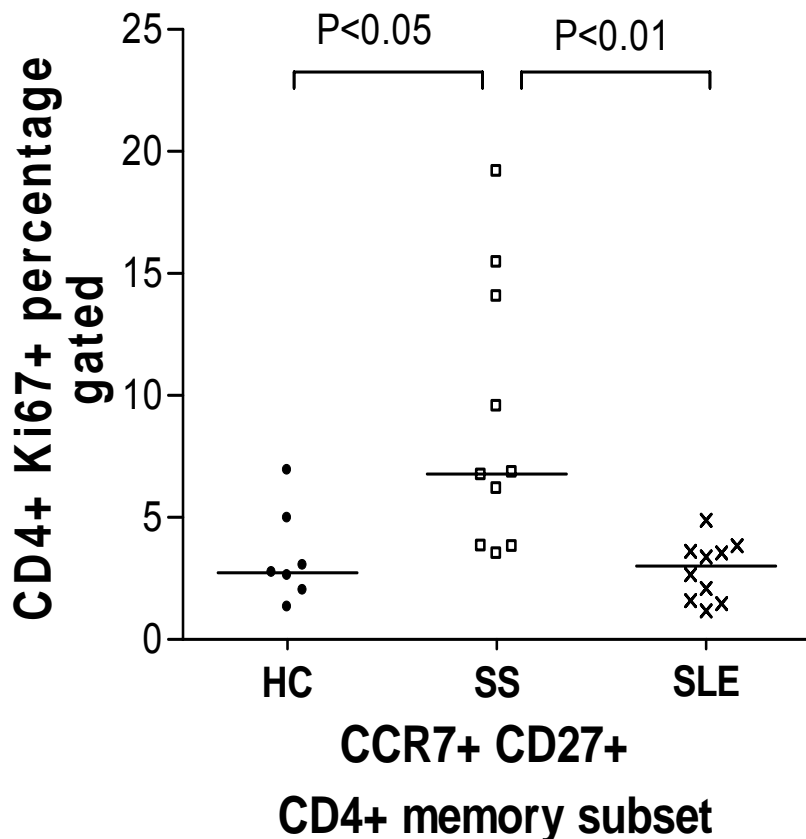


Hellerstein et al., *J. Clin. Invest.* 112:956, 2003

Simplified technology: Busch et al., *J. Immunol. Methods* 286:97, 2004

Further subset analyses by pulse/chase: Ladell et al., in press (this meeting)

A case for studying aberrant T-cell dynamics in patients with primary Sjögren's syndrome



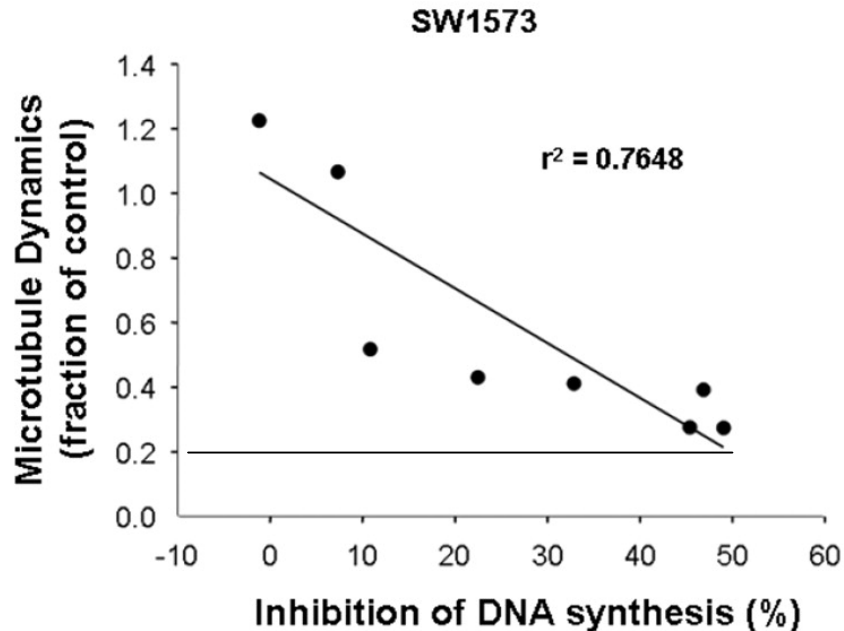
- PSS: autoimmune dysfunction of exocrine glands with characteristic autoAb (Ro/La)
- Some patients have systemic manifestations, ↑ lymphoma
- CD4+ T cells prominent in lymphocytic infiltrates
- CD4+ T lymphopaenia is common and predicts adverse outcomes

