Discussion
Circulating erythrocytes have a life span of 50 to 60 days in mice and 120 days in humans (Goodman and Smith 1961, Horky et al 1978, Piomelli et al 1993, Deiss 1999). Thus roughly 2% and 1% of circulating erythrocytes are destroyed each day in mice and humans respectively. Phagocytosis by macrophages in the reticuloendothelial system in liver, bone marrow and spleen is considered to be the prime mechanism of destruction of erythrocytes in circulation. Factors that earmark erythrocytes for destruction are not clearly understood though several suggestions have been made about changes in aging erythrocytes that may render them susceptible to phagocytosis (Bratosin et al 1998, Kuypers and de Jong 2004, Lutz 2004, Bosman et al 2005). Such studies are hampered by lack of a good method to identify erythrocyte populations of defined age group in blood.

*In vivo* biotinylation of erythrocytes provided a good technique to study aging of erythrocytes *in vivo* (Suzuki and Dale 1988, Hoffmann-Fezer et al 1993). Three daily intravenous injections of biotinylation reagent were shown to label all erythrocytes in circulation (Hoffmann-Fezer et al 1993). Fresh erythrocytes that appeared in blood after the biotinylation step were not biotinylated. Therefore the biotin-ve population isolated from blood at any time point after the first biotinylation step, represented erythrocytes released into blood stream after the biotinylation step. Variations of this technique could be used to identify either very old or very young erythrocytes in a given mouse (Suzuki and Dale 1988, Fossati-Jimack et al 2002) but the technique could not be used to simultaneously identify old as well as young erythrocytes.

In the present thesis, we have modified and improved the above mentioned *in vivo* biotinylation technique. The new technique allows us to label a cohort of erythrocytes of a defined age group and track age related changes on this cohort of cells in blood circulation. This modification comprises a second biotinylation step that could be carried out at any time point after the first biotinylation step. To take an example, if the second biotinylation is done 5 days after the first biotinylation, the erythrocytes generated fresh during this window of 5 days would be without biotin label and will get biotinylated as a result of the second biotinylation step. The important point is that we used a lower dose of the biotinylation reagent so that the intensity of biotin label on erythrocytes labeled during the second biotinylation step
remains significantly lower than the biotin label on erythrocytes that got biotinylated in the first step. Biotin\textsuperscript{high} erythrocytes thus represent erythrocytes generated before the first biotinylation step and biotin\textsuperscript{low} population represents freshly made erythrocytes released in blood stream between first and second biotinylation steps.

Second biotinylation step could also result in additional biotinylation of the biotin\textsuperscript{high} population but because this population was already labeled to saturation, no further increase was observed as a result of the second biotinylation step. Hoffmann-Fezer et al. (1991, 1993) have demonstrated that biotin label on erythrocytes is very stable and does not change significantly with age of labeled erythrocyte in circulation. Our results confirm their observation and further show that the lower biotin label resulting from the second biotinylation step was also stable for the life time of the labeled erythrocytes. Due to the stability of the biotin label on erythrocytes, the difference in biotin label on biotin\textsuperscript{high} and biotin\textsuperscript{low} populations of erythrocytes was sustained till the labeled erythrocytes were all together lost due to normal turnover (Figure 2). It should be noted that all erythrocytes generated after the second biotinylation step would be biotin negative. Mice subjected to double \textit{in vivo} biotinylation (DIB) procedure, can be sacrificed at any time point after the second biotinylation step and their blood erythrocytes will have a profile as depicted diagrammatically in Figure III. Erythrocyte cohort generated between the first and second biotinylation steps (biotin\textsuperscript{low} population) can be identified flow cytometrically at any given time point and used to study age related changes on this cohort of erythrocytes as it progressively ages in blood circulation. By increasing the time period between the first and second biotinylation steps, simultaneous identification of very young and very old erythrocyte populations becomes possible in a given mouse, as explained in Figure IV.

