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Analysis of Neurogenesis in the Adult Human Brain Using Bomb-Carbon

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Retrospective Birth Dating of Cells in Humans

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Summary

The generation of cells in the human body has been difficult to study, and our understanding of cell turnover is limited. Testing of nuclear weapons resulted in a dramatic global increase in the levels of the isotope ^{14}C in the atmosphere, followed by an exponential decrease after 1963. We show that the level of ^{14}C in genomic DNA closely parallels atmospheric levels and can be used to establish the time point when the DNA was synthesized and cells were born. We use this strategy to determine the age of cells in the cortex of the adult human brain and show that whereas nonneuronal cells are exchanged, occipital neurons are as old as the individual, supporting the view that postnatal neurogenesis does not take place in this region. Retrospective birth dating is a generally applicable strategy that can be used to measure cell turnover in man under physiological and pathological conditions.

Introduction

Many cells have a shorter life span than the organism and are continuously replaced. Cells exposed to a harsh environment, such as in the skin or intestine, are often very short lived. In other tissues there may be less cell turnover under physiological conditions, but regeneration may be activated in response to injury. For many cell types, however, it is largely unknown whether they turn over at all once development is completed.

Cell generation is often studied by analysis of molecular markers of proliferation. This provides a good view of the number of cells in cycle at a given time, but it does not provide insight into the number of mature cells that are generated or added to the tissue. Administration of labeled nucleotides such as ^{3}H-thymidine or BrdU allows prospective labeling of newborn cells and can reveal the generation and integration of mature cells in an organ. It is, however, difficult to detect rare events, and there are several technical caveats with a risk of both false positive and negative results, leaving room for controversy (Nowakowski and Hayes, 2000; Rakic, 2002a). Moreover, it is not possible to retrospectively determine cell turnover in collected material, and the toxicity of labeled nucleotides limits its use for studies in man.

Much of our view on cell turnover in the adult human body is inferred from studies in rodents, which in most cases are only a few months old at the time of analysis. This may not be an ideal model for man, who can live for a century and can potentially have a greater need to replace cells over the life span. Although it may be easy to conceptualize that, for example, a neuron in the brain can live for months, is it possible for the same cell type to be maintained and function for many decades?

Whether a specific cell type is exchanged, and at what rate, is a fundamental question that may have important implications for how we view the human body under physiological and pathological conditions. Alterations in cell turnover are a key feature in several diseases, such as decreased erythrocyte production in aplastic anemia or increased keratinocyte turnover in psoriasis. Distorted cell turnover has been implicated in the pathogenesis of many diseases, for example decreased neurogenesis in depression (Duman, 2004; Santarelli et al., 2003; Sheline et al., 1996) and impaired production of cardiomyocytes in heart failure (Sussman and Anversa, 2004), but it has remained controversial and difficult to study.

Much of the impetus in stem cell research and regenerative medicine is fueled by the prospect of promoting cell replacement. Stimulating blood-cell production with erythropoietin or G-CSF are successful and established therapies (Richard and Schuster, 2002), and it has been suggested that similar pharmacological therapies may be developed to influence cell replacement in other organs (Ding and Schultz, 2004; Lie et al., 2004; Lindvall et al., 2004). Without knowing if a specific cell type is renewed in the healthy or pathological situation, it remains uncertain whether it may be realistic and rational to modulate this process.

The lack of methods to study cell turnover in man prompted us to develop a novel strategy to approach this issue. Inspired by ^{14}C-dating in archeology, we sought to develop a way to retrospectively determine the age of cells without the need for delivering any chemical to the individual prior to the analysis. ^{14}C levels on earth have remained relatively constant over long time periods, and the radioactive decay of the isotope is used to retrospectively date biological material in archeology. The resolution in modern time is poor due to a half-life of 5730 years. However, extensive above ground testing of nuclear weapons between the mid 1950s and early 1960s resulted in the generation of large quantities of ^{14}C (Figure 1A), which rapidly distributed evenly in the atmosphere around the globe (De Vries, 1958; Nydal and Lovseth, 1965). Since the test ban treaty in 1963, there has been no high-yield, aboveground, nuclear detonation leading to significant ^{14}C production (Levin and Kromer, 2004). Thus, the levels have dropped thereafter exponentially, not primarily be-

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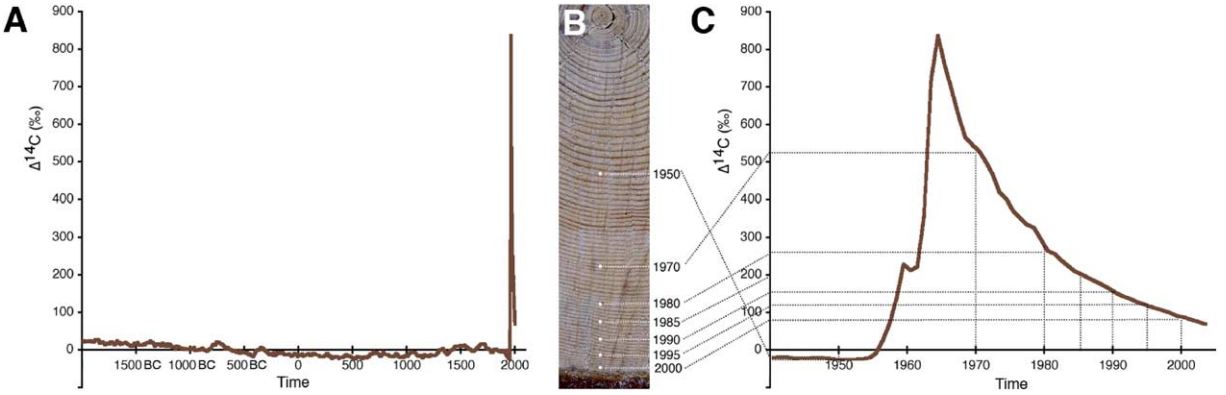

Figure 1. ^{14}C Levels in the Biotope

The levels of ^{14}C in the atmosphere have been stable over long time periods, with the exception of a large addition of ^{14}C in 1955–1963 as a result of nuclear bomb tests ([A], data from Levin and Kromer, 2004; Stuiver et al., 1998). ^{14}C levels spanning the last decades in the geographical area of the study (Sweden) were measured in cellulose taken from annual growth rings of local pine trees (B), which were subjected to accelerator mass spectrometry, and were found to closely correspond to mid-latitude northern hemisphere ^{14}C levels (C). Only a fraction of representative measurements are displayed. ^{14}C levels from modern samples are by convention given in relation to a universal standard and corrected for radioactive decay, giving the $\Delta^{14}C$ value (Stuiver and Polach, 1977).

cause of radioactive decay, but due to diffusion and equilibration with the oceans and the biosphere (Figure 1A), resulting in a slope decreasing 50% approximately every 11 years after 1963 (Levin and Kromer, 2004).

We have taken advantage of the drastically altered atmospheric ^{14}C levels in modern times to develop a strategy to retrospectively birth date cells in the human body. We use this method to address whether cortical neurons are renewed in the adult human brain and find that these neurons are as old as the person, arguing against adult neurogenesis in this region. This is a generally applicable strategy that can be used to analyze cell turnover in man under physiological and pathological conditions.

Results

^{14}C in Genomic DNA Reflects the Age of Cells

^{14}C in the atmosphere reacts with oxygen and forms CO_2, which enters the biotope through photosynthesis. Our consumption of plants, and of animals that live off plants, results in ^{14}C levels in the human body paralleling those in the atmosphere with a short lag for seasonal growing and harvest cycles (Harkness, 1972; Libby et al., 1964).

Most molecules in a cell are in constant flux, with the unique exception of genomic DNA, which is not exchanged after a cell has gone through its last division. The level of ^{14}C integrated into genomic DNA should thus reflect the level in the atmosphere at any given time point, and we hypothesized that determination of ^{14}C levels in genomic DNA could be used to retrospectively establish the birth date of cells in the human body.