$^2\text{H}_2\text{O}$ labelling: beyond lymphocytes

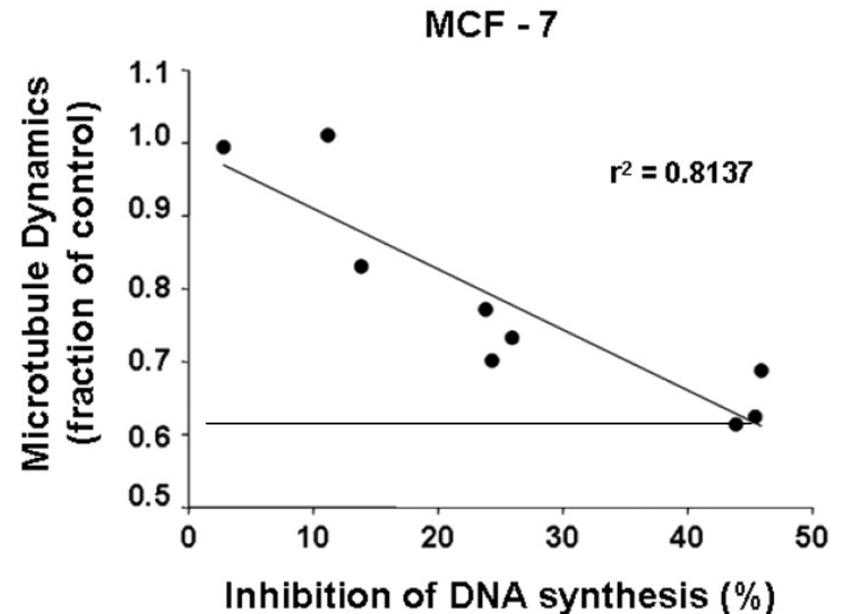
Cell type	Species	Turnover rate ^b
Hippocampal neuronal precursors	Rats, mice	0.2–1.0% d ⁻¹
Hippocampal neurons ^a	Rats, mice	0.2–0.4% d ⁻¹
Brain microglia	Mice	0.3–3.0% d ⁻¹
Vascular smooth muscle cells	Mice	1–2 % wk ⁻¹
Adipocytes/stromal-vascular cells	Humans, rodents	1–3% wk ⁻¹
Myocytes	Rats	0.5% wk ⁻¹
Colonocytes	Rodents	20–60% d ⁻¹
Keratinocytes	Mice	5–7% d ⁻¹
Prostate epithelial cells	Rodents	1–2% d ⁻¹
Pancreatic islet β -cells	Rats, mice	0.5–2.0% d ⁻¹
Endothelial cells (liver, kidney)	Rats, mice	1–3% d ⁻¹
Tumor endothelial cells	Rodents	10–20% wk ⁻¹
Lymphocytes (several subsets) ^d	Humans, rodents	1–15% wk ⁻¹
Mammary epithelial cells	Humans, rodents	0.2–0.7% d ⁻¹
Spermatocytes	Humans	~ 60 d lag time
Monocytes, granulocytes	Humans	> 20% d ⁻¹
Chronic lymphocytic leukemia cells	Humans	1–10% wk ⁻¹

One label – multiple analytes: Effect of taxanes on microtubule dynamics vs. cell proliferation

A



B



Conclusions: Applications

- $^2\text{H}_2\text{O}$ labelling enables *in vivo* proliferation and turnover of long-lived cells from any accessible site to be quantified
- Technical refinements enable novel applications, limited by low label incorporation or low cell numbers per sample
- Entry of ^2H from $^2\text{H}_2\text{O}$ into multiple metabolic pathways enables multidimensional evaluation of disease states
 - Simultaneous analysis of different cell types
 - Immune effectors and target cells (viral infection, autoimmunity, transplantation)
 - Malignant vs. bystander cells
 - Bulk and antigen-specific T cells
 - Cells and their products
 - Multiple myeloma cells and M protein
 - Proliferation and microtubule dynamics in drug-treated cells
 - Hepatocyte proliferation and hepatic fibrosis
- $^2\text{H}_2\text{O}$ labelling studies deliver novel insights into normal lymphocyte homeostasis, mechanisms underlying lymphopaenic states, and the action of immunomodulatory agents

Acknowledgments

Methodology

KineMed, Inc.



Immunomodulation: *Iche Siah, Tracy Gee*, Dan Holochwost, Carole Keim

Protein studies: Patrizia Fanara (microtubules), J. Gardner (fibrosis) Mike Marino (M protein)

Analytical Chemistry: Mohamad Awada, Ablatt Mahsut, Chancy Fessler

Indications Discovery: Mary Wolff, Danielle Kassner, Sarah Laraway

UC Berkeley, Dept of Nutritional Sciences and Toxicology

Marc K. Hellerstein, Richard Neese



Sjögren's/SLE T cells:

Frances C. Hall, *Christine Bryson*, Cyndi Goh

University of Cambridge, Department of Medicine/Rheumatology

Simon Bowman

Selly Oak Hospital, University of Birmingham

HIV1:

Mike McCune, Steve Deeks, Dana Higuera-Alhino, Drina Boban, Mette Hazenberg, Kristin Ladell

UC San Francisco/San Francisco General Hospital

Funding:

Kinemed, Inc.

Cambridge Arthritis Research Endeavour

Stanley Elmore Senior Research Fellowship, Sidney Sussex College