The technique that we have developed would enable researchers for the first time to identify an aging cohort of erythrocytes in blood circulation in an objective manner. So far, erythrocytes belonging to different age groups were isolated by exploiting differences in physical properties like buoyant densities of young and old erythrocytes. The problem with such techniques is that there is considerable overlap between the distribution of such properties within cohorts of erythrocytes of different ages and it is almost impossible to isolate discreet erythrocyte populations of a
Double in vivo Biotinylation (DIB) Technique
Labeling a cohort of erythrocytes

1st Biotinylation step

Wait 5 days

2nd Biotinylation step

After several days

Erythrocyte cohort

TIME

○ Biotin negative RBC  □ Biotin-High RBC  □ Biotin-Low RBC

Figure III
Double in vivo Biotinylation (DIB) Technique
Simultaneous Identification of young and old RBCs

ERYTHROCYTES

1st Biotinylation step

Wait 25 days

2nd Biotinylation step

Wait 15 days

< 15 days 15-40 days > 40 days

TIME

○ Biotin negative RBC  ● Biotin-High RBC  ○ Biotin-Low RBC

Figure IV
defined age group by separation procedures based upon such parameters. DIB technique is highly objective as it labels all those erythrocytes that are present in the blood at the time of labeling, with high or low intensities of biotin, and the distribution of biotin label with in the biotin labeled populations is sufficiently sharp to avoid any mix up in identification of erythrocytes in these two groups. Availability of this technique should enable a proper re-evaluation of some generally held beliefs about age related changes in erythrocytes e.g. changes in buoyant density.

**Discussion**

Using the DIB technique, we first assessed the survival kinetics of blood erythrocytes. Survival kinetics of circulating erythrocytes is of interest as it can be a pointer to the mechanism of erythrocyte destruction. Two extreme models have been considered about the destruction of erythrocytes. Firstly, it is possible that erythrocytes are only destroyed when they reach a certain age in circulation and acquire certain crucial age dependent markers (Eadie and Brown 1953, Clark 1988). Alternatively, erythrocyte destruction could be a random process in which a certain fraction or erythrocytes are destroyed each day, irrespective of their age (Burwell et al 1953, Eadie and Brown 1953, Clark 1988). If the first model is correct, the proportion of biotin$^{low}$ cohort of erythrocytes in a DIB labeled mouse would start to decline in the blood only when this population has aged sufficiently and remain constant before such time. On the other hand, if erythrocyte destruction is random, the proportion of biotin$^{low}$ cohort of erythrocytes would start to fall from the very beginning, irrespective of the age of the cohort. Our results indicate that the proportion of biotin$^{low}$ erythrocytes remained constant for ten days (Phase A in Figure 12) and fell consistently over next 50 days (Phase B and C in Figure 12). From the slope of survival curve of erythrocytes it appears that the rate of decline in biotin$^{low}$ cohort of erythrocytes was relatively lower up to 40 days of age (Phase B), but increased significantly thereafter (Phase C). Interplay of erythrocidal mechanisms that are responsible for the tri-phasic nature of the survival curve of erythrocytes is not clear at present. It is however interesting to speculate that the Phase B may represent random killing of erythrocytes whereas both random as well as age dependent killings of erythrocytes may occur during Phase C.

An age related decline in size of erythrocytes has been suggested (Waugh et al 1992). DIB technique was utilized to track age related changes in forward scatter of
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erythrocytes that is a measure of the size of the cells. It was found that the youngest erythrocytes are largest in size as compared to intermediate and old age erythrocytes (Figure 13). A biphasic decline in forward scatter of biotin^low cohort of erythrocytes was found. An initial decline in average cell size was noticed till 20 days of erythrocyte age, after which the forward scatter appears to remain unchanged for next 15 days. After 35 days of age, another phase of decreasing cell size ensued that lasted till the end of the life of erythrocytes (Figure 14). Reasons behind this pattern of changes in cell size are not clear but it is possible that the earlier phase of decrease in cell size may represent the maturation of reticulocytes into nucleus-free erythrocytes. Later phase of decrease in cell size may be due to progressive shedding of vesicles from aging erythrocytes (Johnstone et al 1987, Waugh et al 1997).