Atmospheric ^{14}C levels have been monitored over time at several locations. As there may be some local variation in ^{14}C levels in different geographical regions, we first established the atmospheric levels of ^{14}C in recent years in the geographical region of the studied population. We dissected year rings from Swedish pine from 1962 to present, as well as several rings from the pre-bomb time. The ^{14}C content of each tree ring was measured by accelerator mass spectrometry. A tree ring is laid down annually and the cellulose is not changed thereafter, thus giving an accurate reflection of the ^{14}C levels in the atmosphere for a specific year. The levels in Sweden closely matched the mid-latitude northern hemisphere levels (Figures 1B and 1C, Supplemental Table S1), in line with the rapid distribution of ^{14}C in the atmosphere. Since our local data closely corresponded to the extensive material of mid-latitude northern hemisphere measurements collected in Germany (Levin and Kromer, 2004), we related all our following analyses to these data.

We next asked whether cells generated at different time points had ^{14}C levels in genomic DNA that correspond to the time they were generated. The sensitivity of accelerator mass spectrometry is rapidly increasing, and ^{14}C levels can today accurately be established with samples containing as little as 30 μg carbon, which corresponds to genomic DNA from 15 million cells. It is critical when extracting the genomic DNA for this application to keep contamination with non-DNA carbon sources to a minimum and not to introduce exogenous carbon that could be isolated with the DNA and thus skew the analysis. We therefore modified established DNA-extraction protocols to minimize the risk of carbon contamination (see Experimental Procedures). DNA samples were analyzed for purity in several ways; in addition to spectrophotometric analysis, the contents of all samples were analyzed by HPLC and the amount of total carbon (^{12}C, ^{13}C, and ^{14}C) was determined during graphite preparation for isotope analysis by accelerator mass spectrometry. The knowledge of the amount of DNA (with a known proportion of the mass accounted for by carbon) and total carbon in each sample allows very sensitive detection of carbon contamination, irrespective of the source, and any sample in which the

total carbon content was higher than calculated from the amount of DNA was excluded from further analysis.

Depletion of hematopoietic stem and progenitor cells by irradiation results in the rapid loss of leukocytes, demonstrating their short life span. We analyzed ^{14}C levels in DNA from nucleated blood cells, representing a population of cells known to be newborn. The measured ^{14}C values were compared to the recorded atmospheric levels to establish at which time point they corresponded. The strategy to establish the age of a cell population by relating ^{14}C concentrations in genomic DNA to atmospheric levels is schematically depicted in Figures 2A and 2B. The levels of ^{14}C in leukocyte DNA did indeed correspond to the contemporary atmospheric levels at the time when the sample was taken (Figures 2C and 2D). We next asked whether cells generated at a known time point much earlier could be accurately dated. To this end, we analyzed DNA from early postnatal brain tissue collected many years earlier. Since the tissue was from postmortem infants, there is a narrow timeframe when the cells could have been generated. ^{14}C levels in DNA from early postnatal brain tissue corresponded to the time shortly after birth (Figures 2E and 2F), in line with brain cells being born both prenatally (mostly neurons) and postnatally (mainly glial cells, which form the majority). This analysis of cells generated at known time points confirms that ^{14}C levels in DNA can be used to establish when cells are born.

Establishing the Age of Cells in Adult Human Organs

We next birth dated cells in different adult organs. We selected intestine, skeletal muscle, and two brain regions, based on the assumption that these tissues have different rates of cell turnover. Intestinal epithelial cells have an average life span of about 5 days (Marshman et al., 2002), whereas many nonepithelial cells in the gut are likely to be long lived. There is thought to be very little cell replacement in the cerebral cortex and cerebellum. All tissues contain blood, but nucleated cells only account for about 1/1000 of cells in peripheral blood, and their effect on the average age of cells in an organ will be negligible. ^{14}C birth dating revealed that the average age of cells in the intestine (jejunum) is 10.7 ± 3.6 years (mean ± SD from three individuals of average age 34.8 years; Figure 3). We quantified the proportion of epithelial cells to other cells in histological sections of jejunum and found that in average 42% ± 3% (n = 5 individuals) of all cells in the specimen were epithelial. Assuming that all epithelial cells are contemporary, the average age of the nonepithelial cells is 15.9 years. Measurement of the level of ^{14}C in DNA from intercostal skeletal muscle from two individuals (37 and 38 years old) indicated an average age of 15.1 years.

The average age of cells in the gray matter of the cerebellum was almost as old as the individual (born at the age of 2.9 ± 1.2 years, Table 1), whereas cells from the occipital-cortex gray matter were substantially younger (Figure 3), indicating more cell turnover in the cerebral cortex than in the cerebellum. Analysis of multiple samples from the same individuals revealed a precision of ±2 years (1 SD) for the dating procedure and a high degree of reproducibility between individuals (Table 1).

An average cell age slightly younger than the individual, as seen for cerebellum, could reflect that the generation of cells continued for a short time postnatally and then stopped, or that most cells were generated before birth but a subpopulation of cells turned over in adulthood. These two possibilities can be distinguished by analyzing tissue from individuals born before the nuclear bomb tests, as schematically depicted in Figure 4A. The strategy to birth date cells builds on the steep slope of ^{14}C decline in the atmosphere after the nuclear bomb tests, and the resolution in time before the bomb tests is very poor. However, the low levels of ^{14}C before the bomb pulse makes the detection of a small population of cells born during or after the bomb tests especially sensitive, and we determined that we could detect such a population constituting down to 1% of the total cell population. Analysis of individuals born pre-bomb demonstrated ^{14}C levels in cerebellar gray matter that correspond to atmospheric levels prior to 1955 (Figure 4; Table 1). The cerebellar gray matter consists of approximately 92% neurons (Supplemental Figure S1), thus these data reflect primarily the average neuronal age. This indicates that cerebellar neurogenesis is completed in the postnatal period and also that there is limited turnover of glial cells.

Isolation of Cell Type-Specific DNA from Archival Material

The analysis of ^{14}C in genomic DNA gives an average age of cells in a tissue. Most tissues are complex and composed of many cell types, and in order to study the potential turnover of a specific cell type, we wanted to separate cells prior to analysis. We focused on the human brain since there is little known about the potential turnover of neurons, and alterations in neurogenesis have been implicated in human disease.

Fluorescence-activated cell sorting (FACS) using antibodies against specific cell-surface epitopes is commonly used to purify distinct cell types. However, the complex morphology of neurons makes it difficult to isolate intact cells from brain tissue. This, together with the lack of pan-neuronal cell surface proteins, prompted us to modify this strategy and instead sort cell nuclei. Nuclei were first isolated from dissociated tissue and then incubated with directly conjugated antibodies to NeuN (Figures 5A–5F), a well-established neuron-specific epitope mainly localized to the nucleus (Mullen et al., 1992). This enabled FACS isolation of neuronal nuclei with high specificity (Figures 5G and 5H). Re-analysis of sorted nuclei revealed that we could obtain >99% neuronal nuclei.

A significant advantage of the method to sort nuclei, rather than whole cells, is that the nuclear sorting is less sensitive to post-biopsy or post-mortem interval and, most importantly, works equally well on fresh tissue and frozen archival material. This makes it possible to study material already collected in biobanks.