In view of the discussion above, two types of erythrocytes may be killed and removed from blood circulation. First would be erythrocytes that are randomly killed and the status of markers that change with the age of erythrocytes should not be important in the recognition of this group of erythrocyte for destruction. The second component of erythrocyte killing is however age related and the clearance mechanisms in this case must be triggered by changes that take place in the membranes of aging populations of erythrocytes. What are these markers that trigger the destruction of aged erythrocytes are not well understood though many suggestions have been made in this regard (Clark 1988, Deiss 1999). A crucial problem arises in identification of such elimination markers on aged erythrocytes because as soon as the aged erythrocytes acquire the elimination markers they may be cleared by the reticuloendothelial system (RES), making it difficult to study such cells. Since macrophages in RES are basically responsible for elimination of aged erythrocytes, we attempted to deplete mice of macrophages in order to enable the aged erythrocyte populations to accumulate in blood circulation and be available for examination. Macrophage depletion was achieved by administering clodronate loaded liposome in mice. These liposomes are taken up by macrophages where by the clodronate in liposomes kills the macrophages (Van Rooijen and Sanders 1994). Barbe et al (1996) and Giuliani et al (2001) have shown that there is a small yet significant increase in blood erythrocyte counts in rats and mice treated with clodronate liposomes to deplete macrophages. This increase could result from accumulation of aged erythrocytes in blood circulation. Macrophage depletion protocol worked well in our hands as
demonstrated by significantly reduced levels of macrophages in clodronate liposomes treated mice (Figure 5). Depletion of macrophages resulted in the blocking of erythrocyte clearance and accumulation of old erythrocytes in the circulation (Table 1). Interestingly, we also observed a significant fall in the proportion of young erythrocytes in macrophage depleted mice (though not much change was noted in the intermediate age group of erythrocytes). Depressed erythropoietic activity as a result of macrophage depletion has been reported (Giuliani et al. 2005), and could have contributed to the decline in the relative numbers of young erythrocytes in clodronate treated mice.

Flow cytometric analysis indicated a significant age related increase in green auto fluorescence (GAF) of erythrocytes in blood circulation. Kinetics of changes in autofluorescence in an aging cohort of blood erythrocytes indicated that significant increase in GAF of aging cells is first seen in erythrocytes above 45 days of age and further goes up relatively steeply till the end of the erythrocyte life span (Figure 9). Depletion of macrophages by clodronate liposome administration resulted in further accumulation in blood of erythrocytes with high GAF (Figure 6-8). Enhanced GAF as a result of oxidative insult / damage of erythrocytes has been demonstrated before (Rahman et al. 1995, Cheng et al. 1999). It is possible that the accumulation of oxidative stress in aged population of erythrocytes was responsible for higher GAF of these cells. These results are however important for another reason. Many studies utilize fluorescent probes like FITC coupled antibodies to study age related changes in expression of specific antigens on blood erythrocytes, e.g. phosphatidylserine expression in old erythrocytes (Singer et al. 1986, Bratosin et al. 1995, 1998, Boas et al. 1998, Fossati-Jimack et al. 2002, Manodori and Kuypers 2002). Since the background gate in flow cytometric studies is set by using whole blood erythrocytes, higher autofluorescence of old erythrocytes is interpreted as an increase in the expression of antigen under study. Our present results show that such increases could primarily or at least partially be due to higher autofluorescence of the old erythrocytes and necessitate a re-look at the inferences about enhanced PS expression on old erythrocytes that were drawn in previous studies (Boas et al. 1998, Bratosin et al. 1998, Manodori and Kuypers 2002). Our study thus underlines the importance of using old erythrocytes for setting the background gate in flow cytometric studies of markers on
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old erythrocytes using probes like FITC whose emission spectrum overlaps with the autofluorescence of old erythrocytes.

The clearance of erythrocyte is a multi factorial process that involves the trapping of erythrocytes in the spleen followed by their phagocytosis by the macrophages of the reticuloendothelial system. Entrapment of erythrocytes in spleen may be determined by physical changes in erythrocytes e.g. alterations in deformability of erythrocytes (Clark 1988). Additionally, membrane expression of CD147 marker may play an important role in determining the recirculation of erythrocytes that enter spleen from blood circulation. Lower expression of CD147 marker on erythrocytes appears to facilitate their entrapment inside the spleen (Cost et al 2001). A significant decrease in the CD147 was seen in the old age erythrocytes as compared to the young erythrocytes (Figure 16), and in time kinetics studies a steady decline in CD147 expression was observed on aging cohort of erythrocytes (Figure 17). Interestingly, the fall in CD147 expression was most steep during the first ten days, a phase when the erythrocyte loss had not yet started according to the survival curve. It is possible that the relatively faster loss of CD147 during early stages could be related to the shedding of vesicles from erythrocytes. Alternatively, or in addition, the decline in CD147 expression could be a feature associated with the differentiation of reticulocytes into erythrocytes, though these possibilities need further examination. As expected, old erythrocytes in the spleen had lower average expression of CD147 as compared to blood erythrocytes (Table 2) This may indicate that the old erythrocytes with low CD147 expression were preferentially trapped inside the spleen. Depletion of macrophages by clodronate liposome treatment of mice resulted in a fall in the mean CD147 expression on the blood erythrocytes that was likely outcome of the accumulation of CD147 low old erythrocytes in blood. The effect of macrophage depletion on the reduction of CD147 expression was more pronounced on the spleen erythrocytes as compared to the blood erythrocytes (Figure 21), which suggests that after macrophage depletion erythrocytes with reduced CD147 could be getting trapped inside the spleen but are not lysed or phagocytosed. This observation was further confirmed by the result of experiments where the kinetics of accumulation of CD147 low erythrocytes in the blood and spleen was examined in macrophage depleted mice (Figure 22). The accumulation of CD147 low erythrocytes in blood was only
marginal whereas a time dependent progressive accumulation of was seen for erythrocytes isolated from spleens of macrophage depleted mice.

Erythrocytes earmarked for destruction must express some unique markers that may activate macrophages to initiate the phagocytic process. For example, opsonization by autoantibodies that bind old erythrocytes and the attendant complement fixation may provide a positive signal to activate macrophages (Clark 1988, Kay 2004, Lutz 2004). In recent years a qualitatively different type of interaction between erythrocytes and macrophages has also been recognized. Molecules like CD47 and sialic acid residues may interact with their corresponding receptors on macrophages and send a negative signal restraining the macrophages from initiating a phagocytic response (Bratosin et al 1995, Lutz 2004, Oldenborg 2004). According to the currently held view of erythrocyte-macrophage interaction, phagocytosis of a given erythrocyte depends upon the net positive and negative signals it sends to macrophages. Thus erythrocyte destruction can result from enhanced positive signal as well as a decreased negative signal originating in erythrocytes, or both. Alterations in both types of signals have been implicated in the destruction of erythrocytes in a variety of autoimmune hemolytic disorders as well in the physiological turnover of erythrocytes (Lutz 2004, Oldenborg et al 2002, Oldenborg 2004).

Using DIB technique we found that the CD47 expression on erythrocyte cohorts starts to decrease soon after the cells are released into circulation and continues through out the subsequent aging process (Figure 24). Overall there occurred a 30% fall in CD47 expression as erythrocytes aged in blood circulation. Old erythrocytes in the spleen had even lower CD47 expression then the old erythrocytes of the blood circulation (Table 4). Our results thus suggest that the intensity of the negative signal that erythrocytes send to macrophages declines gradually on aging population of erythrocytes. In order to further understand if this decrease in CD47 expression is correlated with macrophage mediated destruction of erythrocytes, the effect of depletion of macrophages was studied on CD47 expression on blood erythrocytes. A significant decrease in the average CD47 expression on circulating erythrocytes was observed in macrophage depleted mice (Figure 26). This decline could result from accumulation of CD47low erythrocytes in blood that were slated for
destruction. Since the fall in CD47 expression was a continuous process, it was necessary to examine if accumulation of CD47\textsuperscript{low} erythrocytes in macrophage depleted mice occurred uniformly in all age groups of erythrocytes or was different for different age groups of erythrocytes. Results of these experiments indicated that the average fall in CD47 expression was comparable in all age groups of blood erythrocytes from macrophage depleted mice. There was however a substantial accumulation of old erythrocytes in blood of macrophage depleted mice that may result from extended survival of erythrocytes that would otherwise have been destroyed. Our results also demonstrate a time dependent accumulation of CD47\textsuperscript{low} erythrocytes occurred in macrophage depleted mice. A substantially greater accumulation of CD47\textsuperscript{low} erythrocytes in spleen as compared to blood of macrophage depleted mice may be due to the fact that aging erythrocytes move to spleen for destruction. It should be noted that our definition of CD47\textsuperscript{low} erythrocytes was only empirical. We defined CD47\textsuperscript{low} erythrocytes as the ones that had less than half the mean CD47 expression on whole erythrocyte population. This assumption was made just for relative enumeration of CD47\textsuperscript{low} erythrocytes and we got essentially similar results if we fixed the window defining CD47\textsuperscript{low} erythrocytes at 30\% or 70\%, rather than 50\% of the mean CD47 expression (results not shown).