Cortical Neurons Are as Old as the Individual

Contrary to the long-standing dogma that neurons cannot be generated in the adult brain, it is now firmly

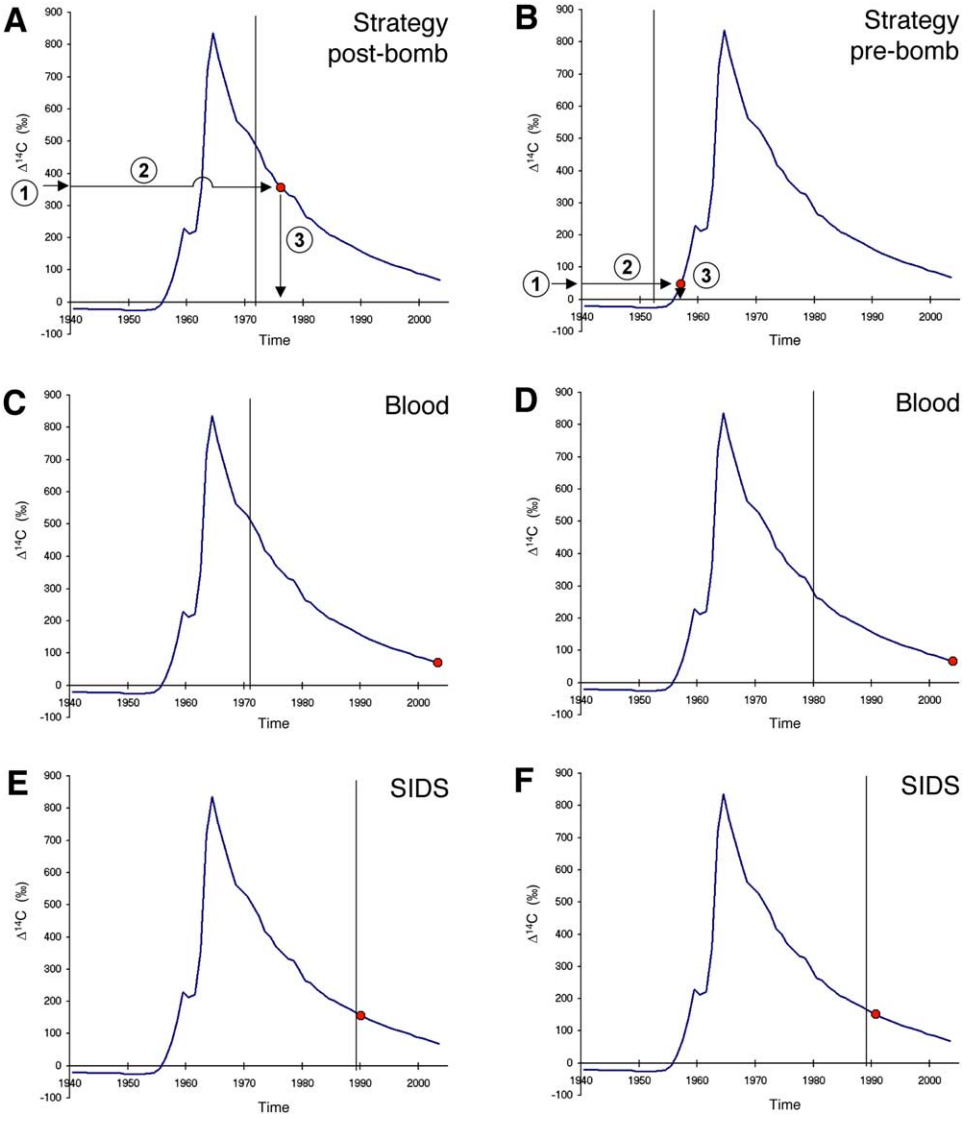

Figure 2. The Level of ^{14}C in Genomic DNA Reflects when a Cell Was Born

(A and B) Schematic images depicting the strategy to establish the birth date of cells. The individual in (A) is born after the period of nuclear bomb tests and the individual in (B) is born before. The time of birth of the person is indicated by a vertical line in each plot. First, the ^{14}C concentration in genomic DNA from the cell population of interest is established by accelerator mass spectrometry. Second, the measured ^{14}C value is related to the recorded atmospheric levels to establish at what time point they corresponded (indicated by red dot). Third, the year is read off the x axis, giving the birth date of the cell population. All the following data are presented in this way, with each plot representing one individual.

(C and D) ^{14}C levels in genomic DNA of nucleated peripheral blood cells, which are very short lived with a high turnover rate, correspond to contemporary atmospheric levels at the time of sampling (2004), irrespective of when the person was born (indicated by vertical line). The levels of ^{14}C in genomic DNA of cells known to be older (brain cells from archival specimen from sudden infant death syndrome patients, SIDS) correspond to the time when the cells were generated (E and F). The error bars for the accelerator mass spectrometry readings are too small to be visualized in this and the following graphs. Each graph represents one individual.

established that neurogenesis continues in discrete brain regions throughout life in all studied mammals (Altman and Das, 1965; Eriksson et al., 1998; Gould et al., 1998; Kaplan and Hinds, 1977). The establishment of a strategy for the retrospective birth dating of cells together with FACS isolation of neuronal nuclei makes it possible to study neuronal turnover in the adult human brain.

Technical difficulties have contributed to conflicting data regarding the distribution and extent of adult neurogenesis in experimental animals. One of the most controversial issues is whether neurogenesis persists in the cerebral cortex. Studies in several species have indicated that this may occur under physiological or pathological conditions (Chen et al., 2004; Dayer et al., 2005; Gould et al., 1999; Gould et al., 2001; Kaplan, 1981; Magavi et al., 2000), whereas other studies have failed to detect adult cortical neurogenesis (Kornack

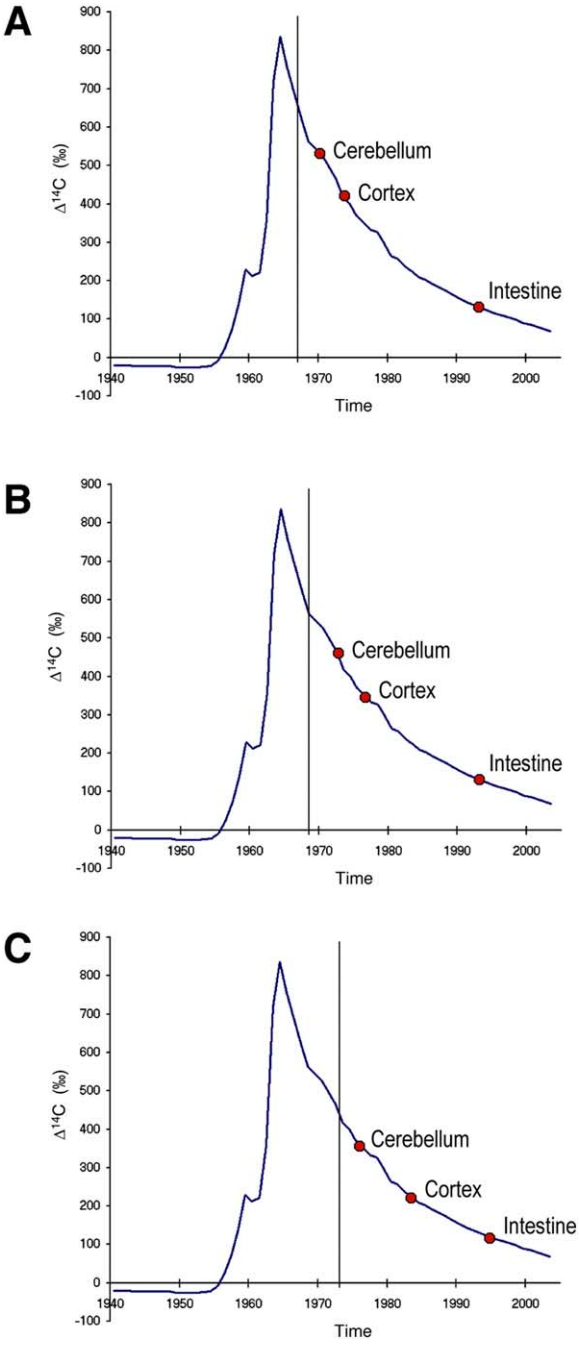

Figure 3. Cell Age in Different Adult Human Organs

Analysis of ^{14}C levels in genomic DNA from cerebellar gray matter, occipital-cortex gray matter, and small intestine (jejunum) from three representative individuals (A–C) born at different times (indicated by vertical lines) reveals the differing turnover rates of cells in different tissues.

and Rakic, 2001). It has been suggested that some positive results were due to technical shortcomings, including unspecific labeling with BrdU or mistaken colocalization due to low-resolution microscopy (Kornack and Rakic, 2001; Nowakowski and Hayes, 2000; Rakic, 2002b). Perhaps the strongest indication of adult cortical neurogenesis under physiological conditions, from a technical point of view, comes from electron microscopic detection of [^{3}H]thymidine neurons in the occipital cortex of the rat (Kaplan, 1981).