An important question is whether up to 30\% decrease in CD47 expression in aging erythrocytes, is sufficient to influence their susceptibility to phagocytosis by macrophages. Olsson \textit{et al} (2007) have recently shown that the expression of CD47 on erythrocytes of heterozygous CD47\textsuperscript{+/−} mice is 50\% of the CD47 levels on homozygous CD47\textsuperscript{+/+} mice and opsonized CD47\textsuperscript{+/−} erythrocytes were markedly more susceptible to phagocytosis by macrophages. And in our study also we found that a 25\% reduction in CD47 expression on mouse erythrocyte induced by treatment with deoxy-glucose is associated with a marked increase in rate of clearance of these cells \textit{in vivo}. Thus graded decline in CD47 expression may influence their clearance. Overall susceptibility of erythrocytes to macrophages is determined by the net positive and negative signals originating from erythrocytes. Our observations support the hypothesis that CD47 expression on erythrocytes may be one such important factor determining the susceptibility of aging erythrocytes for destruction by macrophages.
Narcosis and apoptosis constitute the two basic mechanisms for killing aged or un-needed cells in biology. In nacrosis the target cells is lysed whereas in apoptosis the dying cell is recognized and phagocytosed by macrophages. Apoptosis, or the programmed cell death, is a phenomenon well recognized for nucleated cells because some of the basic features of apoptotic death involve the nucleus of the cell. DNA fragmentation and the fragmentation of nucleus are key features in an apoptotic response. Another prominent feature is the flipping of phosphatidylserine (PS) from the inner layer of the cell membrane lipid bilayer, to the outer layer. Apoptotic cells thus become PS\(^+\) whereas normal healthy cells are PS\(^-\). Even though erythrocytes are not nucleated cells, they have been shown to go through a process similar to programmed cell death. Erythrocyte subjected to stress decrease in size and show PS externalization. (Bratosin et al 2001, Lang et al 2006 b). Programmed cell death of the erythrocyte is called eryptosis to distinguish it from the apoptosis in nucleated cells (Lang et al 2005). It has been suggested that PS exposing erythrocytes may be recognized and phagocytosed by the macrophages and this may be a mechanism for normal turnover of erythrocytes in blood (Schroit et al 1985, Boas et al 1998, Bratosin et al 1998, Lang et al 2005).

Using DIB technique, we examined age related PS externalization in erythrocytes but found no significant difference in PS expression on erythrocytes in different age groups. Except for very young erythrocytes that had significantly greater PS expression, PS expression in all subsequent age groups of erythrocytes remained below 1%. These results appear to contradict the above mentioned paradigm of removal of aged cells by virtue of their greater expression of PS. An early steep fall in PS expression on erythrocytes could be due to the differentiation of reticulocytes with higher PS expression to erythrocytes with negligible PS expression. After this phase, no evidence was found for an increase in PS expression in old erythrocytes. It may be argued that old erythrocytes that express PS may immediately be phagocytosed by macrophages and this may be the reason for not seeing an increase in PS expression on old erythrocytes. To test this possibility, PS expression on erythrocytes was examined in macrophage depleted mice, but even after blocking the macrophage mediated clearance of erythrocytes, no increase was noticed on PS expression on old erythrocytes. We therefore found no evidence for a role of PS exteriorization in the
process of clearance of aged erythrocytes from blood circulation. Lutz (2004) and Arese et al (2005) have also stated similar reservations regarding the role of PS externalization in the normal clearance of erythrocytes from the circulation. While earlier studies in humans (Bratosin et al 1998), rabbits (Boas et al 1998) and mice (Manodori and Kuypers 2002) did show a positive correlation in the PS exposure and age of erythrocytes, the basic problem with these studies was the use of Annexin-FITC conjugate for assessing PS expression on erythrocytes. We found an increased green autofluorescence (GAF) associated with old erythrocytes that has not been reported in literature. Since GAF overlaps with the green fluorescence of FITC, we think that the increased PS expression reported in some of the studies mentioned above could be an artifact due to GAF of aged erythrocytes. We used Annexin-APC conjugate for assessing the PS externalization. Since the APC fluorescence does not overlap with the GAF of old erythrocytes, the artifact of autofluorescence could be avoided in our study.