We therefore set out to retrospectively determine the age of neurons in the adult human occipital cortex, to establish whether there is any appreciable addition of neurons in this brain region postnatally. ^{14}C levels in unsorted total occipital cortex showed an average age substantially younger than the individual, pointing to cell turnover (Figures 3 and 6). To specifically analyze the age of neurons and nonneuronal cells, we isolated NeuN-positive and -negative cells by flow cytometry. ^{14}C levels in NeuN-positive neurons from individuals born after the bomb pulse demonstrated levels corresponding to the average age of the cells being as old as the individual (Figures 6A and 6B). We can currently establish the age of a cell population with a precision of ±2 years, and the time of birth of the individual was within this error margin in all measurements of NeuN-positive neurons from occipital cortex. The NeuN-negative cells, which are mainly glial cells, were substantially younger (Figures 6A and 6B). We additionally analyzed cells from individuals born before the bomb pulse, which provides a very sensitive measure of postnatal cell turnover. The ^{14}C levels in genomic DNA from NeuN-positive neurons corresponded to those in the atmosphere prior to the nuclear bomb tests (Figures 6C and 6D), which suggests that adult neurogenesis does not occur in this region.

Discussion

Cell turnover may be necessary under physiological or pathological conditions to maintain certain organs, yet our knowledge regarding this process in most human tissues is scarce due to a lack of means to study this process. We describe here a general strategy to study cell renewal in man. The dramatic increase in atmospheric ^{14}C levels and the subsequent exponential decline have resulted in different amounts of ^{14}C being integrated into the DNA of cells depending on the time point the DNA was synthesized. We all therefore have a date-mark in our DNA, and we show here that this can be used to establish the age of cells.

DNA as a Time Capsule

Most molecules in a cell are in constant flux, and DNA is likely to be the most stable molecule after a cell has undergone its last mitosis. It is important to consider the stability of DNA for the validity of the current technique since turnover of nucleotides in the absence of cell division would give a false impression of a cell population being younger than its actual age. DNA damage, followed by nucleotide exchange, mainly occurs during DNA replication (Nouspikel and Hanawalt, 2002). Since DNA repair during cell division will not influence the current analysis, it is most important to consider the potential turnover of DNA in very rarely dividing or postmitotic cells. DNA repair in postmitotic cells is limited to transcribed genes, and untranscribed DNA in terminally differentiated cells, including human neurons, does not appear to be repaired even after massive experimental

Table 1. Resolution and Reproducibility of Retrospective Birth Dating

Case	DOB of Person	n	Δ^{14}C% Mean (SD)	Cell Birth Date (SD)	Average Birth Date of Cell after DOB
ND014	1968.2	4	462 ± 95	1972.6 ± 3.3	4.4
ND004	1972.6	10	382 ± 42	1975.1 ± 1.9	2.5
ND009	1973.2	17	357 ± 48	1976.2 ± 1.5	3
ND002	1973.8	4	373 ± 4	1975.4 ± 0.8	1.6
				Average (SD)	2.9 ± 1.2
ND017	1952.0	2	–8.9	1955.0 ± 1.5	3
ND018	1953.4	3	0.5	1955.8 ± 1.2	2.4
ND007	1955.0	2	–8.5	1955.5 ± 0.2	0.5
ND008	1958.1	2	381	1962.6 ± 2.2	4.5
				Average (SD)	2.6 ± 1.7

Accelerator mass spectrometer analysis of genomic DNA from cerebelli from individuals born after (upper half) or before (lower half) the nuclear bomb tests. n indicates the number of independent analyses of different samples for each individual. The cell birth date is the time point when the measured Δ^{14}C corresponded to the atmospheric value. The resolution before the nuclear bomb tests is in general very poor, with the exception of the years just before the testing. DOB is date of birth.

damage (Nouspikel and Hanawalt, 2002). Nucleotide exchange, even in transcribed DNA, appears limited in terminally differentiated cells. The mutation rate of transcribed genes in rarely dividing human lymphocytes has been measured using different techniques and estimated to be 1.4–2/10^9 per day and locus, which corresponds to 5.2–7.3/10^5 events over a century (Bridges, 1997; Green et al., 1995; Morley, 1996). Since these frequencies are obtained for transcribed genes, the rate is likely to be substantially lower in total DNA. Thus,

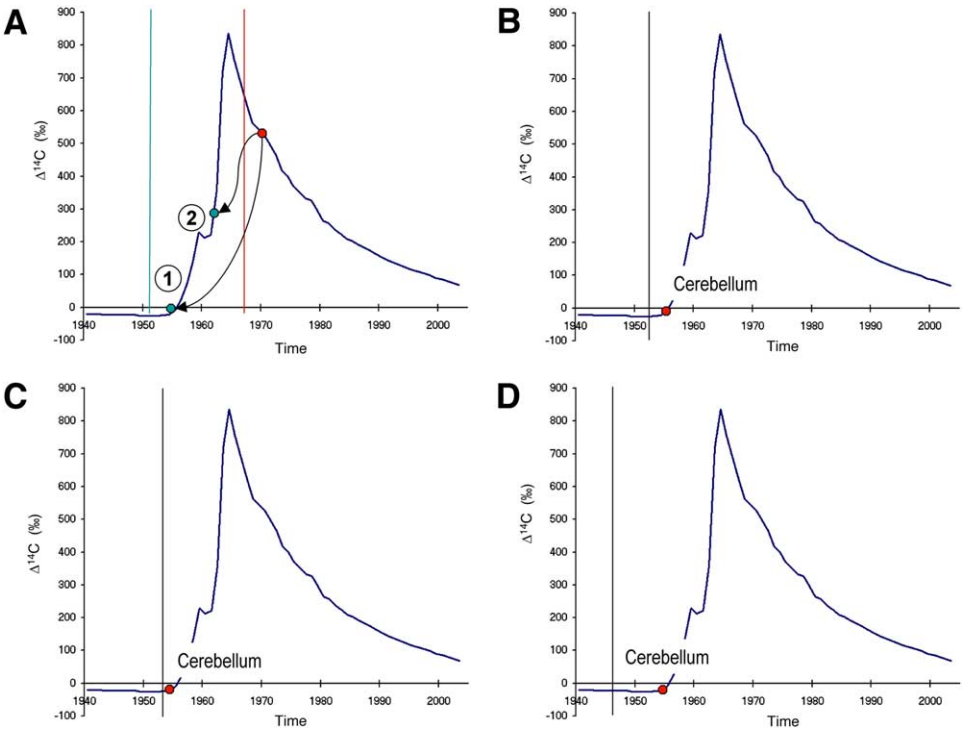

Figure 4. Analysis of Tissues from Individuals Born before the Nuclear Bomb Tests

(A) Schematic representation of how the integration of information from individuals born before and after the nuclear bomb tests can provide further information on the kinetics of a population. A ^{14}C value corresponding to the generation of a cell population shortly after birth (red dot) in an individual born after the nuclear bomb tests (red vertical line indicates birth date) can arise in several ways. The two extreme possibilities are that either (scenario 1) all cells are born up to shortly after birth and no cells are generated after that or (scenario 2) that one cell population is generated prenatally and another population is born substantially later. Analysis of ^{14}C in genomic DNA of cells from individuals born before the nuclear bomb tests (indicated in green in [A]) offers a sensitive way to detect postnatal generation of cells and can distinguish these possibilities.

(B–D) Cells from cerebellar gray matter have ^{14}C levels corresponding to the time before 1955, indicating no or very limited generation of cells in this region after the perinatal period. Each graph represents one individual and is a representative example.

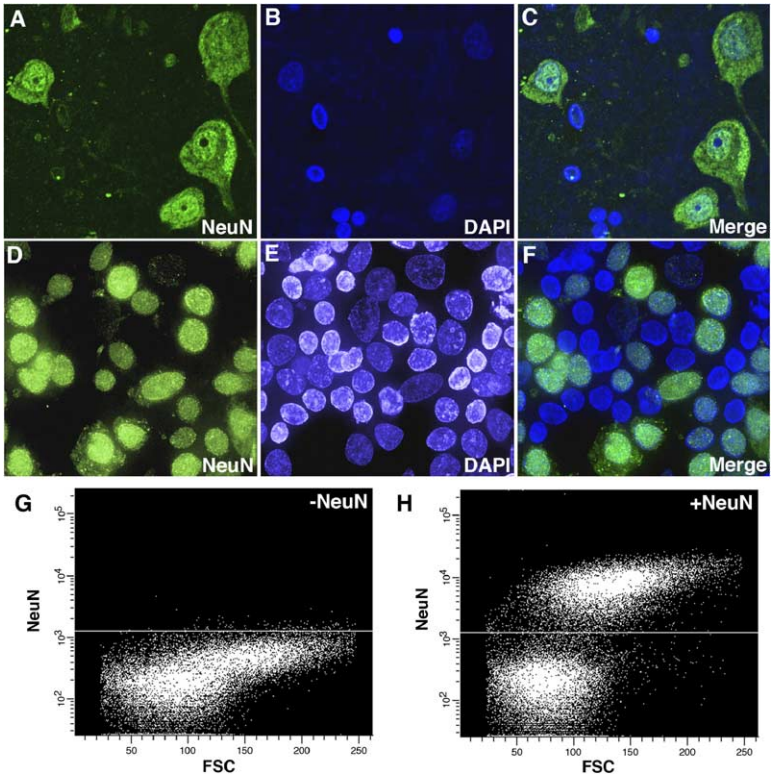

Figure 5. Isolation of Neuronal Nuclei from the Adult Human Brain

Immunohistochemical identification of neurons with antibodies against the neuron-specific epitope NeuN in tissue sections (A–C) and nuclear isolates (D–F) from adult human cerebral cortex. All nuclei are labeled with DAPI. (G) and (H) show specific isolation of NeuN+ neuronal and NeuN– nonneuronal nuclei by FACS sorting.

available data indicate extremely little nucleotide turnover in rarely dividing and postmitotic cells, well below levels that would influence the current method. Our finding that cerebellar cells and cortical neurons have ^{14}C levels corresponding to the time of birth of the person corroborates that nucleotide exchange in postmitotic cells is very limited and below the detection level with the method employed.

Another source of carbon exchange in the genome is methylation, which constitutes an important mechanism in transcriptional regulation (Bird, 2002). Methylation is restricted to cytosine residues 5′ to guanosine in CpG stretches, and the degree of methylation varies in different organs between 0.7%–1% of nucleotides in DNA (Ehrlich, 1982). Even if all methyl groups were contemporary, addition of one carbon atom by methylation of 1% of nucleotides (which contain in average 9.75 carbon atoms) could maximally skew the analysis by about 1‰ and is thus negligible.

Sensitivity of Retrospective Birth Dating

How many cells have to be generated postnatally for turnover to be detected? The resolution in time and number of cells depends on when the person was born in relation to when the new cells were generated. In an individual born just before the nuclear bomb tests, the relative difference in ^{14}C concentration is highest between the time of birth and the period of and just after the nuclear tests and drops thereafter. The sensitivity for detecting cell generation is therefore highest for the childhood period. A person born 20 years before the bomb pulse will instead have the largest relative difference in ^{14}C levels between the time up to adolescence and early adulthood. In contrast, analysis of cells from a person born after the bomb tests will have the highest sensitivity for the latest born cells. Taking these differences into account, the highest total sensitivity is reached by analysis of several individuals born at different time points in relation to the nuclear bomb tests. This allows for the detection of relatively small additions of new cells and may also provide information as to when in time a cell population is generated. The detection limit, when comparing time points with the largest difference in ^{14}C levels with the current sensitivity of accelerator mass spectrometry, is about 1% newborn cells within a population of old cells.

The current method provides an average age for a cell population. The sensitivity of accelerator mass spectrometry is increasing rapidly, making it likely that successively smaller populations can be analyzed in the future. Single-cell resolution will, however, never become possible since there is only one ^{14}C atom in the DNA of less than every 15^{th} cell (6.4×10^{10} carbon atoms in nucleotides of the human genome and 1 out of 10^{12} C is ^{14}C).

The current analysis provides a qualitatively different type of information than with ^{3}H-thymidine and BrdU in experimental animals. Labeled nucleotides are given in a short pulse, and analysis of labeled cells at different time points provides information at the single-cell level about newly generated cells of a certain phenotype. Since the labeled nucleotide is given as a pulse, it is difficult to detect rare events as well as to gain a view of the proportion of cells that are exchanged over a longer time period. The current method is unique in that

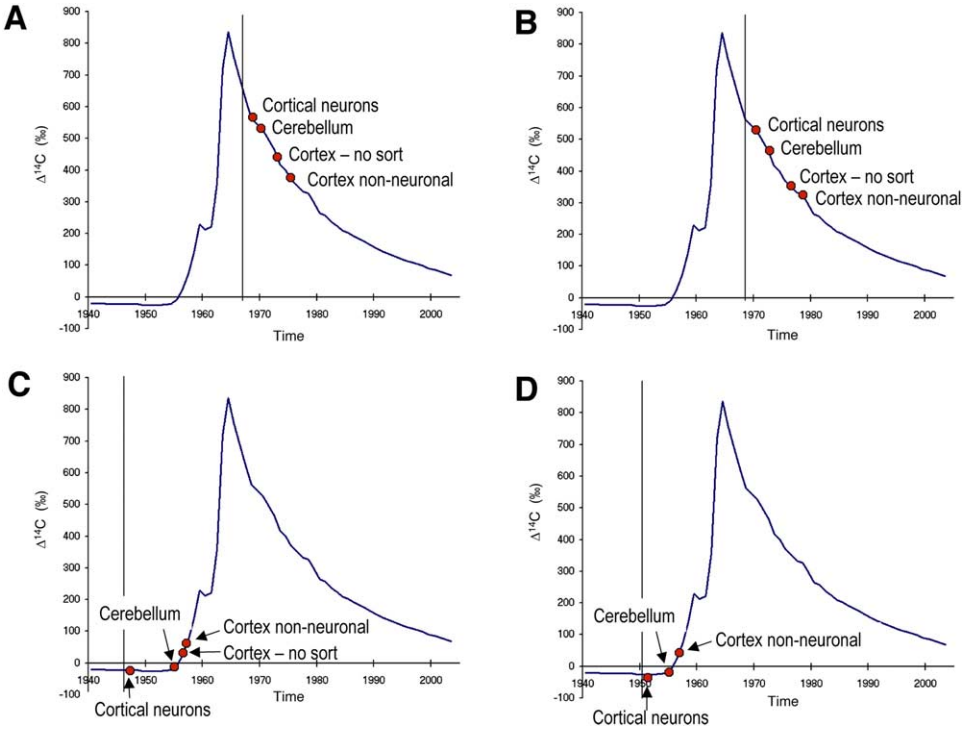

Figure 6. Cortical Neurons Are as Old as the Individual

The ^{14}C levels in genomic DNA of FACS-isolated NeuN+ neurons and NeuN– nonneuronal cells from occipital cortex of individuals born after (A and B) or before (C and D) the period of nuclear bomb tests indicate specific exchange of nonneuronal cells and no or minimal turnover of neurons.

it provides information over the whole life span of the individual rather than at a single time point, which can give good resolution even for rare events. For example, if 1/100,000 cells would be replaced per day in a certain structure, it would be far from reliably detectable by labeled nucleotides, but over a decade it would amount to 3.65% of the cells, which is easily detected with the current methodology.