While a role of PS externalization is unlikely to be the mechanism of normal turnover of old erythrocytes from blood circulation, our studies pointed out to a possible role of PS externalization in removal of stress damaged erythrocytes. Stress was induced in erythrocytes by a variety of procedures like (a) incubation of erythrocytes in HBS (Hepes Buffered saline) in absence of serum, (b) incubation with deoxyglucose (DOG) that prevents glucose utilization by erythrocytes (Klarl et al 2006), and (c) incubation with calcium ions and calcium ionophore. All these treatments resulted in significant PS externalization as well as a decrease in cell size assessed through forward scatter measurements on a flow cytometer. Interestingly, in all cases of subjecting erythrocytes to in vitro stress, maximum effect on PS externalization as well as on forward scatter was seen with the youngest group of erythrocytes whereas old populations of erythrocytes were relatively resistant to these effects.

PS externalization on erythrocytes is an active process, which involves many enzymes. Abrogation of enzymes responsible for the maintenance of lipid asymmetry may not result in loss of membrane asymmetry with respect to PS expression (Diaz and Schroit 1996), but activation of enzyme scramblase seems to be required for PS externalization response (Zhou et al 1997). The role of protein kinases and caspase
Discussion

also has been suggested (Mandal et al 2002, 2005; Karl et al 2006). Since erythrocytes lack nuclear machinery, they are unable to synthesize proteins needed for repairing any damage due to insults and stress. It is possible that the cellular machinery that brings about PS externalization (eryptosis) in response to stress is gradually lost from erythrocytes as they get old. This may be the reason for a decreased ability of older erythrocytes to externalize PS in response to stress.

As compared to PS negative erythrocytes, PS+ erythrocytes were cleared rapidly when re-infused into mice. These results demonstrate a crucial role of PS externalization on the clearance of stress damaged erythrocytes. However factors other than PS externalization may also have a role in the clearance of stress exposed erythrocytes. DOG treated erythrocytes infused back into mice were cleared at very rapidly and were later found to be localized in spleen macrophages (Figure 35, 37). Interestingly, almost all the DOG treated erythrocytes were cleared within 4hrs of re-infusion into mice even though only 20% of the DOG treated erythrocytes were PS positive. These results point to a role of factors other than PS expression in the clearance of stress damaged erythrocytes. One such factor could be the expression levels of CD47 on erythrocytes. In addition to the effect seen on PS expression as well as on the cell size, stress inducers also induced a loss of CD47 expression on erythrocytes (Figure 30-32). Since CD47 expression protects erythrocytes from macrophage attack, stress induced loss of CD47 expression may have a role in clearance of stress damaged erythrocytes.

Taken together our results with respect to stress induced changes in erythrocytes suggest two important points. Firstly, younger erythrocytes are significantly more prone to PS externalization (eryptosis) in response to stress, than the older erythrocytes. Secondly, PS expressing erythrocytes are cleared rapidly from the blood circulation. Taking these two points together, we can speculate that PS externalization may have a role to play in the clearance of stress damaged erythrocytes in blood circulation. On the other hand PS externalization is unlikely to have a role in the regular removal of old erythrocytes from blood circulation. Even though old erythrocytes having gone through the rough and tumble of longer stay in blood circulation as well as having experienced greater accumulated stress may be expected to externalize PS, but that does not seem to happen perhaps because of a
simultaneous age dependent loss of the relevant enzymes required for bringing about PS externalization. It is interesting to speculate that the random killing of erythrocytes for which we have the evidence of the survival kinetics, may occur through the mechanism of stress induced PS externalization, especially in younger erythrocytes.

In the end we reiterate that the work contained in this thesis has resulted in the development of a new technique for studying erythrocyte turnover *in vivo*. Using this technique we have been able to gain some crucial insights into the factors that regulate the clearance of erythrocytes that may either have suffered stress induced damage or have become too old to function optimally. Homeostasis of erythrocyte turnover is crucial to the sustenance of life itself. A variety of conditions that alter this homeostasis result in a variety of anemic conditions, many of which are poorly understood. It is hoped that the availability of this new technique will open new avenues to explore the patho-physiology of erythrocyte turnover in a variety of mouse models.