Atmospheric levels of ^{14}C rapidly equalized around the globe after the nuclear bomb tests, making the method generally applicable in different countries. ^{14}C levels may, however, be slightly lower in extremely polluted industrialized regions due to excessive burning of ^{14}C-depleted fossil fuels such as coal, oil, and natural gas (Pawelczyk and Pazdur, 2004). If an individual lived mainly off of food derived from the directly polluted area, ^{14}C values could be depressed and cell age underestimated. Our measurement of ^{14}C in tree rings in the region where the individuals studied in our analysis lived excluded pollution skewing our results, but this may be important to consider in more heavily industrialized areas.

The Age of Cortical Neurons

The cerebral cortex is the site of the highest cognitive functions. There is clearly a considerable degree of plasticity in the cortex, enabling memory formation and adaptation to new conditions (Chklovskii et al., 2004). Much of the plasticity can be accounted for by modulating preexisting connections, but there are also structural changes in the adult human cortex in response to certain conditions resulting in detectable changes in volumes in distinct areas (Draganski et al., 2004). It is important to understand if neuronal turnover may contribute to cortical plasticity.

The cerebral cortex spans a large area, and although structurally similar, different cortical areas have distinct functions. We selected to study the occipital cortex since the strongest evidence, from a technical point of view, for neurogenesis in animals under physiological conditions has been reported from this area (Kaplan, 1981). Our analysis revealed that neurons from the adult human occipital cortex have ^{14}C levels in their genomic DNA corresponding to the time when the individual was born, lending little support to postnatal cortical neurogenesis in man.

Kaplan (1981) reported 1/10,000 cortical neurons in the adult rat, and Gould and colleagues (1999) found approximately 1/100,000 labeled cortical neurons in the adult primate being newborn after single injections of labeled nucleotides. It is difficult to infer the rate of cell replacement over time from pulse labeling with ^{3}H-thymidine or BrdU, but even with conservative estimates these frequencies would amount to a large fraction of neurons being generated during adulthood (Nowakowski and Hayes, 2000); the fraction is very easily detectable by retrospective birth dating. Our data establish that neuronal turnover of this magnitude leading to long-term stable integration of new nerve cells does not

occur in the adult human occipital cortex. This does, however, not rule out the possibility of neurogenesis in other cortical areas or that neurogenesis may be induced in response to injury (Chen et al., 2004; Magavi et al., 2000).

Applications for Retrospective Birth Dating

The rapid development of accelerator mass spectrometry has made sensitive ^{14}C analyses increasingly accessible and affordable, enabling scientists in many fields to use it to answer specific questions. The steady decrease of ^{14}C in the atmosphere will make retrospective birth dating less sensitive with time and limits the time period in which this technique may be used on new material. However, the existence of tissue banks, where material has been collected from many pathologies, and the applicability of the method on archival material, allows for analyses of already collected material. In some regards though, the technique becomes even more powerful in the coming decades; as people born around the nuclear bomb tests get older, its usefulness for the study of age-related pathologies, such as dementia, will increase.

The possibility to determine the age of cells can give us a map of the human body from a cell-renewal perspective. The stability or turnover of cells in different tissues is a fundamental feature that may influence the response of different organs to insults and the aging process. Analysis of cell turnover in different pathologies may further the understanding of certain diseases.

Experimental Procedures

Tissue Collection

Tissues were procured from cases admitted during 2003 and 2004 to the Department of Forensic Medicine, Karolinska Institute for autopsy, after consent from relatives. Ethical permission for this study was granted by the Karolinska Institute Ethical Committee. Five individuals born before and five born after the nuclear bomb tests were included in this study. Tissues were frozen in 1 g samples and stored at –80°C until further analysis.

Nuclei Isolation

Tissue samples (1 g) were defrosted and Dounce homogenized in 10 ml lysis buffer (0.32 M sucrose, 5 mM $CaCl_2$, 3 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl [pH 8.0], 0.1% Triton X-100, 1 mM DTT). Homogenized samples were gently suspended in 18 ml of sucrose solution (1.8 M sucrose, 3 mM magnesium acetate, 1 mM DTT, 10 mM Tris-HCl [pH 8.0]), layered onto a cushion of 10 ml sucrose solution, and centrifuged at 30,000 g for 2.5 hr at 4°C. The isolated nuclei were resuspended and stored overnight at 4°C.

Immunohistochemistry

Brain tissue was immersion fixed in 4% formaldehyde in PBS and cut on a cryostat. Tissue sections were incubated overnight at 4°C with mouse monoclonal antibodies against NeuN (Mullen et al., 1992) at a dilution of 1:1000.

Flow Cytometry

Neuronal nuclei were isolated by FACS of nuclei labeled with the neuron-specific monoclonal antibody NeuN. NeuN antibodies were directly conjugated with Zenon mouse IgG labeling reagent (Alexa 488, Molecular Probes) as follows: 10 μl of Alexa 488 conjugate was added to 100 μl blocking mix (PBS/0.5% BSA/10% NGS) and 300 μl NeuN mix (6 μl NeuN in 1.5 ml PBS) and incubated for 5 min at room temperature (RT). One milliliter of nuclei mix (cerebellum, 83 mg starting tissue/ml; all other brain regions, 167 mg starting tissue/ml) was added to NeuN-conjugated mix and incubated at 4°C for a minimum of 45 min.

To ensure that only single nuclei were sorted (and not aggregates), nuclei were stained with a red-fluorescent cell-permeable DNA probe, DRAQ5, according to manufacturer's instructions (Biostatus). Single nuclei were easily discerned from doublets, triplets, and potential higher-order aggregates based on their fluorescence intensity (FL1), and a gate was set so that only single nuclei were sorted. Using this technique it was calculated that 98% or more of all nuclei are singlets. DRAQ5 binds tightly to DNA and therefore becomes a source of carbon contamination to the extracted DNA. To avoid this problem an aliquot of nuclei was labeled with DRAQ5 and the singlet population plotted as a function of forward scatter width (FSC-W) versus forward scatter height (FCS-H). Using these parameters it is easy to determine single nuclei from doublets, triplets, and potential higher-order aggregates as well as background noise (Wersto et al., 2001). Selective isolation of single-nuclei events was performed using this technique for every sort.

Nuclei were sorted based on purity, and the purity of all sorts confirmed by reanalyzing the sorted populations. Nuclei pellets were collected by centrifugation and frozen at –80°C in readiness for DNA extraction. All FACS analysis and sorting was performed using a FACS Vantage DIVA (BD).

DNA Extraction

The following extraction protocol was optimized for minimal introduction of carbon and maximal DNA yield and purity. Two milliliters of 1% SDS, 5 mM EDTA-Na_2, 10 mM Tris-HCl (pH 8.0), and 100 μl of protease solution (2 mg/ml, Sigma) was added to each sample of isolated nuclei and the tube was inverted several times and incubated at 65°C for 40 min (tubes were inverted periodically). After this incubation, 20 μl RNase cocktail (Ambion) was added to each sample. The samples were inverted again and incubated 20 min. Three milliliters of sodium iodide solution (7.6 M NaI, 20 mM EDTA-Na_2, 40 mM Tris-HCl [pH 8.0]) was added to each sample followed by repeated inversion, after which 6 ml filtered 99.5% ethanol was added and tubes inverted repeatedly until the DNA precipitated (Wang et al., 1994). The DNA was washed in 70% ethanol for 15 min, and this was repeated three times. Finally, the DNA was quickly rinsed in a water bath, for 5 to 10 s, and transferred to a glass vial. Once the DNA sample had dried, it was resuspended in water and left in the incubator at 65°C for at least three days, with constant inversions, in order to fully dissolve the DNA in the water. DNA was quantified using spectrophotometry and purity was analyzed by spectrophotometry and HPLC.

For DNA isolation from whole tissue, fresh or defrosted tissue was minced and mechanically dissociated using a Stomacher-80 Biomaster in plastic bags containing 1.2 ml digestion buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8], 25 mM EDTA [pH 8], 0.5% SDS, 0.1 mg/ml proteinase K) per 100 mg tissue. Samples were incubated shaking at 50°C for 3–12 hr, until fully digested. Nucleic acids were extracted at RT using an equal volume of phenol/chloroform/isoamyl alcohol and centrifuged for 10 min at 1700 g in a swinging bucket rotor. This step was repeated 2–6 times as necessary (as determined by achieving a protein-free interface between the aqueous upper phase and the phenol-protein containing lower phase). The aqueous layer was transferred to a new tube and DNA precipitated by adding half the volume of 7.5 M ammonium acetate and 2 volumes (of the original amount of top layer) of 100% ethanol. DNA was washed thoroughly in 70% ethanol, air dried, and resuspended in nuclease-free DDH_2O.

DNA purity for all samples was analyzed by spectrophotometry and HPLC. A minimum of 15 million cells is required for ^{14}C analysis with the current sensitivity of accelerator mass spectrometry. The amount of tissue that is required for an analysis varies from region to region, depending upon region-specific cell densities.

Accelerator Mass Spectrometry

All accelerator mass spectrometry analyses were performed blind to age and origin of the sample. Purified DNA samples suspended in water and whole-tissue samples were transferred to quartz combustion tubes and evaporated to dryness in a convection oven maintained at 90°C–95°C. Excess copper oxide (CuO) was added

to each dry sample, and tubes were evacuated and sealed with a H_2/O_2 torch. Tubes were placed in a furnace set at 900°C for 3.5 hr to combust all carbon to CO_2. The evolved CO_2 was purified, trapped, and reduced to graphite in the presence of iron catalyst in individual reactors (Vogel et al., 1987). Large C samples (>500 μg) were split and $\delta^{13}C$ was measured by stable isotope ratio mass spectrometry. Graphite targets were measured at the Center for Accelerator Mass Spectrometry at Lawrence-Livermore National Laboratory.

Few of the DNA samples were large enough to get $\delta^{13}C$ splits, but those measured ranged from –19‰ to –23‰, values similar to the whole tissues. We used a $\delta^{13}C$ correction of –21 ± 2‰ for all samples. Corrections for background contamination introduced during sample preparation were made following the procedures of Brown and Southon (Brown and Southon, 1997). The measurement error was determined for each sample and ranged between ±2‰ and ±10‰ (1 SD) $\Delta^{14}C$. All ^{14}C data are reported as decay corrected $\Delta^{14}C$ following the dominant convention of Stuiver and Polach (1977). This convention established for reporting radiocarbon data in chronological and geophysical studies was not developed to deal with post-bomb data, but it is the most common pending the adoption of a standard nomenclature for post-bomb data (Reimer et al., 2004).

Preparation of Tree Rings

A wedge was sliced from each cross-section of pine tree, and the surface was sanded to remove surface contamination. Year rings were counted inward from the outer edge. For years of interest, a subsample was selected which integrated a whole growth year. Wood samples were prepared by rinsing with dilute acid-base-acid followed by distilled water and then dried as described by Love et al. (2003). The samples were placed in quartz tubes with excess CuO, sealed, and combusted to CO_2. The CO_2 was then reduced to graphite over iron catalyst and analyzed by accelerator mass spectrometry.

Supplemental Data

Supplemental data include one figure and one table and can be found with this article online at http://www.cell.com/cgi/content/full/122/1/133/DC1/.

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BRIEF COMMUNICATIONS

Age written in teeth by nuclear tests

A legacy from above-ground testing provides a precise indicator of the year in which a person was born.

Establishing the age at death of individuals is an important step in their identification and can be done with high precision up to adolescence by analysis of dentition, but it is more difficult in adults. Here we show that the amount of radiocarbon present in tooth enamel as a result of nuclear bomb testing during 1955–63 is a remarkably accurate indicator of when a person was born. Age is determined to within 1.6 years, whereas the commonly used morphological evaluation of skeletal remains and tooth wear is sensitive to within 5–10 years in adults.

The amount of carbon-14 isotope (^{14}C) in the atmosphere remained relatively stable until 1955, when above-ground nuclear bomb tests caused it to rise dramatically[1,2]. Although the bombs were detonated at only a few locations, the additional ^{14}C in the atmosphere rapidly equalized around the globe. Since the Test Ban Treaty in 1963, atmospheric ^{14}C has been dropping exponentially (Fig. 1a). This is not primarily because of radioactive decay (the half-life of ^{14}C is 5,730 years) — it is also due to diffusion from the atmosphere[3]. Atmospheric ^{14}C reacts with oxygen to form carbon dioxide, which is incorporated into plants by photosynthesis; by eating plants, and animals that feed on plants, the ^{14}C concentration in the human body closely parallels that in the atmosphere at any given time[4–6].

The enamel of individual teeth is formed at distinct, well characterized times during childhood[7,8] and it contains 0.4% carbon. There is no turnover of enamel after it has been laid down, so the ^{14}C concentration reflects that in the atmosphere at the time of enamel formation. We measured the ^{14}C content of tooth enamel (for methods, see supplementary information) and related it to the known concentrations in the atmosphere in different years to establish the year of tooth formation. This date was then related to the known age for enamel deposition of individual teeth[7] to establish the person's year of birth (Fig. 1a).

We found that this method gave a remarkably precise estimate of age for 22 individuals ($R^2 = 0.99$ from regression shown in Fig. 1b; for details, see supplementary information). The average systematic deviation from the correct value was +0.2 years, and the average absolute error for individual measurements was 1.6 ± 1.3 years (s.d.). This indicates that the precision is substantially higher than that obtained by other available methods[9].

The final formation of enamel is for the wisdom teeth at 12 years of age. For individuals born before 1943 (12 years before the onset of nuclear bomb testing), we can therefore conclude by this method only that birth occurred before that year, albeit with a high degree of certainty (100% correct in our analysis (Fig. 1b); $n = 4$). In any case of ambiguity as to whether birth occurred before or after the peak of nuclear bomb testing, it is necessary to analyse two teeth that were formed at different ages: this distinguishes whether the ^{14}C measurements relate to the rising or falling part of the ^{14}C curve (Fig. 1a).

The sensitivity of our method is mainly determined by variation between individuals in their age at tooth formation, and the precision of the ^{14}C measurement. The degree of inter-individual variation is different for different teeth, so selection for ^{14}C measurement of teeth with the least variation[8] and of several teeth from the same individual should give a more accurate date of birth. With regard to measurement precision, we cannot exclude the possibility that differences in diet or in local conditions might contribute some variability in the amount of ^{14}C incorporated into tooth enamel. Although such an effect is not supported by results from comparative analyses of different foodstuffs produced in rural and industrial areas[10], the method will need to be verified on a larger number, and perhaps on a wider geographical range, of cases before it can be applied to forensic work.

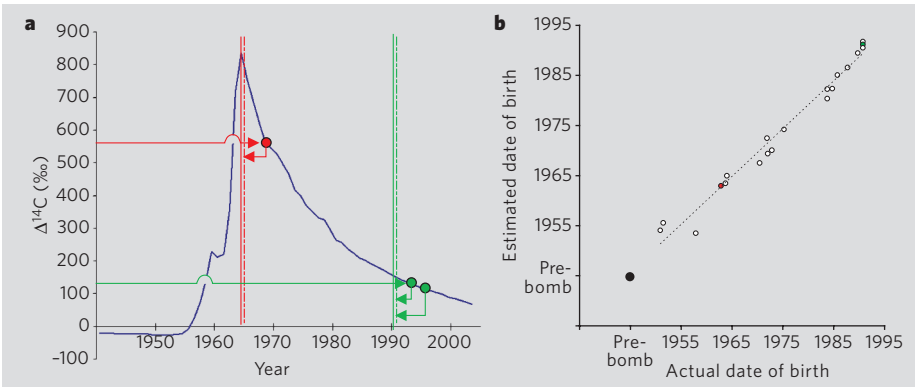

Figure 1 | Date of birth determined from ^{14}C in teeth. **a,** Nuclear bomb tests during 1955–63 produced large amounts of ^{14}C, which have since declined exponentially (blue line). The ^{14}C:C ratio has ranged from 1.15×10^{-12} to 2.20×10^{-12} since 1950. $\Delta^{14}C$ represents the ^{14}C value corrected for radioactive decay and ^{13}C fractionation (see supplementary information). To estimate an individual's date of birth, the ^{14}C concentration measured in their tooth enamel is plotted on to the curve of atmospheric $\Delta^{14}C$ against time (blue) to find the year of enamel synthesis (right-pointing arrows), and the known age at enamel formation for individual teeth was then subtracted from the year obtained to give the date of birth (left-pointing arrows; dashed vertical lines). Two representative cases are shown (red and green); two teeth were analysed for the case depicted in green. Solid vertical lines, actual dates of birth. **b,** Relation between estimated and actual dates of birth. Each point corresponds to one individual, except for the 'pre-bomb' point, which represents four individuals; coloured points are cases shown in **a**.

Although the nuclear bomb tests were conducted several decades ago and the resulting change in atmospheric ^{14}C is now decreasing only slowly (Fig. 1a), the method described here should allow precise age determination for a long time to come because techniques for ^{14}C measurement are becoming increasingly sensitive. In addition, accelerator mass spectrometry for ^{14}C analysis has become more accessible and inexpensive, making the potential application of our dating method no more difficult than other methods now used in routine forensic examinations.

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BOTANY

Floral fluorescence effect

The way flowers appear to insects is crucial for pollination[1–3]. Here we describe an internal light-filtering effect in the flowers of *Mirabilis jalapa*, in which the visible fluorescence emitted by one pigment, a yellow betaxanthin, is absorbed by another, a violet betacyanin, to create a contrasting fluorescent pattern on the flower's petals. This finding opens up new possibilities for pollinator perception as fluorescence has not previously been considered as a potential signal in flowers.

We investigated the spectra and distribution of the pigments in the multicoloured, strikingly patterned flowers of *M. jalapa* (Nyctaginaceae), which open only in the late afternoon. This and related plants, such as *Bougainvillea*, *Celosia*, *Gomphrena* and *Portulaca*, contain pigments known as betalains. These comprise the yellow, fluorescent betaxanthins[4] and violet betacyanins, of which betanin (betanidin-*O*-β-glucoside) is the most common.

We extracted and purified the pigments of *M. jalapa* flowers and analysed them by high-performance liquid chromatography, as previously described[5]. The analysis confirmed that the pigmentation pattern on the flowers was due to a mixture of betaxanthins and betanins. Measurement of the fluorescence-emission spectrum of dopaxanthin and the absorbance spectrum of betanin indicates that the light emitted by the fluorophore is strongly re-absorbed (Fig. 1). Addition of increasing concentrations of betanin to the dopaxanthin solution reduced the intensity of its fluorescence, until only 30% of the initial fluorescence was detectable at a ratio of 8.5:1. (For details and methods, see supplementary information.)

This internal light-filtering effect between the two types of betalain plant pigment causes a fading of visible fluorescence on parts of the flower where both types are present; areas containing only betaxanthins appear yellow under white light because of a combination of fluorescence and reflectance of non-absorbed radiation (Fig. 2a). The effect can be demonstrated in a system designed to visualize green fluorescence, which filters the incident light to blue and causes betaxanthins in the flower to fluoresce by emitting green light (Fig. 2b).

Detailed images of different zones of petal coloration were obtained by using light and fluorescence microscopy. A brightfield image under white light shows some cells containing only betaxanthins (Fig. 2c, yellow), others with betacyanins (Fig. 2c, deep-red spots), and some with both pigments together (Fig. 2c, orange).

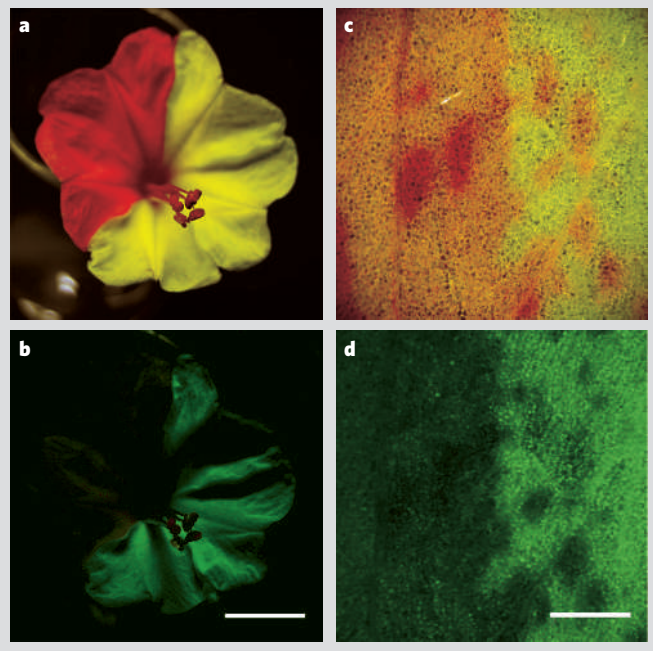

Figure 2 | Visible fluorescence in *Mirabilis jalapa* petals. **a, b,** Flower with areas of red or yellow coloration under white light (**a**); only the yellow areas emit green fluorescence when excited by blue light (**b**) (scale bar, 1.5 cm). **c, d,** Light micrographs of a section of a single red-and-yellow petal, showing brightfield (**c**) and fluorescent (**d**; excitation wavelength, 450–490 nm) images (scale bar, 500 μm). Green fluorescence is due to betaxanthins; dark areas correspond to orange areas in **c**, where light emitted from the fluorescent pigment is absorbed by betanin.

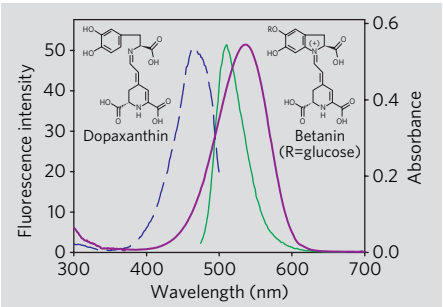

Figure 1 | Spectra of dopaxanthin and betanin. Dopaxanthin is used as a model betaxanthin because of its structural (insets) and biochemical similarity to betacyanins. When excited by blue light, betaxanthins emit green fluorescence[4]. Fluorescence spectra (blue line, excitation spectrum; green line, emission spectrum) for natural dopaxanthin (6.0 μM, in water) are shown; violet line, absorbance spectrum of pure betanin (8.4 μM, in water). Note the overlap of the emission and absorbance spectra of the pigments.

The fluorescence micrograph shows that fluorescence is inhibited in areas where betaxanthins coexist with betanin (Fig. 2d) — the dark area corresponds to the orange area in Fig. 2c.

Fluorescence can be an important signal in mate choice for budgerigars[6] and possibly in mantis shrimp[7], and it may be that in flowers it attracts pollinators. The patterns arising from the internal light-filtering effect between betalain pigments described here could encourage bees[1] and bats[8], which have visual receptors that are sensitive to green light and can detect bright targets better than dim ones[9]. Variation in light emission by flowers at visible wavelengths also modifies their colour, which would enhance their visibility to pollinators[10].

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